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CUPRINS

REPRODUCTION ACTIVITY OF SHEEP IN THE NORTH OF MOLDOVA

Ana RACOVITĂ [1], Ion RACOVITĂ [1], Ştefan CIORNEI[2] ,Liviu RUNCEANU[2], Dan DRUGOCIU[2], Petru ROŞCA [2], Gherasim NACU [2], Vlad PĂDURARU[2]
[1]CSVSA Brodina, Suceava; [2]Facultatea de Medicină Veterinară, Iaşi
stefan_ciornei@yahoo.com

Abstract
In sheep, sexual inactivity period and its distribution during the year depends heavily on each individual race and the conditions for growth and dependent. The study was developed on 5581 sheep, the area N of Moldova, which were monitored over a year, and the data were reported meteoclimatici seasonal indices. With weather-time during the main breeding season in sheep (9.4 ° C average temperature, precipitation amount of 2.03 mm / m and cloudiness code 4.6), 91.2% of sheep have shown estrus and libido. In multiparous, of the total, 90.8% of ewes showed estrus and were mounted by rams. In sheep, expression of estrous phase was evident, their share is over 96%. At the end of parturition 85.6% of sheep have lambed one or more products of conception. Batch of sheep showed a higher calving rate by 1.4% compared to multiparous (86.9).
Keywords: sheep, estrus, fertility, prolificacy.

Sexual season in sheep is placed by end summer and early autumn, and its duration is variable from 80 to 150 days (1, 7).

Merinos of Palas has a long breeding season, sexual season begins in early August and can last until late November. Turcana breeds and pans sexual season is much shorter, it starts in September and lasts 1.5 to 2 months.

There are races that season anestrul is absent, showing estrus throughout the year, as D'man breeds, Tarasconnaise and Limousin. Regardless of breed sheep have sexual season shorter than adult sheep (3.6).

All breeds of sheep have seasonal variations in sexual activity. Characteristic of sheep is there a period of anoestrus (spring) present in all breeds of sheep. Even sperm production is reduced to a minimum in winter and spring. These seasonal variations are as dependent alternation of long days and short days (fotoperiodism). This dependence has been well demonstrated in a series of experiments that used different models fotoperiodice: annual variations photoperiod reverse alternation of long days and short days every 3 or 4 months. (2.5).

At the same time, sexual inactivity period and its distribution during the year depends heavily on each individual race and the conditions for growth and maintenance. Consequently period of sexual inactivity shows a variation in duration and time of event.

Material and metods

The study was conducted on 5581 sheep, the area N of Moldova. Maintenance and feeding conditions were employed in standarzi parameters, semi-intensive system of breeding. Lactating adult sheep were in number 4598, the number of 341 sheep, young sheep in the year 491. Rams were actually rent this for 151.

Climatic data were centralized months and seasons, and the data used were provided by the State Meteotrologică Suceava (INMH). Area for which data are collected, refer to the area, time and exact date of exercise of sheep farms that have been research.

Results and discussions

Of all races, 66.9% (3736/5581) were bred sheep pan and 33.1% (1845/5581) belonged to race Turcana. Young sheep breed distribution was 5.5% (308/5581) to race pan and 3.2% (183/5581), the total number of sheep (Table 1.).

The main breeding season (September-November) were monitored breeding activities on the main categories of sheep (sheep, ewes and rams) (Table 2.).

Table 1. Major indices in sheep breeding, the main breeding season in the North of Moldova

Specification	Total effectively sheep		Sheep in oestrus		Sheep lambed (farrowing rate)		prolificity	
	nr	%	nr	%	nr	%	Nr miei	%
Total females	4939/ 4939	100	4504/ 4939	91,2	3856/ 4504	85,6	4666	121
multipara	4598/ 4598	100	4175/ 4598	90,8	3570/ 4175	85.5	4354	122
primipara	341/ 341	100	329/ 341	96,5	286/329	86.9	312	109

Total herd of female sheep (ewes and ewe) monitored between September 2010-August 2011 was 4939 sheep and sheep, and 151 rams.

Of the total number of females, 93.1% (4598/4939) were sheep and 6.9% (341/4939) were sheep.

Sheep in heat detection and estrous manifestation feasible, try to do with rams. Considering that by taking sexual reflexes manifestations of rams, sheep are in heat, and occurred in sheep fitted with dye marked on the device rams. Mention so that the sheep were marked did not show estrus (heat did not enter) and there has been no mount.

Event monitoring during oestrus ewe to a number of 435 female estrous signs were not detected. We believe that the sheep used for breeding, a value of 8.8% (435/4939) has not been in heat.

With weather-time during the main breeding season in sheep (9.4°C average temperature, precipitation amount of 2.03 mm / m and cloudiness code 4.6), 91.2% of sheep have shown estrus and libido.

In multiparous, of the total, 90.8% (4175/4598) of sheep have shown estrus, and were mounted by rams. In sheep, expression of estrous phase was evident, their share is over 96% (96.5% - 329/341).

Weather conditions were reported and pooled by season and mean months of the season as a whole (Table 2).

Temperature values and hints registered during this period were down, coparativ with summer 2010, averaging 9.4 ° C, providing thermal comfort u closer degree of thermal homeostasis. Rainfall during this period was increased (by an average of 2.03 mm / m), coupled with a higher degree of cloud (4.6 degree), and most of the time the sky was covered.

Table 2. Condiţiile meteorologice medii înregistrate pe perioada studiului

Date	T. max. monthly (°C)	T. min. monthly (°C)	T. medie monthly (°C)	Mean quantity of precipitation (mm/mp)	level cloudiness (cod 0 – 9)
09/2010	17.7	10	13.8	1,9	5,4
10/2010	9.7	2.8	6.2	1,6	4,1
11/2010	12.5	4.4	8.4	2,6	4,3
averages	13.3	5.7	9.4	2,03	4,6

Thus, these conditions were to decrease light intensity of the land, and local atmospheric temperature, which resulted in triggering hormonal cascade of waves cortical axis - pituitary - ovary of sheep, leading to natural timing of the event estrous, the presence of libido, and ovulation.

The weather phenomena - were obvious behavioral Turcana race compared to the pan, which is not so influenced space weather conditions - the time of the N - Moldova.

At the end of parturition in sheep 4504, 85.6% (n = 3866), have given birth one or more products of conception. Lot of sheep that calved for the first time (sheep) had a higher farrowing rate by 1.4% compared to multiparous (86.9).

The total average prolificacy was 121%, obtaining the number of lambs 4666. We note that 21% of calving calved twin lambs.

Number twin parturition were observed with greater frequency in sheep (a prolificity of 122%). Instead the ewe prolificacy was 109%, and were obtained a number of 312 lambs.

Conclusions

1. The weather in the North of Moldova, during breeding season in sheep (9.4 ° C average temperature, precipitation 2.03 mm / m and cloudiness code 4.6), 91.2% of sheep have expressed estrus and libido.
2. In multiparous, of the total, 90.8% (4175/4598) of sheep have shown estrus, and were mounted by rams. In sheep, expression of estrous phase was evident, their share is over 96% (96.5% - 329/341).
3. At the end of parturition in sheep 4504, 85.6% (n = 3866), have given birth one or more products of conception. Lot of sheep that calved for the first time (sheep) had a higher farrowing rate by 1.4% compared to multiparous (86.9).
4. The total average prolificacy was 121%, obtaining the number of lambs 4666. We note that 21% of calving calved twin lambs.
5. Turcana breed was found satfel a more focused group of oestrus, especially in the autumn (94.2%).

References

1. Bîrţoiu, A., Seicu,F., (2004) - *Tratat de reproducţie la animale*. Ed. All, Bucureşti.
2. Drugociu D., Runceanu L., 2004- Optimizarea reproducţiei la ovine. Ed. Ion Ionescu de la Brad, Iaşi, IOSBN 973-7921-36-4.
3. Hafez E.S.E. (2002) - *Anatomy of female reproduction in reproduction in animals*, 7th edition. Ed. Hafez E.S.E., Hafez b.
4. Mircu C., (2001) - *Elemente de reproducţie asistată la animalele domestice*, Ed. Brumar, Timişoara.
5. Runceanu, L., Drugociu, D., Roşca, P., (2008) – *Reproducţie, obstetrică şi andrologie clinică.* Casa de editură Venus, Iaşi
6. Salamon S, Maxwell WMC. 2000: Storage of ram semen. Anim Reprod Sci 62, 77-111.
7. Zamfirescu Stela, Sonea A., 2004 –Biotehnologii de reproducere la rumegătoarele mici, Ed. Ex Ponto, ISBN: 973-644-113-X.

MONITORIZAREA DINAMICA VOLUMULUI MATERIALULUI SEMINAL DE BERBEC ÎN CONDIŢIILE SEZONULUI NATURAL DE MONTĂ

Ana RACOVITĂ [1], Ion RACOVITĂ [1], Ştefan CIORNEI[2], Liviu RUNCEANU[2], Dan DRUGOCIU[2], Petru ROŞCA [2]
[1]CSVSA Brodina, Suceava; [2]Facultatea de Medicină Veterinară, Iaşi
stefan_ciornei@yahoo.com

Abstract
In rams, sexual activity is influenced and controlled by a series of external and internal factors. Research has been conducted on a total of 12 rams of different ages, race Turcana and pan. Semen was collected in the months breeding season and beyond. Research on ejaculation volume revealed a larger volume to harvested rams in natural mating season (1.12 ml) than those harvested in the season (0.77 ml). In the age rams, a larger volume of semen was recorded in male 5-year higher values than the other rams. The purpose of this study is to evaluate the sexual activity in rams are mounted in season, and in season, compared to age and environmental factors (climate), in the North of Moldova.

Keywords: rams, semen volume, weather

Weather and climatic factors influencing the production of sperm by variations in temperature and light (2). Research supports the finding that unlike sheep rams exhibit mounted libido and can perform all year, but the number and quality of the ejaculate may be obtained from rams varies by month and season, autumn is the greatest (3, 5).

There is a reduction in sperm quality due to high temperature in summer months. The high temperature associated with long light of day can reduce the desire to pair up to her disappearance (1, 4, 6).

Sperm quality is influenced by season. The external environmental temperature on fertility varies by species, breed, age, height, its influence can be eliminated by selective growth. Also, and meteorological factors and other factors influence the sexual behavior of breeding males and sperm quality such as: humidity, pressure, UV emanations (2, 6).

Materials and methods

12 rams were selected to prepare the monthly dynamics spermogramelor. Rams selected for monitoring reproductive function belong to two races: Aries the race pan, and rams of the breed Turcana. Their age ranged from 1.5 to 4 years. Two groups were composed of rams, the breed, so a group of six rams of the breed represented pan, and one of 6 rams of the breed Turcana.

From each ram of the two groups was collected semen breeding season months and beyond. Thus, in the months September to November, and March-May, semen was collected twice a week, every Monday and Thursday of the week. Month is natural sheep breeding seasons. The semen was collected from each lot in the months from December to February and June-August, a period considered in breeding season. During this period rams were semen collected once a week, namely on Monday.

Climatic data were centralized months and seasons, and the data used were provided by the State Meteotrologică Suceava (INMH). Area for which data are collected, refer to the area, time and exact date of business fermelelor sheep that have been research.

Mean values of key indicators of weather-monitoring period spermogramelor lots of rams are given in the table below (Table 1).

Table 1. Mean indicators weather - climate of the study period

No	Climatic indicators	U.M.	The reproduction season			Extrasesons		
			September, October, November	**March April more**	**average season**	**December January, February**	**June July August**	average Low season
1	Minimum temperature	(°C)	5,1	5,9	5,15	- 3,9	18,6	**7,35**
2	Maximum temperature	(°C)	20,7	18,6	19,6	4,6	29,9	**17,25**
3	Mean temperature	(°C)	12,9	12,2	12,5	0,3	24,2	**12,25**
4	level of rainfall	mm/m^2	1,2	0,9	1,05	2,1	2	**2,05**
5	**level cloudiness**	**cod 0 - 9**	**4,7**	**4,6**	**4,65**	**6,3**	**3,5**	**4,9**

Table 2. Dynamics of average volume of ejaculate, the race pan according to season

	age	Season	
		Natural (autumn, Spring)	Extraseson (winter, summer)
1	1,5	1,12	***0,76***
2	2	1,5	**0,65**
3	2,5	*1,03*	***0,63***
4	3	*1,35*	***0,76***
5	3,5	1,1	**0,64**
6	4	1,09	**0,75**
x		**1,19**	**0,69**

Table 3. Dynamics of average volume of ejaculate, the breed Turcana, depending on season

	age	Seazons	
		Natural (autumn, Spring)	Extraseson (winter, summer)
1	1,5	1,6	***0,5***
2	2	*1,4*	***0,5***
3	2,5	1,5	**0,6**
4	3	*2,3*	**0,7**
5	3,5	1,6	**0,6**
6	4	1,5	***0,9***
Medie		**1,65**	**0,63**

Results and discussion

Semen analysis was performed in a laboratory organized sheep farms. Semen volume was estimated directly in collector glass flask. Mean semen volume breed rams pan by age and type of season are presented in Table 2, 3.

The natural The reproduction season (autumn - spring 3 months -3 months) were examined in the group of 318 rams of the breed Tigaie ejaculate, their average volume, by age, ranging from 1.03 ml (2.5 years) and 1.35 ml (3 years) and mean volume of the entire natural breeding season is of 1.19 ml per ejaculate. In season (winter and summer) were the group examintate breed rams 170 Tigaie ejaculate, mean volumes accestora, by age, ranging from 0.63 ml (2.5 years) and 0.76 ml (1.5 years) with a season average of 0.69 ml whole sperm per ejaculate.

From the 6 rams of the breed group Turcana the normal breeding season were harvested a total of 318 ejaculate, the average limits on age, being of 1.50 ml (2.5 years) and 2.3 ml (3 years) and mean volume throughout the exploited (the natural breeding season) is 1.65 ml. per ejaculate.

In season an average volume of 0.63 ml sperm per ejaculate, the examination of 150 ejaculate with average limits on age, from 0.5 ml per ejaculate (1.5 and 2 years) and 0.9 ml per ejaculate (four years).

Making differences of mean values of ejaculate and their statistical significance between the two seasons, natural season is noted that the highest value is obtained naturally reporducţie season months (Table 3, 4). Ejaculate average values of the two races, Turcana and pans in terms of differences between natural season and season to breed Turcana shows that they are larger, ranging between 0.6 ml (4 years) and 1.6 ml (3 years), while the breed differences are between 0.34 mL Tigaie (4 years) and 0.85 ml (3 years), which shows that the breed Turcana breeding seasonality is well marked in character race.

Table 4. Differences in mean values of ejaculate between natural and season to season two races

No	age	bred Turcana			bred Tigaie		
		reproduction season	**Extraseson**	**Average volume differences**	**reproduction season**	**Extraseson**	Average volume differences
1	1,5	1,6	0,5	***1,1***	1,12	0,76	*0,36*
2	2	1,4	0,5	***0,9***	1,5	0,65	*0,85*
3	2,5	1,5	0,6	***0,9***	1,03	0,63	*0,4*
4	3	2,3	0,7	***1,6***	1,35	0,76	*0,59*
5	3,5	1,6	0,6	***1***	1,1	0,64	*0,46*
6	**4**	**1,5**	**0,9**	*0,6*	**1,09**	**0,75**	*0,34*

Table 5. Diferențe ale valorilor medii ale ejaculatelor la cele două rase pe sezoane

No	age	Sezon natural			Extrasezon		
		bred Turcana	**bred Tigaie**	**Average volume differences**	**bred Turcana**	**bred Tigaie**	Average volume differences
1	1,5	1,6	1,12	**0,48**	0,5	0,76	-0,26
2	2	1,4	1,5	**-0,1**	0,5	0,65	-0,15
3	2,5	1,5	1,03	**0,47**	0,6	0,63	-0,03
4	3	2,3	1,35	**0,95**	0,7	0,76	-0,06
5	3,5	1,6	1,1	**0,5**	0,6	0,64	-0,04
6	**4**	**1,5**	**1,09**	0,41	**0,9**	**0,75**	0,15

Conclusions

1. Research on ejaculation volume revealed a larger volume to harvested rams in natural mating season (1.12 ml) than those harvested in the season (0.77 ml).
2. In the age rams, a larger volume of semen was recorded in male 5-year higher values than the other rams.
3. Assessment of macroscopic parameters can reveal qualitative aspects do not match.

References

1. Bîrţoiu, A., Seicu, F., - Tratat de reproducţie la animale. Ed. All, Bucureşti (2004).
2. Drugociu D., Runceanu L., - Optimizarea reproducţiei la ovine. Ed. Ion Ionescu de la Brad, Iaşi, 2004 IOSBN 973-7921-36-4.
3. Hafez, E.S.E., - Anatomy of female reproduction in reproduction in animals, 7th edition. Ed. Hafez E.S.E., Hafez b(2002).
4. Mircu, C., - Elemente de reproducţie asistată la animalele domestice, Ed. Brumar, Timişoara (2001).
5. Runceanu, L., Drugociu, D., Roșca, P., – Reproducţie, obstetrică şi andrologie clinică. Casa de editură Venus, Iaşi (2008)
6. Zamfirescu, STELA, Șonea, A., –Biotehnologii de reproducere la rumegătoarele mici, Ed. Ex Ponto, 2004, ISBN: 973-644-113-X.

STIMULATION OF GOAT OESTROUS IN SEASON

Ion RACOVITĂ [1], Ana RACOVITĂ [1], Ştefan CIORNEI[2] ,Liviu RUNCEANU[2], Dan DRUGOCIU[2], Petru ROŞCA [2],
[1]CSVSA Brodina, Suceava; [2]Facultatea de Medicină Veterinară, Iaşi
stefan_ciornei@yahoo.com

Abstract
Stimulation of estrus in goats is an important method in bitehnica reproduction in small ruminants. Oestrus synchronization generally apply to group artificial insemination and calving, all with the aim of having lactating goats throughout the year. Were introduced in synchronization regimen (Progesterone, prostaglandin F2α and PMSG), a group of 25 goats in the Carpathian breed. Goats that have responded to treatment and were entered in heat number 23, represented by 95.8%. Thus from 23 goats fitted a number of 14 of them became calving, representing 60.7%. %. Percentage of twin births following estrus synchronization in goats in the experimental group was 28.6% (4/14).
Keywords: goats, synchronisation, estrus, fertility

Synchronization heats during the breeding season in goats is mainly used to facilitate artificial insemination (3, 6, 9). Conventional protocols are based on a schedule aimed for 10 -14 days administration of progesterone.

Recent data on follicular dynamics in cows, suggests that the timing prozocoalele heats are necessary to manage the dominant follicles, in order to achieve maximum fertility. Protocols as progesterone vaginal inserts, now became a routine treatment in sheep and goats, for artificial insemination and embryo transfer (2, 5).

Current information on ovarian follicular dynamics in small ruminants, we show that every 4-5 days develops a follicular wave, 3-4 follicles. Ovarian activity aims to end the dominant follicle, having a size of 5-7 mm in diameter (Hafez ESE 2002).

Materials and methods

The study was conducted on 25 goats breed Carpantină. Maintenance and feeding conditions were employed in standarzi parameters, semi-intensive system of breeding. System that allows access to pasture, the paddock to rest, and also access to dietary supplements and fresh water. To synchronize heats were used intravaginal progesterone implants for 14 days. The extraction (removal) vaginal sponges received PG F2α injection at a dose of 0.125 mg. Detection was carried out with goats heats, 8 hours daily.

Results and discussion

Of the 25 goats, the experimental group subjected to estrous synchronization, a total of 24 goats were identified progesterone vaginal inserts. Thus, following the therapeutic protocol established in 24 of 25 goats could end Cronogest regimen, PG F2α and Folligon, a group represented 96%.

The progesterone released by sponges and absorbed gradually into circulation, is to block the hypothalamus of goat and stop natural secretion of Gn RH. The removal of the source of progesterone (after 12-14 days), will be circulated in sufficient quantity Gn RH naturally capable of producing a response adenohipofizar expressed by ginadotropi hormone

secretion (FSH and LH). Follicle stimulating hormone, are released, they have a direct impact on the target represented by evolutionary ovarian follicles. Thus, the regimen used, it produces waves trigger a hormonal cascade that results in ovarian follicular effect. Follicles enter the stage of development grow and develop to maturity (follicle graaf).

Taking a dose of 0.125 mg Proliz makes evemtualilor action to suppress existing corpus luteum on the ovaries at the time. Prostaglandin F2α action is known, producing luteoloză, the phenomena of contractility of the ovarian stroma and corpus luteum vessels leading to vasoconstriction.

Taking Folligon (Intervet) at a dose of 400 IU, intra muscular progesterone vaginal inserts extraction makes to supplement natural drought ade of pituitary FSH. P.M.S.G. was decomposed in a fraction eletroforetic FSH and in two qualitatively different parts of ICSH

PMSG's physiological action occurs in the regulation of ovarian follicular activity during pregnancy, and corpus luteum formation. P.M.S.G. the powerful effect F.S.H. weak effects L.H. Interstitial cells of the ovary stimulates division and development of several follicles. PMSG's single ovulation can occur, requiring an amount of LH exogenous or endogenous.

The analysis shows that tebelului a regimen used to synchronize estrus in goats, was based on Progesterone, prostaglandin F2α and PMSG

At 25 goats were inrtoduşi intravaginal sponges impregnated with progesterone (Chronogest / Intervet). For 14 days these were retained intravaginal inserts. On the 15th, the sponges were tracţionarea Remove the string, present in the lower corner of the vulva.

At 4% (1/25), the experimental group could not be identified vaginal inserts.

When removing the sponges, they were examined to detect any local vaginal secretions. The same day, the 24 goats received intramuscular injection two hormone preparations: 0.125 mg PG F2α (Proliz), and 400 IU P.M.S.G. (Folligon - Intervet / Germany).

Some goats were observed slight serous discharge, or seromucoase less or more abundant. Character present in sponges and possibly secretions from the vulva, has removed the possibility of uterine disorders. But still, 25% (6/25) of goats were observed reduced secretion, possibly due to transient local inflammatory, stimulating reflex inserts data. We believe that a possible local defense reaction.

Within 3 to 5 days, since the introduction of goats, and the end timing hormonal regimen, goats began to show estrus.

Goats that have responded to treatment and were entered in heat number 23, represented 95.8% (23/24).

Most goats showed estrus within 3 days. Estrous signs were detected and clinical (behavioral and morpho-physiological), and try to help you make the leap goats on 83.3% (20/24), the goats in the first three days. Analyzing and behavioral surveillance goats were found three goats was identified between 3 and 5 days estrus poorly expressed clinically, with a rate of 12.5% (3/24) of goats.

Table 1. indicators obtained through of reproduction goats synchronization scheme

Goats introduced in therapy		**Goats came out of therapy**		**Goats in oestrus**		**prolificacy**	
no	%	no	%	no	%	no. kids	%
25	100	24/25	96	23/24	95,8	18	128,6

We note that 4.16% (1/24), the goats did not show estrous signs, accepting or jump, or the presence of goats us the 9o - 100 days after artificial insemination, could appreciate Clinically 82.6% (19/23), the goats were pregnant. We appreciate that fecundity in the present experiment had a value of 82.6%.

Assess the degree of prolificity was established after parturition and seeking assistance from the total number of goats parturientelor mounted and the number of lambs produced. Thus from 23 goats fitted a number of 14 of them became calving, representing 60.7%.

Prolificacy of the goats, is determined by the number of products of conception obtained by the number of goats kidded. From the 14 goats kidded, we obtained a total of 18 kids, so prolific in the experimental group was 128.6%. Percentage of twin births following estrus synchronization in goats in the experimental group was 28.6% (4/14).

Conclusions

1. The regimen used, it produces waves trigger a hormonal cascade that results in ovarian follicular effect. Follicles enter the stage of development grow and develop to maturity (follicle grraf).
2. In 25% of goats were observed reduced secretion, possibly due to transient local inflammatory, stimulating reflex inserts data. We believe that a possible local defense reaction.
3. Within 3 to 5 days, since the introduction of goats, and the end timing hormonal regimen, goats began to show estrus. Goats that have responded to treatment and were entered in heat number 23, represented by 95.8%.
4. Percentage of twin births following estrus synchronization in goats in the experimental group was 28.6% (4/14).

References

1. Bogdan, L. M., Groza I., (2009) – Obstetrică veterinară. Ed. Academic Press, Cluj-Napoca, ISBN 978-973-744-164-5, p. 1-20.
2. Ciornei Şt.G., Drugociu D., Roşca P., - coordonator -RUNCEANU L., (2008) - Biosecuritatea însămânţărilor artificiale prin spermograma microbiologică, Taurine şi Suine, Ed. Ion Ionescu De Le Brad, Iaşi, ISBN 978-973-147-011-5.
3. Drugociu D., Runceanu L., 2004- Optimizarea reproducţiei la ovine. Ed. Ion Ionescu de la Brad, Iaşi, IOSBN 973-7921-36-4.

4. Hafez E.S.E. (2002) - Anatomy of female reproduction in reproduction in animals, 7th edition. Ed. Hafez E.S.E., Hafez b.
5. Liciu Gh, Rosca Ovidiu (1988)-Ghid tehnic privind insamantarea artificiala la ovine si caprine, Editura Ceres, Bucuresti
6. Mircu C., (2001) - Elemente de reproducţie asistată la animalele domestice, Ed. Brumar, Timişoara.
7. Opris Ioan (2007) - Ovicultura dobrogeana, Editura Ex Ponto, vol I, Constanta
8. Roşca, P., (2004) – Reproducţia animalelor domestice – fiziologie şi patologie. Ed. Tehnopress, Iaşi.
9. Salamon S, Maxwell Wmc. 2000: Storage of ram semen. Anim Reprod Sci 62, 77-111.
10. Tafta V, (2008) - Cresterea ovinelor și a caprinelor, Editura Ceres, ISBN 978-973-40-0800-1, Bucuresti.
11. Zamfirescu S. (1987)-Productia, ameliorarea si reproductia ovinelor, Editura Ceres, Bucuresti

TEH SPERMOGRAM OF GOAT ORDER TO PRESERVATION

Ion RACOVITĂ [1], Ana RACOVITĂ [1], Ştefan CIORNEI[2] ,Liviu RUNCEANU[2], Dan DRUGOCIU[2], Petru ROŞCA [2],
[1]CSVSA Brodina, Suceava; [2]Facultatea de Medicină Veterinară, Iaşi
stefan_ciornei@yahoo.com

Abstract
Assessment of sperm to rent animals, has a special value, especially when semen is for preservation and storage of sperm bank. Examination of sperm (semen analysis) must be made as complete as required to determine volume, sperm concentration and mobility. Ejaculate were evaluated 80 taken from Saanen goats, 60 goats breed ejaculate from Alpine and 40 Saanen crossbred goats ejaculate from the Carpathian breed. The volume of semen collected Saanen breed was on average 1.7 ± 0.25 ml, the Alpine breed of 1.8 ± 0.27 ml, and the crossbred group showed the average value of 1.5 ± 0, 25ml. Motility after harvest was 94 ± 5.3 Saanen breed, 92 ± 7.2 to ± 87 breed Alpine and crossbred 9.1. Sperm concentration in seminal cells of the following values: 3.7 ± 0.85 billion sperm / ml in Saanen breed, 3.5 ± 0.63 at Alpine breed, and 2.9 ± 0.21 to half-breeds.
Keywords: goats, semen analysis, conservation

Small ruminant in male sexual activity is influenced and controlled by a series of external and internal factors. Knowledge of seasonal dynamics, plays an important role in the production and sperm quality. Environmental factors represented by temperature, light and rainfall, affect spermatogenesis, libido and sexual reflexes (2, 8, 10).

The literature reveals that male goats goats in contrast to females, showed libido and can perform fitted all year, but the number and quality of ejaculate you can get from rams varies by month and season, autumn is the greatest common value (1, 5, 7, 9). The purpose of this study is to evaluate the quality of goat semen in preservation by freezing (3, 4, 6).

Materials and methods

The research was conducted at I.C.D.O.C. Palas Constanta during 2006 to 2010. Were evaluated 80 ejaculate collected from 4 Saanen goats, 60 ejaculate from 4 Alpine breed goats and 40 crossbred Saanen goats ejaculate from the Carpathian breed.

Sperm collection was done by artificial vagina technique. Semen volume was measured directly in the glass collector was made by the classical mobility between the blade and blade. And sperm concentration was performed using sperm analyzer SPERMAQE.

Table 1. Semen indicators of the three categories of goats studied

Bred	n	No. ejac	Vol ejac/ml x±Sx	M1% x±Sx	Concentration $x10^9$/ml x±Sx	Doses in stock
Saanen	4	80	1,7±0,25	94±5,3	3,7±0,85	3000
Alpină	4	60	1,8±0,27	92±7,2	3,5±0,63	3000
Metis *	4	40	1,5±0,25	87±9,1	2,9±0,21	1500

Note: n = number of goats, M1 = gross sperm motility * Metis Saanen x local goat Carpathian.

Number of ejaculate was collected and analyzed 80 Saanen breed, 60 and 40 crossbred Alpine.

The volume of semen collected Saanen breed was on average 1.7 ± 0.25 ml, the Alpine breed of 1.8 ± 0.27 ml, and the crossbred group showed the average value of 1.5 ± 0, 25ml.

The data analysis presented in the table, we see that the average size apple has been obtained from Alpine breed goats (1.8 ± 0.27 ml), 0.1 ml which is higher than the average of the Saanen breed (1.7 ± 0.25 ml), and 0.3 ml higher than average volume of crossbred (1.5 ± 0.25). Motility after harvest was 94 ± 5.3 Saanen breed, 92 ± 7.2 to ± 87 breed Alpine and crossbred 9.1. Sperm concentration in seminal cells of the following values: 3.7 ± 0.85 billion sperm / ml in Saanen breed, 3.5 ± 0.63 at Alpine breed, and 2.9 ± 0.21 to half-breeds.

After harvest and evaluation, semen was consrevat. Frozen goat semen for distribution and storage in the ground criobancă cogelare technique was performed using sperm without seminal plasma.

As solvents were used ecremat milk, Tris and sodium citrate.

The results of freezing sperm from 4 Saanen goats from each breed, half breed with Saanen, Alpine are shown in Table 1.

Conclusions

As a result of work of collecting, processing and freezing of semen doses are processed and stored in the broadcast that is 3000 doses of semen of Saanen goats, 3000 dose from Alpine breed and 1500 doses from x Saanen crossbred goats Carpathian. Frozen semen has the following indicators:

- Number of sperm per dose (0.25 ml straw of semen) is 80-125 x 106
- Motility after thawing is on average between 51-67%
- Viability by vital staining with eosin-Nigrosine spirit is on average 54-72%
- 62-78% survival index

References

1. Bogdan, L. M., Groza I., (2009) – Obstetrică veterinară. Ed. Academic Press, Cluj-Napoca, ISBN 978-973-744-164-5, p. 1-20.
2. Ciornei Şt.G., Drugociu D., Roşca P., - coordonator -RUNCEANU L., (2008) - Biosecuritatea însămânţărilor artificiale prin spermograma microbiologică, Taurine şi Suine, Ed. Ion Ionescu De Le Brad, Iaşi, ISBN 978-973-147-011-5.
3. Drugociu D., Runceanu L., 2004- Optimizarea reproducţiei la ovine. Ed. Ion Ionescu de la Brad, Iaşi, IOSBN 973-7921-36-4.
4. Hafez E.S.E. (2002) - Anatomy of female reproduction in reproduction in animals, 7th edition. Ed. Hafez E.S.E., Hafez b.
5. Mircu C., (2001) - Elemente de reproducţie asistată la animalele domestice, Ed. Brumar, Timişoara.
6. Opris Ioan(2007)-Ovicultura dobrogeana, Editura Ex Ponto, vol I, Constanta
7. Roşca, P., (2004) – Reproducţia animalelor domestice – fiziologie şi patologie. Ed. Tehnopress, Iaşi.
8. Salamon S, Maxwell WMC. 2000: Storage of ram semen. Anim Reprod Sci 62, 77-111.
9. Tafta V, (2008) - Cresterea ovinelor și a caprinelor, Editura Ceres, ISBN 978-973-40-0800-1, Bucuresti.
10. Zamfirescu S et al, (1987) - Productia, ameliorarea si reproductia ovinelor, Editura Ceres, Bucuresti

INFLUENCE OF FREEZING RATE UPON POST-THAW QUALITY OF DOG SEMEN EXTENDED WITH CANIPRO FREEZE

Manuela STĂNESCU (PASCAL), Dorin Iulian ŢOGOE, Dana Simona DRUGOCIU, Alin Ion BÎRŢOIU
University of Agronomical Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine of Bucharest; manuelastanescu@hotmail.com

Abstract
The aim of the study was to assess the effect of freezing rate on post-thaw quality of frozen canine semen. For this purpose, the sperm-rich fractions of ejaculates from 4 healthy dogs were collected. The semen was examined and diluted with a comercial extender (CaniPRO Freeze), following the protocol suggested by the producer. The straws were divided into two groups and frozen on a styrofoam box as follows: group I at 4 cm above the liquid nitrogen and group II at 6 cm and stored at -196^{o}C. After 1 week of storage, thawed semen samples were assessed for motility using the computer assisted sperm analyzer SpermVision (Minitüb, Germany), morphology and acrosome status (Spermac stain). Motility characteristics were significantly improved for the samples frozen at 4 cm above the liquid nitrogen (group I). Morphology and acrosome status were not significantly differen between the two freezing rates.

Key words: Semen, cryopreservation, CaniPRO Freeze

Introduction

Cryoconservation of canine semen has become a very useful reproductive biotechnology for the genetic amelioration of this species. There are many protocols developped for the cryopreservation of canine semen. Still the fertilizing results after insemination with frozen-thawed semen are variable (Linde-Forsberg et al., 1999; Thomassen et al., 2006). There are a many known factors that influence this results: technique of semen collection, the extender and the final concentration of spermatozoa (Okano et al., 2004; Pena and Linde-Forsberg, 2000), semen processing (Nothling and Shuttleworth, 2005; Rijsselaere et al., 2002), the combination of extender and cooling rate during the freezing procedure (Pena and Linde-Forsberg, 2000; Schafer-Somi et al., 2006; Silva and Verstegen, 1995; Sirivaidyapong et al., 2000), the thawing technique (Pena and Linde-Forsberg, 2000; Strom et al., 1997) and the use of a thawing medium (Oettlé, 1986; Okano et al., 2004; Pena and Linde-Forsberg, 2000; Pena et al., 2003). Individual factors that makes individual dogs or individual ejaculates more resistant to freezing and thawing damage of spermatozoa are also important (Holt, 2000; Pena et al., 2003; Thurston et al., 1999).

The objective of this study was to compare the effects of two freezing rates upon the quality of frozen-thawed dog semen.

Materials and methods

Four privately-owned stud dogs (two Cane Corso, one Mioritic Sheperd and one Caucasian Sheperd) were selected for this experiment. The dogs ranged between 4 and 6 years old.

Semen was collected by manual stimulation into pre-warmed glass tubes (+37^{o}C) and separated into the three different fractions as described by Kutzler (Kutzler, 2005). Each

sperm rich fraction was assessed immediately after collection and the following parameters were determined: volume, motility (computer assisted sperm analyzer, CASA, SpermVision, Minitüb, Germany), concentration (SpermVision, Minitüb, Germany), morphology and acrosome status (Spermac stain).

The following motility parameters were assessed with CASA:

1) Curvilinear velocity (VCL, μm/s), the instantaneously recorded sequential progression along the whole trajectory of the spermatozoon per unit of time.
2) Linear velocity (VSL, μm/s), the straight trajectory of the spermatozoa per unit of time.
3) Mean velocity (VAP, μm/s), the mean trajectory of the spermatozoa per unit of time.
4) Mean coefficient (STR, %), which indicates the linearity of the mean trajectory and is defined as (VSL/VAP) x 100.
5) Linear coefficient (LIN, %), the ratio of the straight displacement in the sum of elementary displacements during the time of the measurement and it is defined as (VSL/VCL) x 100.
6) Wobble coefficient (WOB, %), which indicates the oscillation of the curvilinear trajectory upon the mean trajectory and is defined as (VAP/VCL) x 100.
7) Frequency of head displacement = beat cross frequency (BCF, Hz), the number of lateral oscillatory movements of the sperm head around the mean trajectory.
8) Amplitude of lateral head displacement (ALH, μm), which is the mean width of sperm head oscillation.
9) Distance curved line (DCL, μm), the actual distance that the sperm cell moved during the analysis period.
10) Distance straight line (DSL, μm), the distance from the point in which the cell was first found in the analysis to the location of the cell at the last frame of the analysis in a straight line.
11) Distance average path (DAP, μm), the measured distance using a smoothed line as a reference.
12) Average orientation change (AOC, degrees), the average number of degrees that the head of the sperm moved from left to right during the analysis.

The CASA was used as described by Schäfer-Somi and Aurich (Schafer-Somi and Aurich, 2007): temperature of analysis 37^{o}C, dimension of sperm heads 5 μm x 7 μm, frame rate 60 s^{-1}, total motility VCL >15 μm/s, linear STR > 0.9, LIN > 0.5, immotile AOC < 9.5.

Morphological defects were determined by staining with Spermac® stain kit (Stain Enterprises, Onderstepoort, South Africa). A drop of semen was placed on a glass slide and a thin smear was prepared and air-dried for 5 min on a warm plate at 37^{o}C. The slide was then fixed for 5 min and washed with distilled water 5-6 times. Excess water was removed with a piece of absorbent paper and the slide was placed into stain solution A for 1 min. This procedure was repeated for solutions B and C. Finally, the slide was air dried. 200 spermatozoa were evaluated for abnormal acrosome, head, mid-piece and tail forms under a light microscope at x1000 magnification. Under the microscope, the acrosome is dark green, the nucleus is stained red, the equatorial region is pale green and the midpiece and tail are green. Morphological abnormalities were classified as primary and secondary (Johnston et al., 2001).

Only ejaculates that met minimal quality requirements were used for further investigations.

We used fresh chicken eggs for the preparation of egg yolk. The eggs were cleaned with an alcohoolic solution. The egg yolk was separated and then filtrated on a paper filter.

The sperm rich fraction was diluted 1:1 with a mix of CaniPRO Freeze - part A and 20% egg yolk (prewarmed at room temperature), then cooled at 4°C for 2 hours. CaniPRO Freeze – part B with 20% egg yolk and pre-cooled at 4°C was slowly added (1 part CaniPRO Freeze B to 1 part semen). The extended semen was packed in pre-cooled 0.5 ml straws and kept at 4°C for 1 hour. The resulted straws were divided into two groups: I and II and frozen in a styrofoam box with liquid nitrogen (LN) for 10 minutes. The rack was placed at 4 cm above the LN for group I and at 6 cm for group II and a distance of 1 cm was mantained between straws. Finally, the straws were plonged in LN and stored in containers with LN.

After 1 week of storage, the straws were thawed 30 seconds at 37°C in a water bath. Motility (SpermVision, Minitüb, Germany), concentration (SpermVision, Minitüb, Germany), morphology and acrosome status (Spermac stain) were determined. Results are presented as mean ± standard deviation (SD).

Results and discussions

Statistical analyses were performed with IBM SPSS software (ver. 19 for Windows; IBM, New York, USA) by unpaired t-test. The results are presented as mean values ± SD and a p value < 0.05 was considered statistically significant.

The fresh semen of the dogs was white in color with a milky viscosity. The volume of the sperm-rich fraction was 1.20 ml, with a sperm concentration of 432.0 x 10^6 spermatozoa/ml. Sperm motility was 82,38%, with 72,86% progressive motility. The rate of sperm morphologic abnormalities was 15.2, of which 1.45 were primary and 13.75 secondary. All characteristics were in agreement with values described for normal canine semen (Johnston et al., 2001).

After thawing the following parameters were determined: motility (computer assisted sperm analyzer, CASA, SpermVision, Minitüb, Germany), morphology and acrosome status (Spermac stain).

For the results of post-thaw motility for the two groups, the following parameters determined by the CASA system were compared: progressive motility (PM), beat cross frequency (BCF), velocity average pathway (VAP) and velocity straight line (VSL) since it was demonstrated that this are significantly correlated with the fertilizing ability of a semen sample (Silva et al., 2006).

Table 1. Post-thaw motility characteristics (individual results, means and standard deviations) for the two groups.

DOG	GROUP I				GROUP II			
	PM	BCF	VAP	VSL	PM	BCF	VAP	VSL
A	29.05	23.83	42.17	29.74	17.79	20.64	55.58	46.26
B	42.39	26.88	62.02	82.34	18.13	21.35	48.52	41.79
C	40.31	25.21	58.13	49.45	25.11	22.87	44.17	34.26
D	39.36	21.43	54.95	45.88	29.44	21.56	42.19	35.44
Mean	37.78	24.34	54.32	51.85	22.62	21.61	47.62	39.44
SD	5.95	2.30	8.60	22.06	5.66	0.93	5.93	5.62

Progressive motility, BCF, VAP and VSL were higher for group I compared to group II, although the difference was statiscally significative only for PM and BCF ($p < 0.05$).

Another study (Dobrinski et al., 1993) used a slower freezing rate (20 cm above the LN), but the distance of 4 cm used in our study is more practical. This study supported the conclusions of various others that dog semen should be frozen at a moderate cooling rate (Rota et al., 1998). By suspending the straws at 4 cm above LN, a cooling rate of -20°C/min is obtained (Linde-Forsberg, 1995). The moderate cooling rate that Olar et al. (Olar et al., 1989) found optimal for a Tris-based extender and various thawing rates took 4 min to cool the semen to -15°C, which was slower than the one used in the current study when straws were placed at 4 cm above the LN. Between -15 and -100°C, the moderate cooling rate of -20°C/min of Olar et al. was similar to the fast cooling rates achieved by suspending straws at 4 cm above the LN. Faster freezing rates during the beginning of the procedure causes less membrane damage because of less crystal formation in the samples. However, very fast freezing rates (-50 to -99°C/min) have been shown to be detrimental for dog semen (Hay et al., 1997; Rota et al., 1998). Very slow freezing rates (-0.5 to -2°C/min) are more harmful than moderate freezing rates because they allow excessive dehydration of sperm cells (Hay et al., 1997; Rota et al., 1998). The results of our study are in contradiction with the study of Nöthling and Shuttleworth (Nothling and Shuttleworth, 2005), but the different results could be explained by the distinct composition of the extenders used in this two experiments.

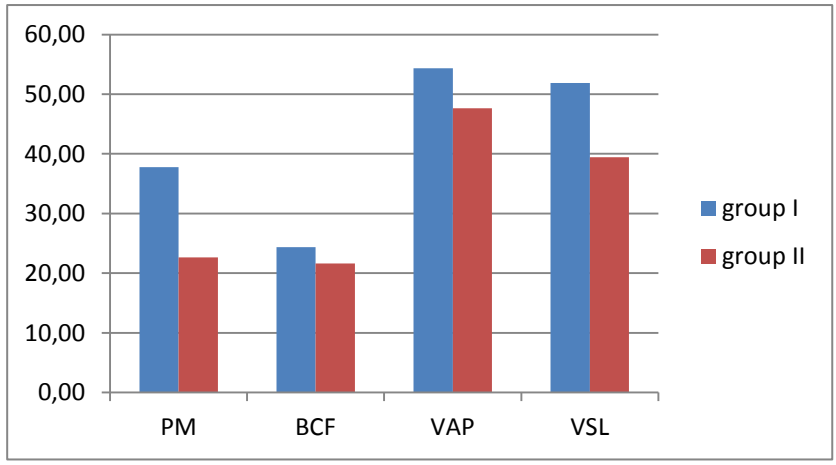

Fig. 1. Comparison of motility characteristics for the two groups

There were no significant differences between the two groups regarding morphology and acrosome status, but there was a significant difference regarding acrosome status between fresh (1,2% acrosome reacted) and frozen-thawed semen (21,34 ± 2,56% acrosome reacted). Cryopreservation of dog spermatozoa it is known to trigger capacitation-like changes (Rota et al., 1999). Freezing and cooling spermatozoa during cryopreservation causes both immediate and delayed ultrastructural changes in the acrosome and plasma membrane which may render the spermatozoa incapable of fertilization and reduce their longevity (Burgess et al., 2001). The number of normal acrosomes in extended and frozen dog spermatozoa is decreased compared to fresh semen (Oettlé, 1986; Sirivaidyapong et al., 1999) and the percentage of acrosome reacted spermatozoa is known to increase in diluents containing high calcium concentrations (Sirivaidyapong et al., 2000). Therefore, constituents of the extender or the

actual dilution of semen could be responsible for the observed changes in the current study, although the percentage of acrosomes reacted is in the range published by the above mentioned authors.

Conclusions

When using the CaniPRO Freeze extender for the cryopreservation of canine semen, a slower cooling rate is recomended. For the manual protocol of freezing canine semen, we recomend placing the equilibrated straws at 4 cm above the surface of liquid nitrogen.

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References

1. Burgess, C. M., Bredl, J. C., Plummer, J. M., and England, G. C. (2001). Vital and ultrastructural changes in dog spermatozoa during cyopreservation. *J Reprod Fertil Suppl* 57, 357-63.
2. Dobrinski, I., Lulai, C., Barth, A. D., and Post, K. (1993). Effects of four different extenders and three different freezing rates on post-thaw viability of dog semen. *J Reprod Fertil Suppl* 47, 291-6.
3. Hay, M. A., King, W. A., Gartley, C. J., Leibo, S. P., and Goodrowe, K. L. (1997). Canine spermatozoa - Cryopreservation and evaluation of gamete interaction. *Theriogenology* 48, 1329-1342.
4. Holt, W. V. (2000). Fundamental aspects of sperm cryobiology: the importance of species and individual differences. *Theriogenology* 53, 47-58.
5. Johnston, S. D., Kustritz, M. V. R., and Olson, P. N. S. (2001). "Canine and feline theriogenology," W. B. Saunders Company, Philadelphia.
6. Kutzler, M. A. (2005). Semen collection in the dog. *Theriogenology* 64, 747-54.
7. Linde-Forsberg, C. (1995). Artificial insemination with fresh, chilled extended, and frozen-thawed semen in the dog. *Semin Vet Med Surg (Small Anim)* 10, 48-58.
8. Linde-Forsberg, C., Strom Holst, B., and Govette, G. (1999). Comparison of fertility data from vaginal vs intrauterine insemination of frozen-thawed dog semen: a retrospective study. *Theriogenology* 52, 11-23.
9. Nothling, J. O., and Shuttleworth, R. (2005). The effect of straw size, freezing rate and thawing rate upon post-thaw quality of dog semen. *Theriogenology* 63, 1469-80.
10. Oettlé, E. E. (1986). Changes in acrosome morphology during cooling and freezing of dog semen. *Anim Repro Sci* 12, 145-150.
11. Okano, T., Murase, T., Asano, M., and Tsubota, T. (2004). Effects of final dilution rate, sperm concentration and times for cooling and glycerol equilibration on post-thaw characteristics of canine spermatozoa. *J Vet Med Sci* 66, 1359-64.
12. Olar, T. T., Bowen, R. A., and Pickett, B. W. (1989). Influence of extender, cryoperservative and seminal processing procedures on postthaw motility of canine spermatozoa frozen in straws. *Theriogenology* 31, 451-61.
13. Pena, A., and Linde-Forsberg, C. (2000). Effects of Equex, one- or two-step dilution, and two freezing and thawing rates on post-thaw survival of dog spermatozoa. *Theriogenology* 54, 859-75.
14. Pena, A. I., Lopez-Lugilde, L., Barrio, M., Becerra, J. J., Quintela, L. A., and Herradon, P. G. (2003). Studies on the intracellular Ca2+ concentration of thawed dog spermatozoa: influence of Equex from different sources, two thawing diluents and post-thaw incubation in capacitating conditions. *Reprod Domest Anim* 38, 27-35.
15. Rijsselaere, T., Van Soom, A., Maes, D., and de Kruif, A. (2002). Effect of centrifugation on in vitro survival of fresh diluted canine spermatozoa. *Theriogenology* 57, 1669-81.

16. Rota, A., Linde-Forsberg, C., Vannozzi, I., Romagnoli, S., and Rodriguez-Martinez, H. (1998). Cryosurvival of dog spermatozoa at different glycerol concentrations and freezing/thawing rates. *Reprod Domest Anim* 33, 355-361.
17. Rota, A., Pena, A. I., Linde-Forsberg, C., and Rodriguez-Martinez, H. (1999). In vitro capacitation of fresh, chilled and frozen-thawed dog spermatozoa assessed by the chloretetracycline assay and changes in motility patterns. *Anim Reprod Sci* 57, 199-215.
18. Schafer-Somi, S., and Aurich, C. (2007). Use of a new computer-assisted sperm analyzer for the assessment of motility and viability of dog spermatozoa and evaluation of four different semen extenders for predilution. *Anim Reprod Sci* 102, 1-13.
19. Schafer-Somi, S., Kluger, S., Knapp, E., Klein, D., and Aurich, C. (2006). Effects of semen extender and semen processing on motility and viability of frozen-thawed dog spermatozoa. *Theriogenology* 66, 173-82.
20. Silva, A. R., Cardoso Rde, C., Silva, L. D., Chirinea, V. H., Lopes, M. D., and Souza, F. F. (2006). Prognostic value of canine frozen-thawed semen parameters on in vitro sperm-oocyte interactions. *Theriogenology* 66, 456-62.
21. Silva, L. D., and Verstegen, J. P. (1995). Comparisons between three different extenders for canine intrauterine insemination with frozen-thawed spermatozoa. *Theriogenology* 44, 571-9.
22. Sirivaidyapong, S., Bevers, M. M., and Colenbrander, B. (1999). Acrosome reaction in dog sperm is induced by a membrane-localized progesterone receptor. *J Androl* 20, 537-44.
23. Sirivaidyapong, S., Cheng, F. P., Marks, A., Voorhout, W. F., Bevers, M. M., and Colenbrander, B. (2000). Effect of sperm diluents on the acrosome reaction in canine sperm. *Theriogenology* 53, 789-802.
24. Strom, B., Rota, A., and Linde-Forsberg, C. (1997). In vitro characteristics of canine spermatozoa subjected to two methods of cryopreservation. *Theriogenology* 48, 247-56.
25. Thomassen, R., Sanson, G., Krogenaes, A., Fougner, J. A., Berg, K. A., and Farstad, W. (2006). Artificial insemination with frozen semen in dogs: a retrospective study of 10 years using a non-surgical approach. *Theriogenology* 66, 1645-50.
26. Thurston, L. M., Watson, P. F., and Holt, W. V. (1999). Sources of variation in the morphological characteristics of sperm subpopulations assessed objectively by a novel automated sperm morphology analysis system. *J Reprod Fertil* 117, 271-80.

THE EFFECT OF HOMOLOGOUS PROSTATIC FLUID ON THE PARAMETERS OF DOG SEMEN EXTENDED AND FROZEN WITH CANIPRO FREEZE

Manuela STĂNESCU (PASCAL), Dorin Iulian ŢOGOE, Ruxandra COSTEA, Alin Ion BÎRŢOIU
University of Agronomical Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine of Bucharest; manuelastanescu@hotmail.com

Abstract
The aim of the study was to assess the effect of autologous prostatic fluid on post-thaw quality of frozen canine semen. The sperm-rich and prostatic fractions of ejaculates from 4 healthy dogs were collected. The semen was examined and diluted with a comercial extender (CaniPRO Freeze), following the protocol suggested by the producer. The third fraction of each ejaculate was collected separately, centrifuged at 1118 x g for 10 minutes and the supernatant was frozen at -18^{o}C until use. The straws were frozen on a styrofoam box at 4 cm above the liquid nitrogen and stored at -196^{o}C. After 1 week of storage, the straws were thawed 30 seconds at 37^{o}C in a water bath. For each dog, six straws were thawed: three straws were diluted 1:2 with autologous prostatic fluid, while the others were not diluted at all. Motility using the computer assisted sperm analyzer SpermVision (Minitüb, Germany), morphology and acrosome status (Spermac stain) were evaluated. Morphology and acrosome status were not significantly differen between the two freezing rates. the addition of autologous prostatic fluid to frozen–thawed dog semen showed a significant effect on some of the parameters that were evaluated in this study, although it did not affect either semen longevity or spermatozoa acrosome status.

Key words: Semen, cryopreservation, prostatic fluid, CaniPRO Freeze

Introduction

Cryoconservation of canine semen has become a very useful reproductive biotechnology for the genetic amelioration of this species. There are many protocols developped for the cryopreservation of canine semen. Still the fertilizing results after insemination with frozen-thawed semen are variable (Linde-Forsberg et al., 1999; Thomassen et al., 2006). There are a many known factors that influence this results: technique of semen collection, the extender and the final concentration of spermatozoa (Okano et al., 2004; Pena and Linde-Forsberg, 2000), semen processing (Nothling and Shuttleworth, 2005; Rijsselaere et al., 2002), the combination of extender and cooling rate during the freezing procedure (Pena and Linde-Forsberg, 2000; Schafer-Somi et al., 2006; Silva and Verstegen, 1995; Sirivaidyapong et al., 2000), the thawing technique (Pena and Linde-Forsberg, 2000; Strom et al., 1997) and the use of a thawing medium (Oettlé, 1986; Okano et al., 2004; Pena and Linde-Forsberg, 2000; Pena et al., 2003). Individual factors that makes individual dogs or individual ejaculates more resistant to freezing and thawing damage of spermatozoa are also important (Holt, 2000; Pena et al., 2003; Thurston et al., 1999).

The prostate is the only accesory gland of the genital system in the dog. As follows, the prostatic fluid (PF) is the main component of seminal plasma. The prostatic fluid of the dog contains a low level of fructose (Bartlett, 1962), acid phosphatases, a high level of arginine esterase (Dube et al., 1985) and 37 proteins of wich 2 are significantly correlated with semen characteristics (de Souza et al., 2007).

During natural mating, the semen is deposited in the cranial vagina of the bitch and the large volume of PF pushes it cranially, trough the cervix, though this action is not indispensable for fertilization (England et al., 2006).

In vivo experiments showed that PF increased fertility of frozen-thawed semen: improved conception rate after intrauterine insemination (Hori et al., 2005), higher fertility rate after intravaginal insemination when PF was added to frozen-thawed semen (Nothling et al., 2005; Nothling and Volkmann, 1993).

In vitro studies showed a negative effect of PF on sperm incubated at 37°C (England and Allen, 1992). PF also has a detrimental effect on semen preservation if it is added to refrigerated (Rota et al., 1995), refrigerated semen followed by freezing (Sirivaidyapong et al., 2001) and when PF is added after thawing (Rota et al., 2007; Yamashiro et al., 2009). Conversely, other in vitro studies demonstrated no effect on frozen-thawed sperm (Koderle et al., 2009) or a positive effect of PF added to frozen-thawed semen on fertility (Nothling et al., 2005; Nothling and Volkmann, 1993).

Spermatozoa undergo capacitation and acrosome reaction inside the female genital tract and the processes then terminate with either fertilization or with cell death. One of the functions of dog PF is to coat sperm membranes, thus masking progesterone receptors and delaying capacitation (Sirivaidyapong et al., 1999). The freezing–thawing process induces destabilization of sperm membranes, which is similar to capacitation (Rota et al., 1999).

The aim of this study was to investigate the effect of prostatic fluid added at thawing to canine sperm frozen with CaniPRO Freeze upon motility, morphology and acrosome status.

Materials and methods

Four privately-owned stud dogs (two Cane Corso, one Mioritic Sheperd and one Caucasian Sheperd) were selected for this experiment. The dogs ranged between 4 and 6 years old.

Semen was collected by manual stimulation into pre-warmed glass tubes (+37°C) and separated into the three different fractions as described by Kutzler (Kutzler, 2005). Each sperm rich fraction was assessed immediately after collection and the following parameters were determined: volume, motility (computer assisted sperm analyzer, CASA, SpermVision, Minitüb, Germany), concentration (SpermVision, Minitüb, Germany), morphology and acrosome status (Spermac stain).

The following motility parameters were assessed with CASA:

1) Curvilinear velocity (VCL, μm/s), the instantaneously recorded sequential progression along the whole trajectory of the spermatozoon per unit of time.
2) Linear velocity (VSL, μm/s), the straight trajectory of the spermatozoa per unit of time.
3) Mean velocity (VAP, μm/s), the mean trajectory of the spermatozoa per unit of time.
4) Frequency of head displacement = beat cross frequency (BCF, Hz), the number of lateral oscillatory movements of the sperm head around the mean trajectory.

The CASA was used as described by Schäfer-Somi and Aurich (Schafer-Somi and Aurich, 2007): temperature of analysis 37°C, dimension of sperm heads 5 μm x 7 μm, frame rate 60 s^{-1}, total motility VCL >15 μm/s, linear STR > 0.9, LIN > 0.5, immotile AOC < 9.5.

Morphological defects were determined by staining with Spermac® stain kit (Stain Enterprises, Onderstepoort, South Africa). A drop of semen was placed on a glass slide and a thin smear was prepared and air-dried for 5 min on a warm plate at 37°C. The slide was then fixed for 5 min and washed with distilled water 5-6 times. Excess water was removed with a piece of absorbent paper and the slide was placed into stain solution A for 1 min. This procedure was repeated for solutions B and C. Finally, the slide was air dried. 200

spermatozoa were evaluated for abnormal acrosome, head, mid-piece and tail forms under a light microscope at x1000 magnification. Under the microscope, the acrosome is dark green, the nucleus is stained red, the equatorial region is pale green and the midpiece and tail are green. Morphological abnormalities were classified as primary and secondary (Johnston et al., 2001).

Only ejaculates that met minimal quality requirements were used for further investigations.

We used fresh chicken eggs for the preparation of egg yolk. The eggs were cleaned with an alcohoolic solution. The egg yolk was separated and then filtrated on a paper filter.

The sperm rich fraction was diluted 1:1 with a mix of CaniPRO Freeze - part A and 20% egg yolk (prewarmed at room temperature), then cooled at 4^{o}C for 2 hours. CaniPRO Freeze – part B with 20% egg yolk and pre-cooled at 4^{o}C was slowly added (1 part CaniPRO Freeze B to 1 part semen). The extended semen was packed in pre-cooled 0.5 ml straws and kept at 4^{o}C for 1 hour. The resulted straws were frozen in a styrofoam box (45cm x 30 cm x 20 cm) with liquid nitrogen (LN) for 10 minutes. The rack was placed at 4 cm above the LN and a distance of 1 cm was mantained between straws. Finally, the straws were plonged in LN and stored in containers with LN.

The third fraction of each ejaculate was collected separately, centrifuged at 1118 x g for 10 minutes and the supernatant was frozen at -18^{o}C until use.

After 1 week of storage, the straws were thawed 30 seconds at 37^{o}C in a water bath. For each dog, six straws were thawed: three straws were diluted 1:2 with autologous prostatic fluid, while the others were not diluted at all.

Motility (SpermVision, Minitüb, Germany), concentration (SpermVision, Minitüb, Germany), morphology and acrosome status (Spermac stain) were determined at 5 min (T_o), 1 hour (T_1) and 2 (T_2) hours post-thaw. Results are presented as mean ± standard deviation (SD).

Results and discussions

Statistical analyses were performed with IBM SPSS software (ver. 19 for Windows; IBM, New York, USA) by unpaired t-test. The results are presented as mean values ± SD and a p value < 0.05 was considered statistically significant.

The fresh semen of the dogs was white in color with a milky viscosity. The volume of the sperm-rich fraction was 1.20 ml, with a sperm concentration of 432.0 x 10^6 spermatozoa/ml. Sperm motility was 82,38%, with 72,86% progressive motility. The rate of sperm morphologic abnormalities was 15.2, of which 1.45 were primary and 13.75 secondary. All characteristics were in agreement with values described for normal canine semen (Johnston et al., 2001).

After thawing the following parameters were determined: motility (computer assisted sperm analyzer, CASA, SpermVision, Minitüb, Germany), morphology and acrosome status (Spermac stain).

Table 1. Post-thaw motility characteristics at T_o
(individual results, means and standard deviations) for the two groups

DOG	CANIPRO FREEZE				CANIPRO FREEZE + PROSTATIC FLUID			
	PM	**BCF**	**VAP**	**VSL**	**PM**	**BCF**	**VAP**	**VSL**
A	29.05	23.83	42.17	39.74	18.25	19.97	54.33	45.03
B	42.39	26.88	62.02	82.34	17.23	21.44	47.21	42.65
C	40.31	25.21	58.13	49.45	24.22	23.15	45.78	32.21
D	39.36	21.43	54.95	45.88	28.67	20.62	40.28	34.55
Mean	37.78	24.34	54.32	54.35	22.09	21.30	46.90	38.61
SD	5.95	2.30	8.60	19.08	5.36	1.38	5.78	6.19

For the results of post-thaw motility for the two groups (CaniPRO Freeze and CaniPRO Freeze with prostatic fluid), the following parameters determined by the CASA system were compared: progressive motility (PM), beat cross frequency (BCF), velocity average pathway (VAP) and velocity straight line (VSL) since it was demonstrated that this are significantly correlated with the fertilizing ability of a semen sample (Silva et al., 2006).

All the motility parameters measured with SpermVision were higher for the group to which PF was not added. At T_o there was a statistically significant difference ($p < 0.05$) for PM and BCF, while at T_1 and T_2 VSL was also significantly affected by the addition of prostatic fluid. A reduction in hyperactivated motility might reduce energy consumption and thus preserve semen functionality for a longer period of time. However, lower motility and slower motion might also mean energy depletion. Our results are partially in agreement with the ones reported by Rota et al. (Rota et al., 2007) who found a lower VCL, but a higher linearity for the samples diluted with PF. Our results contradict the data reported by Hori et al. (Hori et al., 2005) who found that progressive motility after the addition of PF more than doubles. Nöthling et al. (Nothling et al., 2007) reported positive effects of PF for the semen extended with a commercial egg yolk-free extender, but the same negative impact on the semene diluted with an egg yolk extender (similar to CaniPRO Freeze) suggesting an interference of PF and egg yolk.

Table 2. Motility parameters measured with SpermVision at T_o, T_1 and T_2
(means and standard deviations)

TIME	CANIPRO FREEZE				CANIPRO FREEZE + PROSTATIC FLUID			
	PM	**BCF**	**VAP**	**VSL**	**PM**	**BCF**	**VAP**	**VSL**
T_O	37.78 ± 5.95	24.34 ± 2.30	54.32 ± 8.60	54.35 ± 19.08	22.09 ± 5.36	21.30 ± 1.38	46.90 ± 5.78	38.61 ± 6.19
T_1	35.49 ± 4.86	20.23 ± 3.47	49.27 ± 5.45	48.29 ± 11.20	19.88 ± 4.28	17.22 ± 1.56	42.62 ± 2.22	35.48 ± 5.34
T_2	20.32 ± 5.22	14.17 ± 1.80	40.58 ± 6.66	40.73 ± 12.34	10.19 ± 4.76	9.83 ± 2.65	37.94 ± 3.81	28.19 ± 5.40

Our data confirm that PF did not prolong post-thaw semen longevity. Nöthling et al. (Nothling et al., 2005) have reported a similar result, but suggested that PF might postpone the acrosome reaction.

There were no significant differences between the two groups regarding morphology and acrosome status confirming that PF protects spermatozoa to develop the acrosome reaction immediately after insemination.

Conclusions

In conclusion, the addition of autologous prostatic fluid to frozen–thawed dog semen showed a significant effect on some of the parameters that were evaluated in this study, although it did not affect either semen longevity or spermatozoa acrosome status.

Acknowledgements

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References

1. Bartlett, D. J. (1962). Studies on dog semen. II. Biochemical characteristics. J Reprod Fertil 3, 190-205.
2. de Souza, F. F., Barreto, C. S., and Lopes, M. D. (2007). Characteristics of seminal plasma proteins and their correlation with canine semen analysis. Theriogenology 68, 100-106.
3. Dube, J. Y., Frenette, G., Chapdelaine, P., Paquin, R., and Tremblay, R. R. (1985). Biochemical characteristics of the proteins secreted by dog prostate, a review. Exp Biol 43, 149-159.
4. England, G. C. W., and Allen, W. E. (1992). Factors affecting the viability of canine spermatozoa. II. Effects of seminal plasma and blood. Theriogenology 37, 373-381.
5. England, G. C. W., Burgess, C. M., Freeman, S. L., Smith, S. C., and Pacey, A. A. (2006). Relationship between the fertile period and sperm transport in the bitch. Theriogenology 66, 1410-1418.
6. Holt, W. V. (2000). Fundamental aspects of sperm cryobiology: the importance of species and individual differences. Theriogenology 53, 47-58.
7. Hori, T., Hagiuda, K., Kawakami, E., and Tsutsui, T. (2005). Unilateral intrauterine insemination with prostatic fluid-sensitized frozen caudal epididymal sperm in beagle dogs. Theriogenology 63, 1573-83.
8. Johnston, S. D., Kustritz, M. V. R., and Olson, P. N. S. (2001). "Canine and feline theriogenology," W. B. Saunders Company, Philadelphia.
9. Koderle, M., Aurich, C., and Schafer-Somi, S. (2009). The influence of cryopreservation and seminal plasma on the chromatin structure of dog spermatozoa. Theriogenology 72, 1215-20.
10. Kutzler, M. A. (2005). Semen collection in the dog. Theriogenology 64, 747-54.
11. Linde-Forsberg, C., Strom Holst, B., and Govette, G. (1999). Comparison of fertility data from vaginal vs intrauterine insemination of frozen-thawed dog semen: a retrospective study. Theriogenology 52, 11-23.
12. Nothling, J. O., Gerber, D., Colenbrander, B., Dijkstra, M., Bakker, T., and De Cramer, K. (2007). The effect of homologous prostatic fluid on motility and morphology of dog epididymal spermatozoa extended and frozen in Biladyl with Equex STM paste or Andromed. Theriogenology 67, 264-75.
13. Nothling, J. O., and Shuttleworth, R. (2005). The effect of straw size, freezing rate and thawing rate upon post-thaw quality of dog semen. Theriogenology 63, 1469-80.
14. Nothling, J. O., Shuttleworth, R., de Haas, K., and Thompson, P. N. (2005). Homologous prostatic fluid added to frozen-thawed dog spermatozoa prior to intravaginal insemination of bitches resulted in better fertility than albumin-free TALP. Theriogenology 64, 975-91.

15. Nothling, J. O., and Volkmann, D. H. (1993). Effect of addition of autologous prostatic fluid on the fertility of frozen-thawed dog semen after intravaginal insemination. J Reprod Fertil Suppl 47, 329-33.
16. Oettlé, E. E. (1986). Changes in acrosome morphology during cooling and freezing of dog semen. Anim Repro Sci 12, 145-150.
17. Okano, T., Murase, T., Asano, M., and Tsubota, T. (2004). Effects of final dilution rate, sperm concentration and times for cooling and glycerol equilibration on post-thaw characteristics of canine spermatozoa. J Vet Med Sci 66, 1359-64.
18. Pena, A., and Linde-Forsberg, C. (2000). Effects of Equex, one- or two-step dilution, and two freezing and thawing rates on post-thaw survival of dog spermatozoa. Theriogenology 54, 859-75.
19. Pena, A. I., Lopez-Lugilde, L., Barrio, M., Becerra, J. J., Quintela, L. A., and Herradon, P. G. (2003). Studies on the intracellular Ca2+ concentration of thawed dog spermatozoa: influence of Equex from different sources, two thawing diluents and post-thaw incubation in capacitating conditions. Reprod Domest Anim 38, 27-35.
20. Rijsselaere, T., Van Soom, A., Maes, D., and de Kruif, A. (2002). Effect of centrifugation on in vitro survival of fresh diluted canine spermatozoa. Theriogenology 57, 1669-81.
21. Rota, A., Milani, C., and Romagnoli, S. (2007). Effect of post-thaw dilution with autologous prostatic fluid on dog semen motility and sperm acrosome status. Theriogenology 67, 520-5.
22. Rota, A., Pena, A. I., Linde-Forsberg, C., and Rodriguez-Martinez, H. (1999). In vitro capacitation of fresh, chilled and frozen-thawed dog spermatozoa assessed by the chloretetracycline assay and changes in motility patterns. Anim Reprod Sci 57, 199-215.
23. Rota, A., Strom, B., and Linde-Forsberg, C. (1995). Effects of seminal plasma and three extenders on canine semen stored at 4^{o}C. Theriogenology 44, 885-900.
24. Schafer-Somi, S., and Aurich, C. (2007). Use of a new computer-assisted sperm analyzer for the assessment of motility and viability of dog spermatozoa and evaluation of four different semen extenders for predilution. Anim Reprod Sci 102, 1-13.
25. Schafer-Somi, S., Kluger, S., Knapp, E., Klein, D., and Aurich, C. (2006). Effects of semen extender and semen processing on motility and viability of frozen-thawed dog spermatozoa. Theriogenology 66, 173-82.
26. Silva, A. R., Cardoso Rde, C., Silva, L. D., Chirinea, V. H., Lopes, M. D., and Souza, F. F. (2006). Prognostic value of canine frozen-thawed semen parameters on in vitro sperm-oocyte interactions. Theriogenology 66, 456-62.
27. Silva, L. D., and Verstegen, J. P. (1995). Comparisons between three different extenders for canine intrauterine insemination with frozen-thawed spermatozoa. Theriogenology 44, 571-9.
28. Sirivaidyapong, S., Bevers, M. M., and Colenbrander, B. (1999). Acrosome reaction in dog sperm is induced by a membrane-localized progesterone receptor. J Androl 20, 537-44.
29. Sirivaidyapong, S., Cheng, F. P., Marks, A., Voorhout, W. F., Bevers, M. M., and Colenbrander, B. (2000). Effect of sperm diluents on the acrosome reaction in canine sperm. Theriogenology 53, 789-802.
30. Sirivaidyapong, S., Ursem, P., Bevers, M. M., and Colenbrander, B. (2001). Effect of prostatic fluid on motility, viability and acrosome integrity of chilled and frozen-thawed dog spermatozoa. J Reprod Fertil Suppl 57, 383-6.
31. Strom, B., Rota, A., and Linde-Forsberg, C. (1997). In vitro characteristics of canine spermatozoa subjected to two methods of cryopreservation. Theriogenology 48, 247-56.
32. Thomassen, R., Sanson, G., Krogenaes, A., Fougner, J. A., Berg, K. A., and Farstad, W. (2006). Artificial insemination with frozen semen in dogs: a retrospective study of 10 years using a non-surgical approach. Theriogenology 66, 1645-50.
33. Thurston, L. M., Watson, P. F., and Holt, W. V. (1999). Sources of variation in the morphological characteristics of sperm subpopulations assessed objectively by a novel automated sperm morphology analysis system. J Reprod Fertil 117, 271-80.
34. Yamashiro, H., Narita, K., Sugimura, S., Sugawara, A., Hoshino, Y., Sakurai, M., Yokoo, M., Konno, T., Yoshida, M., and Sato, E. (2009). Influence of the prostatic fluid from the first and third fractions of the ejaculates on the cryosurvival of poodle dog sperm. American Journal of Animal Veterinary Science 4, 14-20.

BIOCOMPATIBILITY STUDY ON MTA MIXED WITH HUMAN BLOOD PLASMA

Teodora STEFANESCU [*] , **Zs.T. CZIRJAK** [**] **, Olivia L. BURTA**[*]
[*] University of Oradea, Faculty of Medicine and Farmacy; University of Oradea, Faculty of Environmental Protection, Oradea Romania; prestigedent@yahoo.com

Abstract
Mineral trioxide aggregate (MTA) was developed and recommended initially as an endodontic material having been used for pulp capping, pulpotomy, apexogenesis, repair of root or pulp chamber perforations, or as a root canal filling material. According to it's patent, MTA contains calcium oxide (CaO) and silicon (SiO). When MTA powder is mixed with sterile water, calcium hydroxide and calcium silicate hydrate are formed. Blood plasma was mixed with MTA, instead of distilled water, to track the effects of cellular immunity, by optical microscopy histological analysis. At the same time study aims to analyze the existence and sequence of the inflammatory manifestations initially occurred at the inoculation site.
Keywords: MTA, subcutaneous, histology, Wistar rats, plasma

Introduction

Mineral trioxide aggregate (MTA) was developed and recommended initially as an endodontic material having been used for pulp capping, pulpotomy, apexogenesis, repair of root or pulp chamber perforations, or as a root canal filling material. MTA has been recognized as a biocompatible, bioactive material[1] with hard tissue conductive[2], and inductive properties. It consists in a powder containing fine hydrophilic particles that set in the presence of moisture (sterile water). Several liquids have been used to hydrate MTA powder. According to it's patent[3], MTA contains calcium oxide (CaO) and silicon (SiO). MTA is currently marketed in 2 forms: gray (GMTA) and white (WMTA). The difference between the two is a lower amounts of iron, aluminum, and magnesium in white MTA than in gray MTA[4,5,6,7,8]. MTA was developed from the initial Portland Cement. The primary differences between MTA and PC are a lack of potassium and the presence of bismuth oxide in the first[6]. White MTA is primarily composed of tricalcium silicate and bismuth oxide[5]. When MTA powder is mixed with sterile water, calcium hydroxide and calcium silicate hydrate are initially formed and eventually transform into a poorly crystallized and porous solid gel[9].

Our study was designed to verify and certify the data on the biocompatibility of MTA made some studies. Blood plasma was tested instead of distilled water in place to track the effects of cellular immunity, by optical microscopy histological analysis. At the same time, this study aims to analyze the existence of MTA induced inlammation, sequencing the inflammatory manifestations initially occurred at the inoculation site.

Material and method

This study was conducted at the Medical High Performance Research Center's Biobase, besides the College of Medicine and Pharmacy Oradea.

We used 10 Wistar experimental rats, with age between 3-6 months, weighing between 250-300gr. Our study used white MTA produced by Angelus™ company (Brazil), which comprises 80% of Portland cement and 20% bismuth oxide.

Samples of white MTA were prepared using the following procedure: sterile silicone tube for infusion of 2 mm external diameter was cut from inch to inch. MTA was prepared in aseptic conditions. Silicone Tubes were filled with MTA paste prepared with human blood plasma, group B3 rh+, in aseptic conditions. Aditionaly, a laminar flow hood was used to sterilize samples. The samples were allowed to cure for 15 minutes, as described by themanufacturer.

Rats were anesthetized, then their backs were shaved. Incision site was disinfected with iodized solution and paravertebral incision of 2 cm was made with blade No. 11. In the chamber created submucous, settled one of the samples. Wound suture was made with a polyester or silk thread and needle number 4. Then rats were placed in cages.

The animals were housed in cages with free access to food and water. Food was in the form of fodder grain to make known and analyzed. Health of the animals was monitored daily by trained personnel. Maintenance procedures and handling of animals complied with "Principles of Laboratory Animal Science"[10] and "Guide for the Care and Use of Laboratory Animals"[11].

Sampling method

Rats were sacrificed at 2-4-6, respectively 12 weeks using the following method:

1. Rats were anesthetized,
2. If there are still imposed itself, local shaving was carried out,
3. Local desinfection was made with iodized solution,
4. Samples were discovered using surgical scissors,
5. MTA sample were removed with a 10 mm area submucous tissue from both ends of the tube with MTA,
6. Samples were submersed in 10% chloroform,
7. Wound was sutured with no. 4 silk thread.

Results

In this morpho-pathological study a total of 10 mucosal bioptic samples were taken from the back of 10 Wistar experimental rats. For histopathological analysis of the products, preservation and histological staining was necessary. We used haematoxiline-eozine staining technique. This results in: blue nucleus, pink cytoplasma, orange collagen fibers, red blood cells appear yellow.

Analyzing the control group, a healthy stratified epitelium with it's basal membrane and cells disposed on 4-6 layers, may be observed.(Fig.1)

Analysis after 2 weeks (Fig.2) reveals a mild inflammatory reaction with an increased angiomatous neoformation component. It is remarkable the absence of a limestone deposit in any of the examined samples.

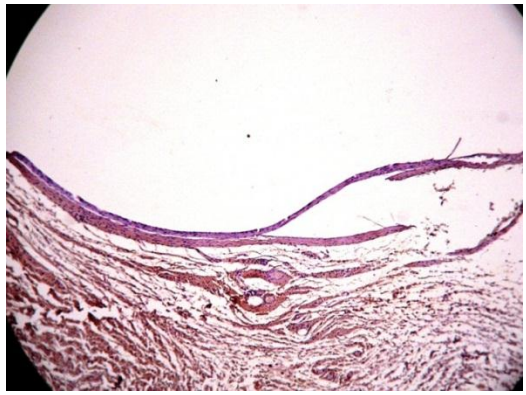

Fig. 1. Healthy mucosa from the control group

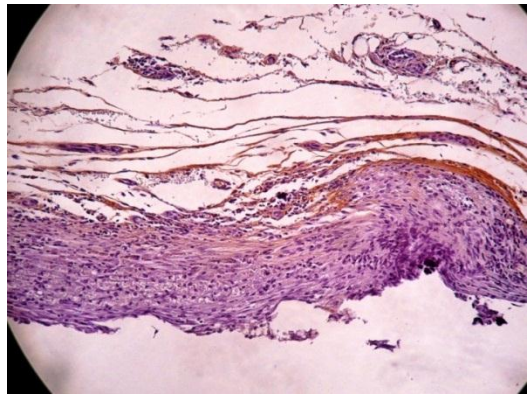

Fig. 2. Medium inflammatory reaction after 2 weeks – HE, 100x

On biopsies from rats sacrificed at 4 weeks (Fig.3), an exacerbatifigon of inflammatory reaction may be noticed. The increased number of lymphocytes and plasmocytes entirely disguises the mucosa and submucosa.

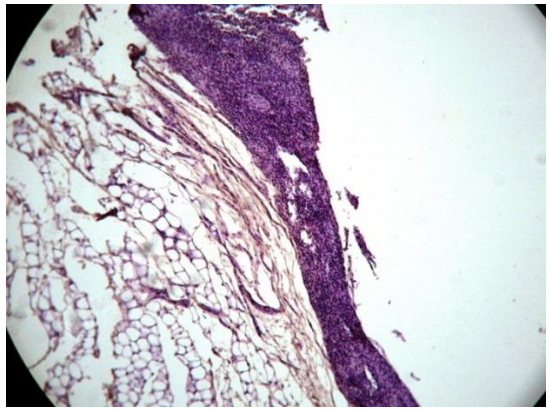

Fig. 3. Severe inflammatory reaction after 4 weeks – HE, 40x

Compared with the control batch, a severe inflammatory reaction was noticed. One explanation could be given by a chemical reaction, resulting from combining MTA with plasma or a reaction to the type and constituents of plasma used in the experiment. The authors have not found a plausible explanation in this case, but noted the existence of inflammatory infiltrate with a tendency to nodular arrangement.

In the analysis of biopsy pieces sampled 6 weeks after inoculation of MTA + plasma (Fig.4), we notice a drastic decreasing in the number of inflammatory cells, and the presence of healing, by a fibrous reaction.

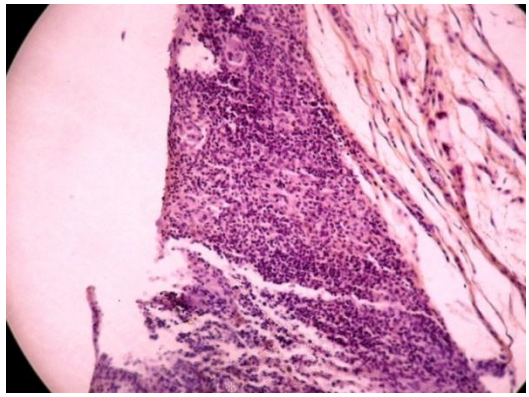

Fig. 4. Medium inflammatory reaction after 6 weeks- HE, 100x

Although the biopsies sampled after 4 weeks, a peak of the inflammatory reaction may be noticed, in case of mucosa harvested at 6 weeks, a shortage of lympho-plasma cells had been remarked. (Fig.4,5)

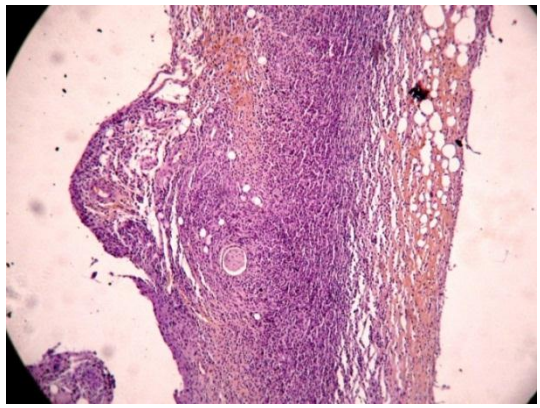

Fig. 5. Medium inflammatory reaction after 6 weeks- HE, 100x

The samples taken after 12 weeks, presented at the morphological examination, an almost complete mucosal healing, although MTA was still present (Fig.6).

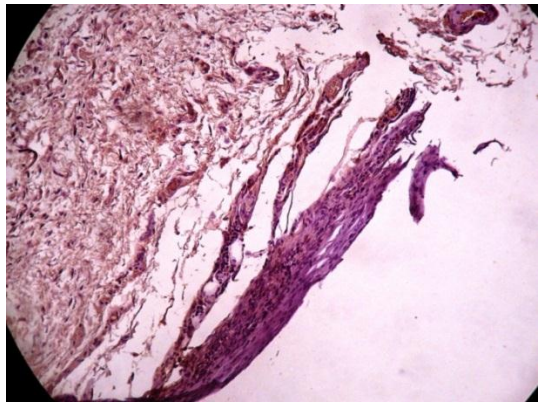

Fig. 6. Mild inflammatory reaction after 12 weeks- HE 40x

Discussion

Sarkar & co[12] suggested that the biocompatibility, the sealing ability and the dentinogenetic activity of MTA, is due to the physico-chemical reactions between the MTA and tissue fluids, during the formation of hydroxyapatite. The presence of various ions in a tissue affects living cells[13]. Although the production of hydroxyapatite is a very desirable phenomenon and a sign of biocompatibility, hydroxyapatite can induce cell death and inhibit their proliferation when the concentration of Ca-P particles is too high.

Gomes-Filho & co[14] compared the subcutaneous reaction of rats towards MTA Angelus and lightcuring MTA. Samples showed a minimal inflammatory response towards MTA Angelus 30 days later, and almost no inflammation 60 days later. Many studies have compared the experimental animal's subcutaneous reaction at MTA and other materials such as amalgam, calcium hydroxide, Super EBA, various root canal sealer, IRM, ZOE, and ethoxybenzoic acid (EBA) [2,15,16,17,18,19,20,21,22,23,24]. Some studies have reported the calcification of tissues, as response to MTA[2,17,18,19,20,21]. Holland & co[20] suggested that all tested materials produce calcified tissue due to the same phenomenon, which is mediated by calcium released by materials, when carbon dioxide is present in tissues.

A recent study showed similar inflammatory tissue reaction, from mild to moderate when CP and white MTA were implanted in the subcutaneous tissue on rats[25]. Another study reported more pronounced tissue necrosis and giant cell formation around the MTA, 7 and 14 days after implantation. Another recent study used Na2HPO4 added to white MTA, reporting a significantly reduced inflammatory reaction compared with white MTA when materials were implanted subcutaneous[26].

These studies have shown that the responses varies from subcutaneous calcification to dystrophic necrosis. In addition, the initial response to MTA produces moderate to severe disorders that reduces in time.

Conclusions

1. 2 weeks after inoculation, striking signs of inflammation can be seen, by the presence of cellular elements characteristic to chronic granulomatous inflammatory reactions of foreign body. Inflammation at 2 weeks, except the control group, is at medium level.

2. 4 weeks after inoculation, the inflammatory response is exacerbating, meaning severe immune reaction, likely to plasma proteins. We cannot explain why this reaction does not occur in the first moments of inoculation.
3. 6 weeks after inoculation a decreasing in number of lymphocytes and plasmocytes was observed, and repair processes become visible. Fibrous healing was also notices. Although in the first weeks after inoculation an explosion in number of lymfo-plasmocytes line was remarked, these reduces after 6 weeks, as it may be noticed on the images taken.
4. 12 weeks after inoculation an almost complete tissue healing was noticed, prooving the biocompatibility of the material, and the acceptance of MTA along with the human blood plasma, by the hosting organism.
5. Remarcable is the way that MTA is generating a stabile compound with an organic liquid, while it is a mineral compound used to be mixed with water. Also, the antigenicity of the plasma is dimmed, making the healing possible. By this point of view, our study is one of it's kind, with a future utility when we are supposed to make mechanical test on it's compounds phisical properties. The goal is to personalize materials based on MTA for each every person, to improve it's bioreactive properties.

References

1. B. Enkel, C. Dupas and V. Armengol *et al.*, Bioactive materials in endodontics, *Expert Rev Med Devices* 5 (2008), pp. 475–494
2. T.R. Moretton, C.E. Brown Jr., J.J. Legan and A.H. Kafrawy, Tissue reactions after subcutaneous and intraosseous implantation of mineral trioxide aggregate and ethoxybenzoic acid cement, *J Biomed Mater Res* 52 (2000), pp. 528–533
3. M. Torabinejad and D.J. White, Tooth filling material and use, *US Patent number 5,769,638* (May 1995).
4. S. Asgary, M. Parirokh, M.J. Eghbal and F. Brink, Chemical differences between white and gray mineral trioxide aggregate, *J Endod* 31 (2005), pp. 101–103
5. J. Camilleri, F.E. Montesin, K. Brady, R. Sweeney, R.V. Curtis and T.R. Ford, The constitution of mineral trioxide aggregate, *Dent Mater* 21 (2005), pp. 297–303
6. J.S. Song, F.K. Mante, W.J. Romanow and S. Kim, Chemical analysis of powder and set forms of Portland cement, gray ProRoot MTA, white ProRoot MTA, and gray MTA-Angelus, *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 102 (2006), pp. 809–815.
7. S. Asgary, M. Parirokh, M.J. Eghbal, S. Stowe and F. Brink, A qualitative X-ray analysis of white and grey mineral trioxide aggregate using compositional imaging, *J Mater Sci Mater Med* 17 (2006), pp. 187–191.
8. S. Asgary, M.J. Eghbal, M. Parirokh, J. Ghoddusi, S. Kheirieh and F. Brink, Comparison of mineral trioxide aggregate's composition with Portland cements and a new endodontic cement, *J Endod* 35 (2009), pp. 243–250.
9. J. Camilleri, Hydration mechanisms of mineral trioxide aggregate, *Int Endod J* 40 (2007), pp. 462–470
10. "Principles of Laboratory Animal Science (LFM van Zutphen, Vera Baumans, Anton C. Beynen, Elsevier, 1993)"
11. Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, National Academy Press, Washington, DC, 1996).
12. Sarkar NK, Caicedo R, Ritwik P, Moiseyeva R, Kawashima I. Physicochemical basis of the biologic properties of mineral trioxide aggregate.J Endod. 2005 Feb;31(2):97-100.
13. Tomson PL, Grover LM, Lumley PJ, Sloan AJ, Smith AJ, Cooper PR. Dissolution of bio-active dentine matrix components by mineral trioxide aggregate. J Dent. 2007 Aug;35(8):636-42
14. J.E. Gomes-Filho, M.D. de Faria and P.F. Bernabé et al., Mineral trioxide aggregate but not light-cure mineral trioxide aggregate stimulated mineralization, J Endod 2008; 34: 62–65.

15. Masuda YM, Wang X, Hossain M, Unno A, Jayawardena JA, Saito K, Nakamura Y, Matsumoto K. Evaluation of biocompatibility of mineral trioxide aggregate with an improved rabbit ear chamber. J Oral Rehabil. 2005 Feb;32(2):145-50.
16. T. Takita, M. Hayashi and O. Takeichi et al., Effect of mineral trioxide aggregate on proliferation of cultured human dental pulp cells, Int Endod J 2006; 39: 415–422.
17. R. Holland, V. de Souza, M.J. Nery, J.A. Otoboni Filho, P.F. Bernabé and E. Dezan Júnior, Reaction of rat connective tissue to implanted dentin tubes filled with mineral trioxide aggregate or calcium hydroxide, J Endod 1999; 25: 161–166.
18. R. Holland, V. de Souza and M.J. Nery et al., Reaction of rat connective tissue to implanted dentin tube filled with mineral trioxide aggregate, Portland cement or calcium hydroxide, Braz Dent J 2001; 12: 3–8.
19. R. Holland, V. de Souza and M.J. Nery et al., Calcium salts deposition in rat connective tissue after the implantation of calcium hydroxide-containing sealers, J Endod 2002; 28: 173–176.
20. R. Holland, V. Souza and M.J. Nery et al., Reaction of rat connective tissue to implanted dentin tubes filled with a white mineral trioxide aggregate, Braz Dent J 2002(13), pp. 23–26.
21. M. Yaltirik, H. Ozbas, B. Bilgic and H. Issever, Reactions of connective tissue to mineral trioxide aggregate and amalgam, J Endod 2004; 30: 95–99.
22. J. Modaresi, S.A. Yavari, S.O. Dianat and S. Shahrabi, A comparison of tissue reaction to MTA and an experimental root-end restorative material in rats, Aust Endod J 2005; 31: 69–72.
23. Sumer M, Muglali M, Bodrumlu E, Guvenc T. Reactions of connective tissue to amalgam, intermediate restorative material, mineral trioxide aggregate, and mineral trioxide aggregate mixed with chlorhexidine. J Endod. 2006 Nov;32(11):1094-6.
24. S. Shahi, S. Rahimi, M. Lotfi, H. Yavari and A. Gaderian, A comparative study of the biocompatibility of three root-end filling materials in rat connective tissue, J Endod 2006; 32: 776–780.
25. Y.C. Hwang, S.H. Lee and I.N. Hwang et al., Chemical composition, radiopacity, and biocompatibility of Portland cement with bismuth oxide, Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2009; 107: e96–102.
26. Lotfi M, Vosoughhosseini S, Saghiri MA, Mesgariabbasi M, Ranjkesh B. Effect of white mineral trioxide aggregate mixed with disodium hydrogen phosphate on inflammatory cells. J Endod. 2009 May;35(5):703-5.

COMPARATIVE HISTOLOGICAL ASPECTS IN SOME NEPHROPATIES IN CAT

V.TIPIŞCĂ, Carmen SOLCAN, Elena-Lavinia NECHITA, Cristina CIORNEI, V. VULPE
University of Agricultural Sciences and Veterinary Medicine, Romania
vlad_tipisca@yahoo.com

Abstract
Renal pathology is very important and knowing the characteristic histological aspect is of great help in kidney pathology diagnosis. To function proper the kidney needs the integrity of both glomerular and tubular structural parts . This paper reaveals some of the most important hitological aspects that appear in hidronephrosis, diabetic nephropaty, PKD(Polokistic Kidney Disease), IRC (Cronical renal insufficiency) and etilen-glicool intoxication, clinicaly diagnosed in five cats. The kidneys were collected and HEA, PAS MASSON'S and Van GISSON stains protocols were conducted, to obtain different views of renal structure. Renal pathology is very important and knowing the characteristic histological aspect is of great help in kidney pathology diagnosis. To function proper the kidney needs the integrity of both glomerular and tubular structural parts . The studies have shown various changes in kidney structure, like tubular necrosis, glomerular sclerosis, intracapsular secretions, fibrosis,and medular cysts.

Key words*: renal, histology, nephropaties*

In all animals the main role of the kidney is the homeostatic control of extracellular fluid composition. To function normally, the kidney requires adequate perfusion with blood, sufficient functional renal tissue and unimpeded urinary outflow. Failure of kidney function can therefore be related to inadequate perfusion (pre-renal), to inadequate processing in the kidney (renal) or to blockage of urinary outflow (post-renal). Each of the four main contributing tissues in the kidney, the blood vessels, glomeruli, tubules and interstitium can be a primary target of disease (1,2).

Once the kidney is fully developed, new nephrons (functional units) are not produced and chronic renal failure with progressive destruction of functional tissue, regardless of initiating cause, leads to a syndrome of salt and water imbalance, acid–base disturbance and accumulation of wastes. Chronic renal failure results in irreversible changes that produce shrunken, fibrosed 'end-stage' kidneys. A wide variety of developmental, circulatory, metabolic, inflammatory and neoplastic conditions can affect the kidneys. Knowing renal histopathological aspects is therefore of great help in kidney pathology diagnosis (3,4,5).

Materials and methods

The study was conducted on 5 cats of different breeds and ages that were clinically diagnosed with renal pathology. The kidneys were colocted from each cat and included in paraffin blocks. After this thin sections of 5 micromethers were obtained at micrhrothome device, displayed on glass slides and stained using PAS(), HEA(), MSSON and Van GISSON stains protochols.After staining, peactures at michroscope were taken.

Results and discussions

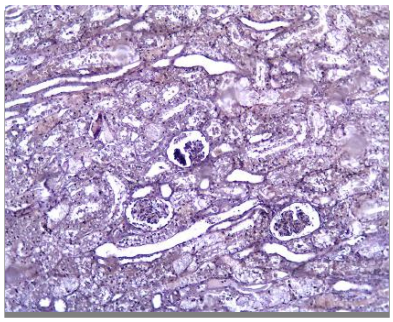

Fig1. Glomerulosclerosis cat with diabetic nephropaty, Van Gieson stain x 100

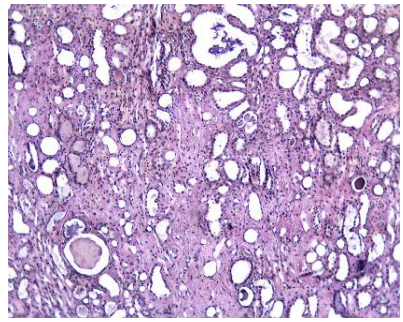

Fig.2 Hislinisation of glomeruli and vascular pole, interstitial fibrosis cat with diabetic nephropaty Van Gieson stain x 100

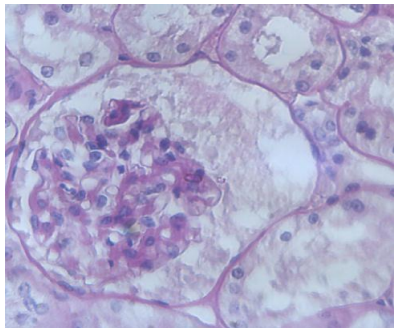

Fig.3 Intracapsular secretions, cat with diabetic nephropaty HEA stain x 400

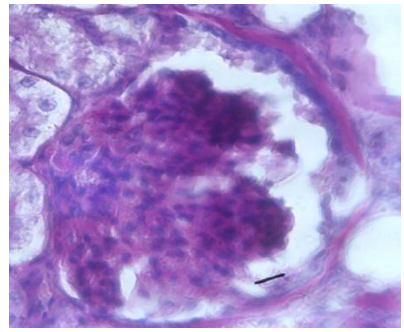

Fig.4 Intercapillary nodular sclerosis (Kimmelstiel-Wilson nodule) HEA stain x 400

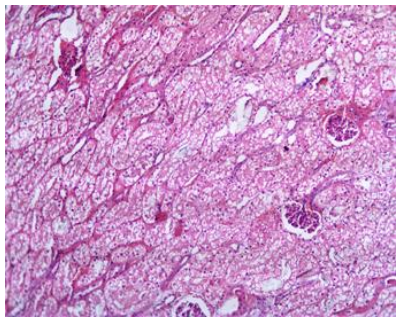

Fig. 5 Etilen glycoolpoisoning in cat the tubules are dilated and lined with flattened epithelial cells, some of which are necrotic HEA stain x 100

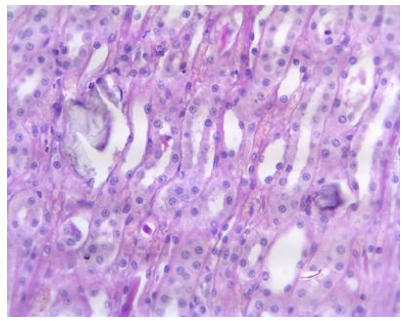

Fig. 6 Etilen glycol poisoning acid oxalic cristal inside renal tubule PAS stain x 400

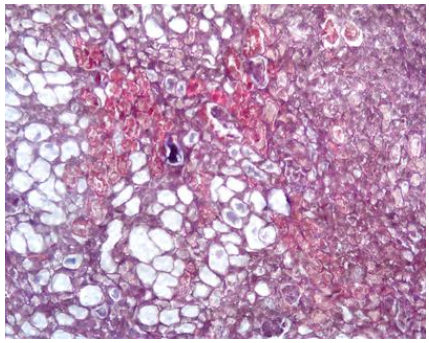

Fig.7 Cat with hidronephrosis, the tubules are dialated and filled with secretion MASSON staining x 100

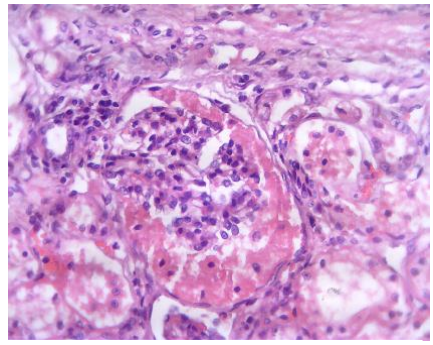

Fig. 8 Cat with hidronephrsis, presence of acellular eosinophilic material in Bowmann space MASSON stain x 400

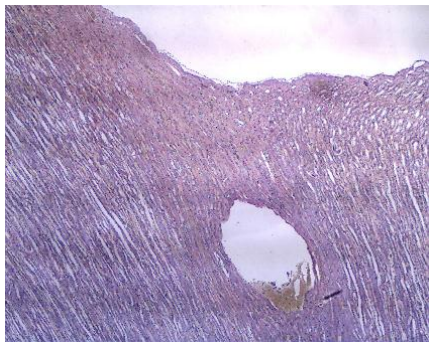

Fig. 9 Cat with PKD, presence of intramedulary cysts, tubular atrophy and interstitial fibrosis Van Gieson stain x 40

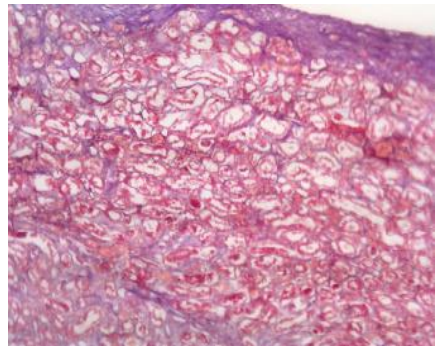

Fig. 10 Cat with PKD, glomerular sclerosis , interstitial infiltrates MASSON stain x 100

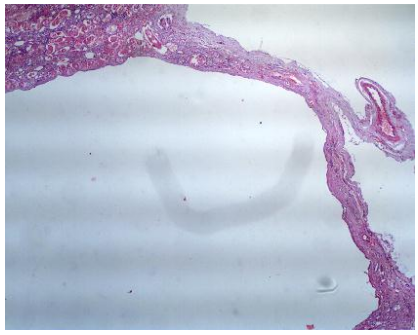

Fig.11 Cat with PKD, cyst wall with tubular atrophy HEA stain x 40

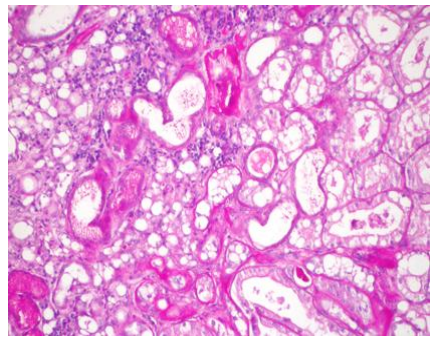

Fig.12 Cat with IRC, severe vacuolization of tubular cels, damaged tubular epithelium PAS stain x 100

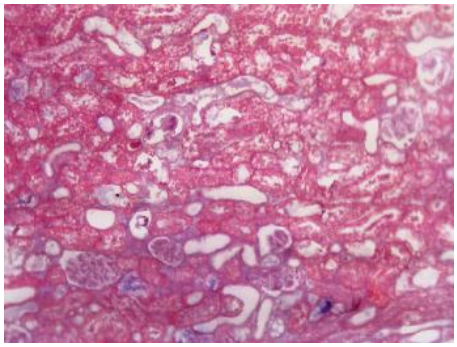

Fig.13 Cat with IRC, necrotic tubular cels, cellular detritus in tubular lumen MASSON stain x 100

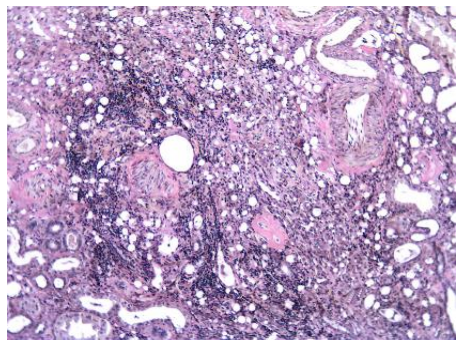

Fig. 14 Cat with IRC, areas of interstitial fibrosis, lymfocite infiltrates Van Gieson stain x 100

Diabetes it's a disease that often affects small animal, aspecialy cats. The glomerulosclerosis in diabetes can be diffuse and nodular and both can appear in the same time, the nodular glomerulosclerosis appears in Kimmelstiel-Wilson syndrome (Fig.4). Other characteristic lesions of diabetes are intracapsular secretions and hyalinization of glomeruli and vascular pole. (Fig. 1,2,3)

In etilen glycool poisoning, the tubules are dilated and lined with flattened epithelial cells, some of which are necrotic (eosinophilic, with loss of nuclear basophilia). Acute tubular necrosis is due to an ischemic or toxic insult. Ethylene glycol is, by itself, harmless to the kidney; however, metabolism of the toxin converts it to oxalic acid, which damages the kidney (Fig. 5,6).

Hidronephosys lesions were consisted in presence of acellular eosinophilic material in Bowmann space, dialation of tubules with the presence of secretion in lumen. In hitrenephrosis, hiperfiltration mey be considered an important factor of these lesions (Fig.7,8).

In PKD(polikystic kidney disease) were observed glomerular sclerosis, interstitial fibrosis, tubular atrophy, (Fig.9,10,11) interstitial infiltrates may appear also, and they are knownn literature to be consisted mainly of CD4 lymfocites and CD8 Macrofages

IRC (cronical renal inssuficiency) patiens presented severe vacuolization that reflects disorganization of intracellular homeostatic mechanism, necrotic tubules with cellular detritus, tubules with excessive cell loss with the membrane very thin and other lesions were interstitial fibrosis and lymfocites infiltrates (Fig. 12, 13, 14).

Conclusions

1. In diabetic nephropaty cases the main lesions were consisted in glomerulosclerosis, intracapsular secretions, and glomerular hyalinization.
2. In etilen glycol poisoning the main factor that damages the kidney is the oxalic acid.
3. In PKD the main lesions were glomerular sclerosis, tubular atrophy and interstitial fibrosis.
4. In hydronephrosis MASSON stain revealed the eosinofilic material in Bowmann space.

5. IRC patients showed lesions like severe vacuolization, tubular necrosis and interstitial fibrosis.

Bibliography

1. 1.Aughey E.,Frye F.— Comparative veterinary histology with clinical correlations, Ed. Manson, 2001.
2. 2.Cotea C.— Histologie speciala, Ed. Tehnopress, 2007.
3. 3.Kemp L. W., Burns K.D. Brown T.G.—Pathology the big picture, Ed. Mc Grau Hill Medical, 2008.
4. 4.Pasca S.— Contributii la studiul morfopatologic al nefropatiilor la caine, Teza de doctorat, USAMV Iasi, 2008.
5. 5.Szende B., Suba Z.— Introduction to Histopathology, Ed. Medicina Publishing, 1999.

PROLIFERATIVE OTITIS EXTERNA IN DOGS: SURGICAL APPROACH

Roxana TOPALĂ, I. BURTAN, M. FÂNTÂNARIU, S. CIOBANU, L.C. BURTAN, Ioana BURCOVEANU
U.S.A.M.V.Facultatea de MedicinăVeterinară-Iaşi
roxanatiron22@yahoo.com

Abstract
The persistence of the inflamation of internal tegument of auricular concha and of auditory tube, determines the emergence of proliferative otitis, the observed clinical manifestations being the stenosis of auditory tube due to proliferations with cauliflower like aspect and decreasing of auditive acuity. Frequently, the proliferative stage is the result of recurrent or relapsing otitis, a common problem in small animal medicine. Each new episode of aural inflammation is more likely to be refractory to medical treatment, therefore surgical management must be considered. The researche was made on dogs with proliferative otitis, presented at Surgery Clinic of Veterinary Medicine Faculty of Iasi, for ambulatory treatment, or hospitalization. The adequate selection of surgical technique was carried out depending of the diagnosed otitis type and the incurred results. Lateral ear canal resection was used to modify the aural environmental, to provide drainage for exudates and moisture and to create access for proliferative tissue removal. Vertical ear canal ablation was used when irreversible proliferative tissues affected all the vertical portion of the ear canal.
Keyword: dog, otitis, proliferative, surgery, therapy

The observations regarding manifestation of external otitis allowed us to work out a classification depending on the evolution stage and prevailing clinical aspect. In this way, clinically, the external otitis in carnivores was classified in: erytematous otitis, ceruminous otitis, exsudative otitis, suppurative otitis, ulcerative otitis and proliferative otitis (Topala, 2007). Frequently, the proliferative stage is the result of recurrent or relapsing otitis, a common problem in small animal medicine (3,4,6). There comes a point when simply managing the infection with cleaning solutions and oral medications is not enough and surgery must be considered (2,5,7,9). There are two techniques commonly used to benefit the patient with chronic otitis: the lateral ear canal resection and the ablation of vertical ear canal (Muller,2007). The lateral ear canal resection is the more conservative approach meant to create access for proliferative tissue removal and resection of vertical ear canal is the surgical approach when irreversible pathological changes affect the vertical ear canal (1,2,9).

Material and methods

The work was carried from October 2010 till October 2011 respectively, on dogs with proliferative otitis, presented at Surgery Clinic of Veterinary Medicine Faculty of Iasi, for ambulatory treatment, or hospitalization. The adequate selection of surgical technique was carried out depending of the diagnosed otitis type and the incurred results. Therefore, surgical techniques used were lateral wall aural resection and vertical ear canal ablation.

Results and discussions

- *Lateral ear canal resection* was used to modify the aural environmental, to provide drainage for exudates and moisture and to create access for proliferative tissue removal.

According to Zepp technique, a probe is insert to ear canal to determine its depth and to guide lateral wall resection (fig.1). Further, a incision is made to lateral ear canal wall, along the probe and two triangualar flaps are removed to provide a large opening. To simplify the technique and to reduce the surgical trauma, we used two hemostatic forceps for triangular flap delimitation and resection (fig. 2). Resection of triangular flap created a large opening necessary for exposer and removal of proliferative tissues, small tumors or polyps (fig.3). If these tissues cause obstruction, they should be excised, and the resultant wound will heal by second intention. The incisions are closed using simple interrupted sutures of skin to internal tegument, with surgical silk(fig. 4).

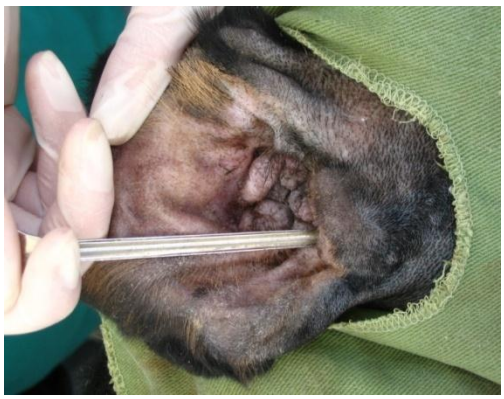

Fig. 1 Determination of ear canal depth

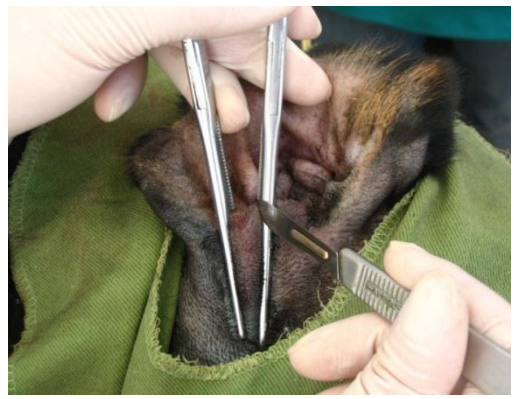

Fig. 2 Delimitation and incisions of triangular flap

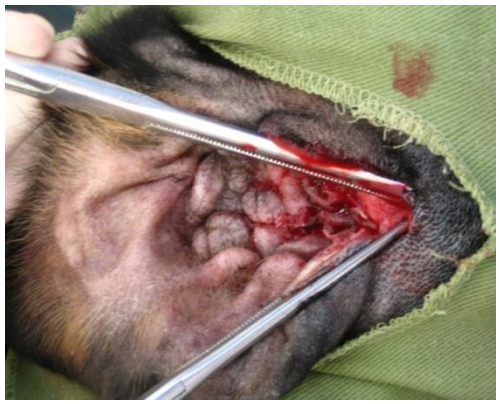

Fig. 3 Triangular flap resection

Fig. 4 Sutures of skin to internal tegument

- *Vertical ear canal ablation* was used when irreversible proliferative tissues affected all the vertical portion of the ear canal. This surgical procedure begins with a T shape incision along the vertical portion of auditive tube (fig.5). This incision mark the limits of two cutaneous flaps, that will be roll up to reveal the vertical ear canal (fig.6). With scissor, this portion of auditive canal was removed along with cauliflower like aspect proliferations (fig.7,

8). After vertical ear canal resection the aperture of horizontal portion was revealed and sutured to cutaneous flaps with surgical silk, using simple interrupted sutures (fig. 9).

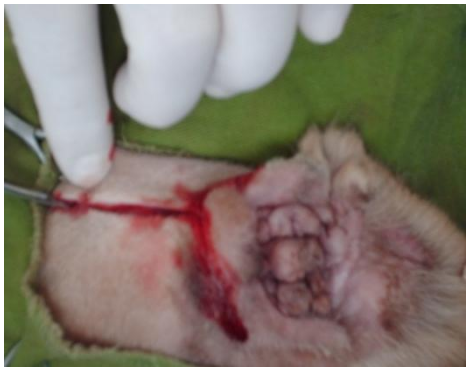

Fig. 5 T shape incision along the vertical ear canal

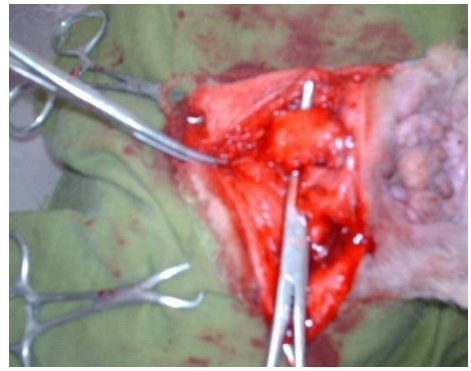

Fig. 6 Revealing the vertical ear canal

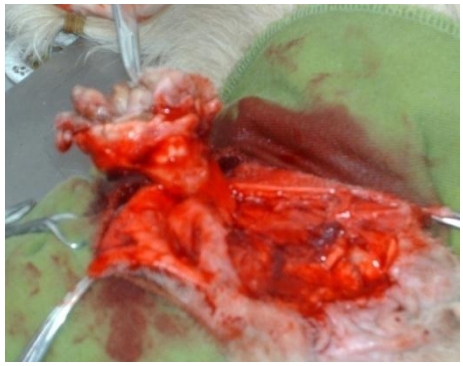

Fig. 7 Vertical ear canal ablation with cauliflower like aspect

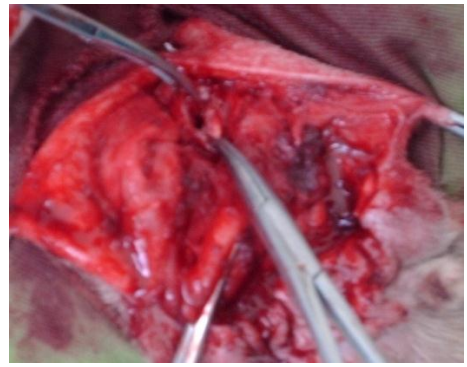

Fig. 8 The aperture of horizontal ear canal

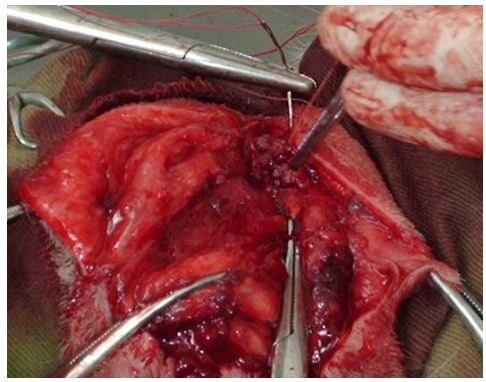

Fig. 8 Suture of horizontal portion to cutaneous flaps

Conclusions

1. The persistence of the inflamation of internal tegument of auricular concha and of auditive tube, determines the emergence of proliferative otitis.
2. Frequently, the proliferative stage is the result of recurent or relapsing otitis, a common problem in small animal medicine .
3. The adequate selection of surgical technique was carried out depending of the diagnosed otitis type and the incurred results.
4. To modify the aural environmental, to provide drainage for exudates and moisture and to create access for proliferative tissue removal, we used lateral ear canal resection .
5. Vertical ear canal ablation was used when irreversible proliferative tissues affected all the vertical portion of the ear canal.

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References

1. Beckman, S., Henry, W., Cecher, P., 1990 – *Total ear canal ablation combining bulla osteotomy and curettage in dogs with chronic otitis externa and media*, Journal of the American Veterinary Medical Association, nr. 196, 84-90
2. Bojrab, M.J., Birchard, S.J., Tomlinson, J.L., 1990 – *Current techniques in small animal surgery*, Lea and Febiger, Philadelphia-London
3. Burtan, I., 2002 – *Chirurgie regională veterinară*, Ed. „Ion Ionescu de la Brad", Iaşi.
4. Gotthelf, L.N., 2005 – *Small Animal Ear Diseases: An Illustrated Guide*, Elsevier Sounders, St. Louis
5. Griffin, C., 2007 – *Otitis topical and systemic*, Internet publisher – www.ivis.org.
6. Hall, J., 2007 – *Medical management of end-stage canine otitis externa*, Internet publisher – www.ivis.org.
7. Little, C., 2005 – *Surgical approaches in the management of chronic otitis externa*, Internet publisher – www.ivis.org.
8. Muller, R., 2007 – *A Practical Approach to Otitis Externa*, Internet publisher – www.ivis.org.
9. Siemering, G., 1980 – *Resection of the vertical ear canal for treatment of chronic otitis externa,* Journal of the American Animal Hospital Association, nr. 16, 753-758
10. Topală Roxana, Burtan, I., Fântănaru, M., Ciobanu S., Burtan, L. C., 2007 – *Clinical signs of otitis externa at carnivores*, Lucr.şt. USAMV Iaşi, vol.50, seria Medicină veterinară, Ed. Ion Ionescu de la Brad, Iaşi, 450-455

THERAPEUTIC MANAGEMENT OF EXTERNAL OTITIS IN DOGS

Roxana TOPALĂ, I. BURTAN, Ioana BURCOVEANU, L.C. BURTAN
U.S.A.M.V.Facultatea de MedicinăVeterinară-Iaşi
roxanatiron22@yahoo.com

Abstract
The main objective of this study was to comparatively estimate some invasive (surgical) and non-invasive (medical) therapeutic methods for setting a therapeutic course adequate to the developing form. Taking into account the etiological polymorphism, in order to set an adequate therapeutic protocol suitable to the evolutionary form, the treatment of otitis externa was classified in preventive, conservative and radical treatment. Regarding the prevention of otitis emergence in carnivores, an essential condition for carrying out this requirement is represented by the periodic cleaning of auricular concha and of auditive tube. In this respect, for setting the periodical hygienic cleaning as a prevention method, we carried out a survey, during January 2011 - April 2012, on a number of 73 dogs of various races susceptible to otitis. Within the conservative treatment, the adequate selection of the products used was conditioned by the results of clinical and paraclinical diagnosis tests. The clinical observations allowed framing into the developing form, by recording local and general signs, framing that formed the basis of the selection of pharmaceutical design of the topic product. The adequate selection of the product composition was carried out by correlating the results of clinical diagnosis with those of the paraclinical diagnosis methods, especially microbiological ones. The external otitis in dogs is a disorder that could give up on medication therapy, in relation to clinical stage but slowing forms without promising response also appear, so that imposing a radical intervention.

Keyword: dog, otitis, therapy

Although it is the most frequent disorder of the statoacoustic apparatus in carnivorous animals, the otitis sometimes could become highly frustrating due to its chronicizing or recurrence following an inadequate treatment application (3,7,8). Taking into account the etiological polymorphism, in order to set an adequate therapeutic protocol suitable to the evolutionary form, the treatment of otitis externa was classified in preventive, conservative and radical treatment. Regarding the prevention of otitis emergence in carnivores, an essential condition for carrying out this requirement is represented by the periodic cleaning of auricular concha and of auditory tube (Nuttal, 2005). Within the conservative treatment, the adequate selection of the products is conditioned by the results of clinical and paraclinical diagnosis tests (1,4,6). Taking into account the etiological polymorphism, commercial products for otitis treatment have a mixture of antibiotic and antifungal in their composition. Antibiotics are used for bacterial infections and antifungals for yeast infections (2,5,8). Glucocorticoids such as dexamethasone are often included in these preparations to reduce the amount of inflammation in the ear.

Material and methods

The researche was perfomed on dogs with varied clinical forms of otitis externa, presented at Surgery Clinic of Veterinary Medicine Faculty of Iasi, between January 2011 and April 2012.

Results and discussions

Taking into account the etiological polymorphism, in order to set an adequate therapeutic protocol suitable to the evolutionary form, the treatment of otitis externa was classified in preventive, conservative and radical treatment.

Regarding the prevention of otitis emergence in carnivores, an essential condition for carrying out this requirement is represented by the periodic cleaning of auricular concha and of auditory tube. In this respect, for setting the periodical hygienic cleaning as a prevention method, we carried out a survey, during January 2011 - April 2012, on a number of 73 dogs of various races susceptible to otitis (Caniche, Cocker, Labrador, Setter). Cases history of the patients revealed that each summer they developed a clinical form of otitis externa. When subjects were presented to consult they had no clinical sign of otitis, but taking into account that every alteration of ear canal microenvironment determine rapid colonization of ear canal, the patients were survey and provide them monthly cleaning of the auditory tube and internal face of the auricular concha. During the surveyed period of time, clinical signs of otitis emerged only on 25 out of 73 dogs, representing 34.2%, demonstrating that a periodic and correctly executed cleaning of the auditory tube and of the internal side of ear tragus can assure the prevention of otitis emergence in 65.8% cases.

Within the conservative treatment, the adequate selection of the products used was conditioned by the results of clinical and paraclinical diagnosis tests. Also, information from a thorough case history facilitated the choice made for improving some predisposing factors such as hypersensitivity states. The clinical observations allowed framing into the developing form, by recording local and general signs, framing that formed the basis of the selection of pharmaceutical design of the topic product.

In this way, in the cases of erytematous and ceruminous otitis, we preferred the use of products in the form solutions and ointments, and in the cases clinically manifested by an increase of the exsudate quantity, by changing its aspect, as well as the presence of ulcers, we used powders, after a previous cleaning of the tube.

The adequate selection of the product composition was carried out by correlating the results of clinical diagnosis with those of the paraclinical diagnosis methods, especially microbiological ones.

To amend auricular and periauricular itching, topical products in whose composition there are anesthetic or analgesic, were chosen. They were chosen also, for patients with painful ear, clinical manifestation for suppurative and ulcerative otitis. In cases of parasitic otitis diagnosed by direct microscopic examination, we used products with ivermectin and lindane.

Establishing antibiotics and antifungal medication was made consecutive microbiological exams. In cases when by microbiological examination, *Malassezia canis* in pure culture was identify, we opted for miconazole şi ketoconazole ointment, and for those with *Malassezia canis* in mixed cultures, we have used comercial products with a mixture of antibiotic and antifungal in their composition. Antibiotic selection was according to antibiogram results. Local treatment efficiency depends on correct cleaning of external ear canal and internal face of auricular concha, before applying the product. Also, in some cases, local treatment was correlated with general therapy to achieve the expected results.

For general treatment antibiotics were selected according to antibiogram results and consisted with that used in topical therapy for clinical forms of suppurative and ulcerative otitis, and to improve cases occurred due to allergic conditions we used Prednisone

1mg/kg/day for 14 days, then 0,5mg/kg/day 14-21 days, or Dexafort 0,2mg/kg/week and antihistamines (Clemastin 0,1mg/kg, 14 days).

Antiparasitic general treatment involved the use of ivomec, in massive infestation with *Otodectes cynotis*, and external insecticides for cases occured consecutive flea bites allergy (Frontline, Decis).

The external otitis in carnivores is a disorder that could give up on medication therapy, in relation to clinical stage but slowing forms without promising response also appear, so that imposing a radical intervention. Although the surgical intervention is more laborious, more traumatizing and requesting a more careful post-operative supervision, the action is imposed in cases of proliferative lesions as well as of supurative-ulcerative ones, stubborn to treatment. Also, surgical treatment was applied to chronic otitis of bacterial origin to improve ear canal microenvironment by lowering temperature and relative humidity, important factors in the growth of microorganisms. The adequate selection of surgical technique is carried out depending of the diagnosed otitis type and the incurred results.

Conclusions

1. Periodic and correctly executed cleaning of the auditory tube and of the internal side of ear tragus can assure the prevention of otitis emergence in 65.8% cases.
2. Within the conservative treatment, the adequate selection of the products used was conditioned by the results of clinical and paraclinical diagnosis tests.
3. The adequate selection of the product composition was carried out by correlating the results of clinical diagnosis with those of the paraclinical diagnosis methods, especially microbiological ones.
4. In the cases of erytematous and ceruminous otitis, we preferred the use of products in the form solutions and ointments.
5. In the cases clinically manifested by an increase of the exsudate quantity, by changing its aspect, as well as the presence of ulcers, we used powders, after a previous cleaning of the tube.
6. To amend auricular and periauricular itching, topical products in whose composition there are anesthetic or analgesic, were chosen.
7. For general treatment antibiotics were selected according to antibiogram results and consisted with that used in topical therapy for clinical forms of suppurative and ulcerative otitis.
8. The external otitis in carnivores is a disorder that could give up on medication therapy, in relation to clinical stage but slowing forms without promising response also appear, so that imposing a radical intervention.

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References

1. Bensignor, E., Grandemange, E., 2007 – *Comparison of an antifungal agent with a mixture of antifungal, antibiotic and corticosteroid agents for the treatment of Malassezia species otitis in dogs*, Internet publisher – www.ivis.org.
2. Burtan I., Fântânaru M., Ciobanu S., Burtan L.C., 2003 – *Utilizarea pudrei Rifaot în terapia otitelor la carnivore*, Revista Rom. de Med.Vet., vol.13(3-4), 330-335.
3. Greek Jean, 2004 – *Canine otitis externa – identification and treatment*, Prat. Med. Chir. Anim. Comp., nr.7
4. Griffin, C., 2007 – *Otitis topical and systemic*, Internet publisher – www.ivis.org.
5. Jackson, H.A., 2002 – *Approach to the pruritic dog*, Waltham Focus, vol.12, nr. 4, 4-10
6. Marignac, G., 2001 – *Gestion medicale d'une otite stenotique*, Le Point Veterinarie, nr.219, 44-48
7. Nuttal, T., Cole Lynette, 2005 – *Ear cleaning: The UK and US perspective*, Veterinary Dermatology, nr.15, 127-136
8. Raugier Sandrine, Borell Daniela, Pheulpine Sandrine, Woehrle, F., Boisrame, B., 2005 – *A comparative study of two antimicrobial/antiinflammatory formulations in the treatment of canine otitis externa*, Veterinary Dermatology, nr.16, 299-307

PRELIMINARY STUDY ON EARLY DIAGNOSIS OF LIPID MOBILIZATION SYNDROME IN TWO BREEDS OF COWS

Alina ANTON[1], Gh. SOLCAN[1], S. CREANGA[2], Elena RUGINOSU[2]
[1]Faculty of Veterinary Medicine, No. 8, Aleea Mihail Sadoveanu, Iasi, Romania
[2]S.C.D.C.B. Dancu, No. 9, Sos. Iasi-Ungheni, Holboca, Iasi, Romania
antonclaraalina@yahoo.com

Abstract
Twenty postpartum lacting cows (10 local Holstein-Friesian mix breed and 10 Romanian Gray Steppe) were studied with the aim to investigate the early diagnosis of lipid mobilization syndrome. Hemograms showed variations in physiological limits, the differences between breeds were not statistically significant (p > 0.05). Regarding the leukograms, the higher values were observed at the local Holstein-Friesian mix breed than Romanian Gray Steppe, but the differences were not statistically significant (p > 0.05). The lower serum concentrations of aspartate aminotransferase, amylase, glucose and the higher values of lactate dehydrogenase, alanine aminotransferase, tryglicerides, cholesterol, than physiological limits, may indicate the evolution of lipid mobilization syndrome in the local Holstein-Friesian mix breed cows.

Key words: haematology, biochemistry, lipid mobilization syndrome, cow

Endocrine and metabolic systems are involved in the metabolic processes of the udder in the initial phase of lactation. In this period, food-intake capacity does not cover the enormous energy and protein needs associated with milk production. This condition promotes a mobilization of animal's adipose tissue stores, stimulates the gluconeogenetic processes and reduces the assimilation of glucose by non-mammary tissues.
The aim of the present study was to investigate the early diagnosis of lipid mobilization syndrome, in the postpartum period, comparing two breeds of cows.

Materials and methods

A survey was conducted in a farm from Iasi country, Romania, on twenty postpartum lacting cows (10 local Holstein-Friesian mix breed and 10 Romanian Gray Steppe) similar for number of lactation (2.25±0.11; mean±SEM). After calving and throughout the following lactation, animals were fed a diet formulated for high-yielding dairy cows. A detailed clinical examination was conducted in all cows, focusing on body condition score (BCS). Blood was collected from the coccigiene vein in tubes with EDTA and clot activator in order to evaluate the haematological profile, serum AST, ALT, LDH, γGT, glucose, amylase, triglycerides, cholesterol, creatine kinase, total proteins and bilirubin. Haematological analyses included red blood cells (RBC), haematocrit (PCV), haemoglobin (Hgb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), white blood cells (WBC) and number of platelets. The EDTA samples were analysed with an automated cell counter (Animal Blood Counter Vet) to obtain the hemograms and clot activator samples were analysed with an automatic analyzer Cormay Accent 200 for serum biochemical profiles. Differential leukocyte count was performed microscopically on Giemsa stained blood film. The results were treated statistically using Student's t-test assessing the mutual statistical difference(Snedecor and Cochran, 1982).

Results and discussion

Cows generally mobilize body lipid reserves in early lactation and regain these reserves during subsequent pregnancy (Friggens et al., 2004). Increased mobilization of body reserves in early lactation has been associated with increased health problems and a reduction in reproductive performance (Friggens et al., 2007).

BCS is an indicator of the energy balance, which evaluates the amount of fat cover on a dairy cows. This evaluation is accomplished by assigning a score to the amount of fat observed on several skeletal parts of the cow (Butler and Beam, 1993). The current body score reflects the last energy balance while the changes of BCS since the last visit reflect the current energy balance. The most commonly used system ranges from 1.0 (thin) to 5.0 (obese) (Zaaijer, 2001). In the present study the values of BCS of cows were fat, ranging from 3.5 to 4, without statistically significant differences ($p > 0.05$) between the two breeds of cows. The range of ideal BCS is: 3.5-4 (calving-older cows), 2.5-3.0 (one month postpartum), 3.0 (mid lactation), 3.25-3.75 (late lactation), 3.5 (calving – first lactation) and 3.5-4.0 (drying-off) (Braun et al., 1987). In agreement with Heuer et al. (1999), cows with a high BCS were more likely to develop an elevated ketone body status than cows in moderate to low body condition. This might be due to a greater mobilization rate of non esterified fatty acid from fat depots in overconditioned than in thin cows.

Regarding the hemograms (table 1), it was observed some variations within their physiological adult reference range. The values of red blood cells, haemoglobin and haematocrit in the local Holstein-Friesian mix breed was lower than Romanian Gray Steppe but the differences were not statistically significant ($p > 0.05$). Also the values of platelets of Romanian Gray Steppe cows were higher compared with the local Holstein-Friesian mix breed group, but differences were not statistically significant ($p > 0.05$).

Table 1. Determination of erythrocyte parameters in Holstein-Friesian mix breed and Romanian Gray Steppe cows

Cows	RBC ($x\ 10^6$ /µL)	Hgb (g/dL)	PCV (%)	MCV (fL)	MCH (pg)	MCHC (%)
H - F	6.19 ± 1.4	8.52 ± 1.05	26.45 ± 3.1	43 ± 3.55	13.9 ± 0.98	32.27 ± 0.23
RGS	6.29 ± 0.51	10.38 ± 0.24	32.4 ± 1.34	51.6 ± 4.44	16.64 ± 1.55	32.1 ± 0.8
Normal values (Kramer, 2000)	5-10	8-15	24-46	40-60	11-17	30-36

H-F - Holstein-Friesian mix breed; RGS - Romanian Gray Steppe

Regarding the leukograms (figure 1), higher values of white blood cells, were observed at the local Holstein-Friesian mix breed (9.1± 0.98 x 10^3/µL) than Romanian Gray Steppe (7.42 ± 1.22 x 10^3/µL), but the differences were not statistically significant ($p > 0.05$). The values of polymorphonuclear leukocytes varied in physiological limits, there were no statistically significant differences ($p > 0.05$) between the two breeds.

Leukopenia with neutropenia and lymphopenia was reported as being possible in cows affected by fatty liver syndrome (Ogilvie, 1998). Pearson and Maas (2002) adds the observation that many cows with this syndrome have leukopenia with a degenerative left shift of Arneth curve, but this issue is not specific to this disease (Tornquist and Rigas, 2010).

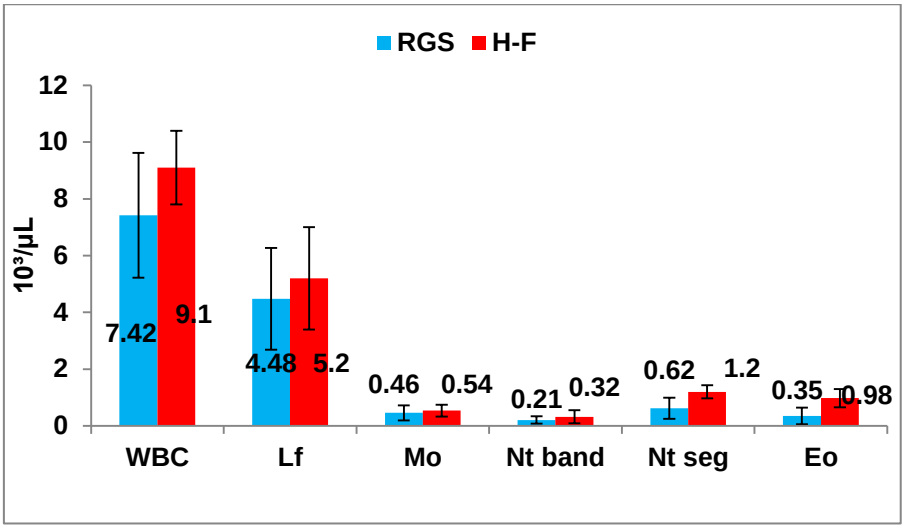

Fig. 1. Differential leucocytes count at Holstein-Friesian mix breed and Romanian Gray Steppe cows
H-F - Holstein-Friesian mix breed ; RGS - Romanian Gray Steppe;
WBC – white blood cells; Lf – lymphocytes; Mo – monocytes;
Nt band – neutrophils band; Nt seg – neutrophils segmented

Regarding biochemical profile (table 2), LDH (1638.4 ± 121.43IU/L), ALT (45.56 ± 0.30IU/L), cholesterol (231.12 ± 2.79 mg/dL) and triglycerides (16.13 ± 2.79 mg/dL) who had values above the upper limit, may indicate deficiency energy, ketosis and lipid mobilization syndrome development in the local Holstein-Friesian mix breed studied. The values were higher than those of Romanian Gray Steppe, but the differences were not statistically significant ($p > 0.05$).

Clinical ketosis in dairy cows usually occurs between the second and seventh week of lactation. Nevertheless, most of cows in this stage of lactation may suffer a subclinical form of ketosis defined as increased blood ketone bodies without any other symptoms but accompanied by considerable decrease in milk yield and susceptibility other diseases (Duffield et al., 1997). Extreme lipid mobilization from adipose tissue exceeds the metabolizing capacity of the liver, leading to an increased accumulation of triglycerides (Herdt, 1988).

Table 2. Determination of biochemical parameters in Holstein-Friesian mix breed and Romanian Gray Steppe cows

Parameters	**H - F**	**RGS**	Normal values
ALT (IU/L)	**45.56 ± 0.30**	28.32 ± 0.23	11-40 (Radostits et al., 2007)
AST (IU/L)	**34.25 ± 4.11**	78.46 ± 8.15	43-127 (Smith, 2009)
LDH (IU/L)	**1638.4 ± 121.43**	987.34 ± 121.43	692-1445 (Radostits et al., 2007)
γGT (IU/L)	21.06 ± 7.25	29.3 ± 4.2	15-38 (Smith, 2009)
Glucose (mg/dL)	**41.33 ± 4.54**	54.8 ± 5.64	45-75 (Radostits et al., 2007)
Amylase (IU/L)	**28.63 ± 11.57**	68.63 ± 9.27	41-98 (Merck Veterinary Manual, 2008)
Tg (mg/dL)	**16.13 ± 2.79**	10.2 ± 1.05	0-14 (Radostits et al., 2007)
Chol (mg/dL)	**231.12 ± 2.79**	89.11± 6.21	62-193 (Merck Veterinary Manual, 2008)
CK (IU/L)	130.2 ± 11.39	126.3 ± 8.52	35-280 (Radostits et al., 2007)
Bilirubin (mg/dL)	0.21 ± 0.02	0.23 ± 0.01	0.01-0.5 (Radostits et al., 2007)

H-F - Holstein-Friesian mix breed; RGS - Romanian Gray Steppe; AST- aspartate aminotransferase; ALT – alanine aminotransferase; γGT – gamma-glutamyltransferase; Chol – cholesterol; LDH - lactate dehydrogenase; Tg – triglycerides; CK – creatine kinase; Chol – cholesterol

During the protocol, no statistically significant differences were noted in the γGT, CK, bilirubin parameters in the local Holstein-Friesian mix breed versus Romanian Gray Steppe cows, remaining within their physiological adult reference range. However, AST, γGT and total bilirubin, are not sensitive indicators of a moderate fatty liver (Ohgi et al., 2005).

In the present study, hypoamylasemie (28.63 ± 11.57 UI/L), hypoglycemia (41.33 ± 4.54 mg/dL) and low AST (34.25 ± 4.11IU/L) may indicates energy deficit, ketosis and lipid mobilization syndrome development in the local Holstein-Friesian mix breed studied. Usually, hypoglycemia is found in the following situations: insufficient food ration in terms of energy or glycogenesis precursors, ketosis with liver damage, starvation and a few days after calving. Serum total proteins values were situated between physiological limits in both breeds of cowsbut near the lower limit. Hipoproteinemie absolute form may occur in liver chronic diseases, malabsorption, nephrotic syndrome, cachexia, endoparasites. Glucose levels may be diminished as well as concentrations of total proteins in fat infiltration into the liver (West, 1990). The affected cows display fat infiltration and degeneration of the liver (Moore and Ishler, 1997). The adipose tissue thus has a critical role in the development of fatty liver and ketosis, because these changes in liver composition and metabolism arise both from

excessive lipolysis in adipose tissue and altered secretion of adipose tissue-derived hormones, which modulate hepatic metabolism (Vernon, 2005).

Conclusions

1. Hemograms showed variations in physiological limits, the differences between breeds were not statistically significant ($p>0.05$).
2. Regarding the leukograms, the higher values were observed at the local Holstein-Friesian mix breed than Romanian Gray Steppe, but the differences were not statistically significant ($p>0.05$).
3. The lower serum concentrations of aspartate aminotransferase, amylase, glucose and the higher values of lactate dehydrogenase, alanine aminotransferase, tryglicerides, cholesterol, than physiological limits, may indicate the evolution of lipid mobilization syndrome in the local Holstein-Friesian mix breed cows.

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References

1. Braun, R.,K., Donovan, G,.A., Tran, T.,Q., Shearer, J.K., Bliss, E,.L., Webb, D.,W., Beede, D.,K., Harris, B .(1987) - Body condition scoring dairy cows as a herd management tool. Comp on Cont Ed Pract Vet 9, F192-F200.
2. Butler, W.,R., Beam S.,W., (1993) - Body condition, days to first ovulation and fertility in lactating dairy cows. J. Anim. Sci., 71: Supl 1, 227.
3. Duffield TF, Kelton DF, Leslie KE, Lissemore KD, Lumsden JH., (1997) - Use of test day milk fat and milk protein to detect subclinical ketosis in dairy cattle in Ontario. Can Vet J , 38, 713-718.
4. Friggens N.C., Ingvartsen K.L., Emmans G., 2004 - Prediction of body lipid changes in pregnancy and lactation. J Dairy Sci; 8, 988-1000.
5. Friggens N.C., Berg P., Theilgaard P., Korsgaard I.R., Ingvartsen K.L., Lřvendahl P., Jensen J., (2007) - breed and parity effects on energy balance profiles through lactation: evidence of genetically driven body energy change, J. Dairy Sci. 90, 5291–5305.
6. Heuer, C., Schukken Y.H., Dobbelaar P., (1999) - Postpartal body condition score and first milk test results as predictors of disease, fertility, production, and culling in commercial dairy herds. J. Dairy Sci. 82, 295–304.
7. Kramer JW. (2000). Normal hematology of cattle, sheep and goats. In: Feldman BF, Zinkl JG, Jain NC, editors. Schalm`s veterinary hematology. 5th edition. Philadelphia: Lippincott Williams and Wilkins, 1075-84.
8. Moore, D. A., Ishler V. (1997) - Managing dairy cows during the transition period: Focus on ketosis. Vet. Med. 92:1061–1072.
9. Ohgi, T. Kamimura S., Minezaki Y., Takahashi M., (2005) - Relationship between fat accumulation in the liver and energy intake, milk fat yield and blood metabolites in dairy cows Animal Science Journal, 76, 549–557.
10. Ogilvie T. H., (1998) - Metabolic disorders: Fat cow syndrome; Primary proteinand energy malnutrition. In: Large animal internal medicine. E.A. Nieginski (ed)1st ed, Williams&Wilkins, Baltimore USA, 220-222.

11. Pearson E.G., Maas J., (2002) - Hepatic lipidosis. In: Large animal internal medicine: diseases of horses, cattle, sheep and goats. B.P. Smith (ed) 3rd ed, Mosby Inc., Missoury USA, 810-816.
12. Radostits O.M., Gay C.C., Blood D.C., Hinchcliff K.W. (2007) - Veterinary Medicine. A textbook of the diseases of cattle, sheep, pigs, goats and horses 10th ed. Saunders W.B. Co.,Philadelphia.
13. Smith B.P. (2009) - Large animal internal medicine 3th. ed. Mosby, London, Philadelphia, Sydney, Toronto.
14. Snedecor, G.W. and W.G. Cochran, (1982) - Statistical Methods. 6th Edn., Iowa State University Press, Ames, USA., 593.
15. Tornquist S.J., Rigas J., (2010) – Interpretation of ruminant leukocyte responses, In: Schalm's veterinary hematology. 6th edition, Blackwell Publishing, Iowa, 307-320.
16. Vernon, R. G., (2005) - Lipid metabolism during lactation: A review of adipose tissue-liver interactions and development of fatty liver. J. Dairy Sci. 72:460–469.
17. West HJ., (1990) - Effect on liver function of acetonaemia and the fat cow syndrome in cattle. Res Vet Sci, 48, 221-227.
18. Zaaijer, D., (2001)- Contrôle des effets de la nutrition sur les caractéristiques de la vache. In : Journée d'étude de la Société Belge Francophone de Buiatrie, 27-30.
19. The Merck Veterinary Manual. Hematologic reference ranges [on line] (2009), URL: http://www.merckvetmanual.com/mvm/index.jps?cfib=htm/bc/ref_00.htm.

EPIDEMIOLOGY OF CORNEAL DISEASES IN DOMESTIC CARNIVORES

Ioana BURCOVEANU, I. BURTAN, Roxana TOPALĂ, L.C. BURTAN, M. FÂNTÂNARIU, S. CIOBANU
Facultatea de Medicină Veterinară, U.Ș.A.M.V. Iași
ioana.burcoveanu@gmail.com

Abstract
Corneal pathology in domestic carnivores summarizes a variety of disorders, which represent real challenges to the veterinary practitioner. It consists in several types of conditions: congenital, traumatic, degenerative, inflammatory and neoplastic. Nevertheless, corneal wounds or ulcers can coexist with uveitis, as well as the persistent pupillary membrane or anterior uveitis can generate modifications to the cornea. Etiologic factors, together with clinical signs specific to each corneal disorder, are extremely important in developing the diagnostic approach. Lacking specialized means, the ophthalmological diagnosis will not be exhaustive, and the treatment will often be a symptomatic one, established by the observers' subjectivism and this way one or some of the pathological components can be omitted. Dogs and cats of any breed, sex or age are predisposed to developping one or even more corneal disease during their lifetime. This paper presents the epidemiological data gathered during a 4 year-period, on the number of domestic carnivores submitted for ophthalmology consultations.

Key words: *corneal pathology, domestic carnivores, epidemiology*

Introduction

Corneal pathology in domestic carnivores summarizes a variety of disorders, which represent real challenges to the veterinary practitioner (3, 5, 6). On one side, he needs to recognize the symptoms and to set, when possible, the etiologic diagnosis, and on the other side, he has to establish local therapy which should not only cure the affection, but also avoid complications, which endanger ocular health and most of all, the vision of pets.

Corneal pathology consists in several types of conditions: congenital, traumatic, inflammatory and neoplastic (3, 6). Nevertheless, corneal wounds or ulcers can coexist with uveitis, as well as the persistent pupillary membrane or anterior uveitis can generate modifications to the cornea.

Literature reviews offer data on corneal diseases that are related to specific disorders, such as indolent ulcers in dogs (1, 3), feline corneal ulcers (4), keratoconjunctivitis sicca or pannus (3), feline corneal sequestra (2, 7).

Etiologic factors, together with clinical signs specific to each corneal disorder, are extremely important in developing the diagnostic approach (3, 6). Lacking specialized means, the ophthalmological diagnosis will not be exhaustive, and the treatment will often be a symptomatic one, established by the observers' subjectivism and this way one or some of the pathological components can be omitted.

The purpose of the scientific approach, represented by this paper, focuses on establishing the relative frequency of the corneal diseases, by presenting the causes for the most frequent disorders in the studied area.

Material and method

The research was undertaken over a period of 4 years, October 2007 - September 2011, on a number of cases submitted to the Surgical Clinic at the Faculty of Veterinary Medicine, Iasi.

For a complex study regarding the epidemiology of corneal disorders in domestic carnivores, there were also used the records of the other Clinics (Internal Medicine, Obstetrics, Parasitology, Infectious Diseases) at the FVM in Iași. By working with a population of domestic carnivores spread on a quite large area, it becomes difficult to estimate the incidence and prevalence of the corneal and uveal tract diseases, because not all sick animals are taken to the veterinarian. That is why these two terms were replaced with relative frequency, which refers only to the presented cases.

In order to undergo an epidemiological study as complete as possible, an *Ophthalmic Observation Sheet* was conceived for pets with ocular diseases.

The epidemiological study permitted to establish the relative frequency of corneal diseases and, by correlating it with different intrinsic factors (animal species, breed, age, sex) and extrinsic (living environment), only where this aspect was relevant to the present study.

Results and discussion

Analysing *table 1*, we notice that from the total number of submitted for consultations, over the 4 year-period, 1504 animals suffered from ophthalmic diseases, located at one structure of the eye.

When we reffer to the species, we can see that the percentages are higher in dogs (1020 animals, 67,82%), than in cats (484 cats, 32,18%).

Table 1. Relative frequency of ocular diseases in domestic carnivores between 2007-2011

Location	**Animals submitted for consultations**	**From which with ophthalmic disorders**		**From which**			
				Dogs		Cats	
		Number	*%*	**No**	*%*	**No**	*%*
Faculty of Veterinary Medicine, Iași	**9651**	**1504**	*15,58*	**1020**	*67,82*	**484**	*32,18*

The results of the epidemiologic investigation revealed the fact that the species does not obviously influence the relative frequency of corneal diseases, as the latter recorded almost equal percentage for the two populations: 21,47% in dogs and 20,24% in cats (*table 2*).

The traumatic injuries (corneal wounds: superficial, deep or penetrating) or their consequences (simple or adherent leukoma) occupy first place in canine corneal pathology, with 31,96%. On the second place come corneal ulcers, with 26,02%, followed by chronic keratitis, that is 18,26% of all dogs. These traumatic lesions take the first place among corneal pathology in cats (51,02%), followed by herpetic keratitis including symblepharon (with 25,52%) and corneal ulcer with 14,28% (*table 3*).

Table 2. General ophthalmic (eye and adnexa) pathology in domestic carnivores between 2007-2011

Species / **Disorder**	**Dogs**		**Cats**		**Total no. of disorders**	
	Number	***%***	**Number**	***%***	**Number**	***%***
Adnexa: *orbit, eyelids, conjunctiv*	545	*53,43*	250	*51,66*	**795**	*52,85*
Cornea, sclera	**219**	*21,47*	**98**	*20,24*	**317**	*21,08*
Uveal tract	**100**	*9,82*	**43**	*8,88*	**143**	*9,52*
Retina	67	*6,56*	30	*6,2*	**97**	*6,44*
Lens	89	*8,72*	63	*13,02*	**152**	*10,11*
Total no. of animals	**1020**	***100***	**484**	***100***	**1504**	***100***

Table 3. Relative frequency of corneal disease in dogs

Corneal disorders in dogs	**Cases**	
	Number	*%*
Corneal dermoid	**2**	*0,92*
Traumatic injuries: wounds, corneal staphyloma, simple or adherent leukoma	**70**	*31,96*
Dystrophies	**25**	*11,46*
Keratoconjunctivis sicca	**16**	*7,3*
Corneal ulcers	**57**	*26,02*
Pannus	**8**	*3,66*
Chronic keratitis	**40**	*18,26*
Primary corneal tumors	**1**	*0,44*
Total	**219**	*100*

Superficial ulcers were most frequent among the canine population (*table 4*), with 54,38%, followed by deep, complicated ones, with 28,08%. The most frequent cause of corneal ulcers in dogs (*table 5*) was the traumatic exogenous one, totalizing up to 47,36 percents, followed by the endogenous trauma – entropion (26,31%) or keratoconjunctivitis sicca (KCS) (8,78%). In cats (*table 4*), superficial ulcers were most frequent (57,14%), followed by the profound ones (35,72%). The most frequent cause for corneal ulcerations was represented by traumas, in 64,28% of cases, followed by the viral one (Feline herpes virus 1), with 21,44% (*table 5*).

Table 4. Relative frequency of different types of corneal ulcers in dogs and cats

Ulcer type	**Corneal ulcers in dogs**		**Corneal ulcers in cats**	
	Number	*%*	**Number**	*%*
Superficial	**31**	*54,38*	**8**	*57,14*
Indolent	**6**	*10,52*	**-**	-
Deep, infected	**16**	*28,08*	**5**	*35,72*
Descemetocele	**4**	*7,02*	**1**	*7,14*
Total	**57**	*100*	**14**	*100*

Table 5. Causes of corneal ulcers in dogs and cats

Causes of corneal ulcers	**In dogs**		**In cats**	
	No.	*%*	**No.**	*%*
Traumatic exogenous: scratches (cat claw, plants)	**27**	*47,36*	**9**	*64,28*
Traumatic endogenous: entropion	**15**	*26,31*	**-**	-
Traumatic endogenous: ectopic cilia	**3**	*5,26*	**1**	*7,14*
Anomalies of the tear film: KCS	**5**	*8,78*	**-**	-
Epitheliotrope virus: Feline Herpes Virus type I	**-**	-	**3**	*21,44*
Carre disease virus (Morbilivirus)	**2**	*3,48*	**-**	-
Long term general treatment with steroidal antiinflamatory drugs (ex: dexamethasone)	**5**	*8,78*	**1**	*7,14*
Total	**57**	*100*	**14**	*100*

As for corneal diseases specific to dogs, our researches revealed epidemiologic data about pannus and KCS. Therefore, most animals (62,5%) brought for consultation for chronic superficial keratitis belonged to this breed (German shepherd) and only 37,5% were half breeds of it. The average age of occurrence was 3-5 years, the majority of the sick animals (75%) being females (*table 6*).

Table 6. Correlation between endogenous factors of pannus in German shepherds

Cases	**Endogenous factors**								
	Breed		**Gender**		**Age (in years)**				
	Pure	Mixed	F	M	1-3	3-5	5-7	7-9	>9
Number	5	3	6	2	-	5	1	1	1
%	*62,5*	*37,5*	*75*	*25*	-	*62,5*	*12,5*	*12,5*	*12,5*

The subjects belonging to the Pekingese breed represented 56,25 percent of KCS affections (*table 7*), followed by Cockers (31,25%). Of all these, intact females were most frequently affected, with 37,5%.

Table 7. Breed and sex disposition to KCS in dogs

Breeds affected by KCS	**Cases**		**From which females**				**From which males**			
			Intact		Spayed		Intact		Neutered	
	Nr.	*%*	**Nr.**	*%*	**Nr.**	*%*	**Nr.**	*%*	**Nr.**	*%*
Pekingese	**9**	*56,25*	**5**	*31,25*	**2**	*12,5*	**-**	-	**2**	*12,5*
Cocker	**5**	*31,25*	**1**	*6,25*	**2**	*12,5*	**1**	*6,25*	**1**	*6,25*
Pug	**1**	*6,25*	**-**	-	**-**	-	**1**	*6,25*	**-**	-
Common breed	**1**	*6,25*	**-**	-	**-**	-	**-**	-	**1**	*6,25*
Total	**16**	*100*	**6**	*37,5*	**4**	*25*	**2**	*12,5*	**4**	*25*

The most frequent cause was represented by the senile degeneration of the lacrimal gland, which stopped the normal tear production, for 31,25% of the subjects. The same percent of animals was offered by traumatic KCS and 25% were diagnosed with KCS secondary to the Carré disease. The congenital (or hereditary) cause occupies the last place in our classification, with 12,5% (*table 8*).

Table 8. Causes of KCS in dogs

Causes	**Cases**	
	No.	***%***
Congenital/hereditary	**2**	*12,5*
Age	**5**	*31,25*
Traumatic	**5**	*31,25*
Infectious (Carré disease virus)	**4**	*25*
Total	**16**	*100*

We diagnosed feline corneal sequestra (*table 9*) exclusively in Persian breed cats, the majority being females (83,34%), with the average occurrence age of the specific symptoms of 2 years and 2 months. The lesions were bilaterally located in just one case, for the other cases the manifestation was of 66,67% for the right eye and 16,67% for the left eye.

Table 9. Correlation between endogenous factors of feline corneal sequestra

Cases	Endogenous factors						
	Breed	**Gender**		**Age (in years)**			
	Persian	F	M	1-3	3-5	5-7	7-9
Number	6	5	1	2	3	-	1
%	*100*	*83,3*	*16,7*	-	*62,5*	*12,5*	*12,5*

Conclusions

1. The epidemiologic researches undertaken during 2007-2011, by consulting 1504 domestic carnivores with ophthalmologic diseases, reveal the relative frequency of corneal affections, which registered 21,47% in dogs, and 20,24% in cats. As for the uveal tract pathology, the relative frequency was 9,82% in dogs and 8,88% in cats.
2. The breeds which gave high percentages of animals with keratopathies in dogs were the common breed (14,62), followed by the half-breeds of different breeds (13,24) and Pekingese dogs (10,5). As for cats, 81,64% of the subjects which presented corneal diseases belonged to the European breed, while the Persian, the Siamese and Birman breeds were poorly represented.
3. The different percentages do not encourage us to affirm a breed predisposition, as the frequency is correlated to the breed predominance in the studied area.
4. Traumas (corneal wounds – superficial, deep, penetrating) or their consequences (simple or adherent leukoma) occupied the first place in corneal pathology in both canine and feline populations, mostly due to their way of life, which exposes them to trauma (dogs living with one or more cats, stray animals, etc.).
5. Corneal ulcers show up on second place in dogs and the third in cats, after herpetic keratitis. The most frequent cause for corneal ulcers was the exogenous traumatic one.
6. The most affected breed by keratoconjunctivitis sicca was the Pekingese breed, followed by the Cocker and the common breed. Of all these, most frequently affected were the intact females, followed by sterilized females and castrated males, without being able to establish a sex or reproductive status predisposition.
7. Most animals presented for consultation with chronic superficial keratitis (pannus) were German Shepherd females. The average occurrence age was 4 years.
8. The feline corneal sequestrum was diagnosed exclusively in Persian cats, most of them being females, the mean occurrence age for the specific symptoms being 2 years. Lesions were seen bilateral in one case, the rest of the animals showing signs of disease in either the right or left eye.

References

1. Bentley Ellison, 2005- *Spontaneous Chronic Corneal Epithelial Defects in Dogs: A Review*, Journal of the American Animal Hospital Association, 41:158-165;
2. Featherstone J. Heidi, Sansom Jane, 2004 - *Feline corneal sequestra: a review of 64 cases (80 eyes) from 1993 to 2000*, Veterinary Ophthalmology, 7, 4, 213-227, available on-line at http://onlinelibrary.wiley.com;

3. Gilger B.C., Bentley Ellison, Ollivier F.J., 2007 – *Diseases and Surgery of the Canine Cornea and Sclera chapter*, in Gelatt K.N. Veterinary Ophthalmology, 4th edition, Blackwell Publishing, Iowa;
4. Hartley Claudia, 2010 – *Aetiology of corneal ulcers. Assume FHV-1 unless proven otherwise. Clinical review*, Journal of Feline Medicine and Surgery, 12, 24-35;
5. Ionaşcu Iuliana, Militaru Manuella, Ciobotaru Emilia, Dinescu Georgeta, Soare T., 2008 - *General overview in ocular lesions of dog*, ESVP 26th Annual Meeting, Programme and Book of Abstracts, Dubrovnik Croaţia, p. 117;
6. Maggs D., 2008 – *Cornea and sclera*. In: Slatter's Fundamentals of Veterinary Medicine, 4th edition, Saunders Elsevier, Missouri;
7. Moore P.A., 2005 – *Feline corneal disease*, Clinical Techniques in Small Animal Practice, 20:83-93.

THE USE OF ENDOSCOPIC EXAMINATION IN THE DIAGNOSIS OF GASTROINTESTINAL DISEASE IN DOGS

R. MALANCUŞ, Gh. SOLCAN, Cristina Maria MALANCUS
FMV Iasi
razvanmalancus@gmail.com

Abstract

The endoscopic examination is a complementary diagnostic method that is used as a final stage when the observation of the gastrointestinal tract is implied. Beside its use as a diagnostic method, the endoscopy also has a therapeutic value, helping removing foreign bodies or different obstructions from the gastrointestinal tract. The current study revealed that within 72 patients 19 of them presented foreign bodies, 59 presented inflammatory disease and in 6 cases were observed neoplasic processes. It must be said that some of these disorders evolve simultaneously , both the presence of foreign bodies and of neoplasia being accompanied by gastric or intestinal inflammatory disease.

Keywords: inflammatory disease, neoplasia, foreign bodies, endoscopy

Introduction

The endoscopic examination is a complementary diagnostic method that is being used as a final stage of diagnosis when the gastrointestinal tract observation is implied.

Beside its use as a diagnostic method, the endoscopy also has a therapeutic value, helping removing foreign bodies or different obstructions from the gastrointestinal tract.

It`s main purpose is to assess the lesions within gastric and intestinal lumen, to gather sample for further analysis and to interpret all the obtained data in order to state a diagnosis.

Matherial and method

The research took place in Small Animal Teaching Hospital, Leahurst, University of Liverpool, where the patients were firstly clinically assessed, afterwards being involved paraclinical diagnostic tools such as hematology, biochemistry, ultrasonography, radiology or endoscopy in order to provide further information regarding the case and to state a diagnosis.

In our study the endoscopic examination presented the utmost interest, gathering data and interpreting it being the main goal of the paper.

The investigation was made on a number of 72 canine patients presented with chronic gastrointestinal disease non-responsive to treatment. The examination took place in special rooms designed for endoscopic purposes, provided with anesthetic machines and all the endoscopic tools necessary for intervention. The system used was an Olympus system with CV 240 video system, Olympus CLV U40 light source and different types of probes depending on the purpose of examination. For gastroduodenoscopy 2 different Olympus probes were used: GIF XP 240 and GIF XP 260 whereas for colonoscopy the Olympus SIF probe was the examination tool. All three probes had 2 mm working channels used for foreign body retrievals and air suction and also presented channels for water insertion and air insufflation.

The main endoscopic investigation took only 30 to 60 minutes depending on patient but the preparation for this kind of intervention is much longer with previous operations such as food diet, enema and anesthesia induction starting with 24 hours prior to endoscopy – food diet – or in the morning of examination for the other two operations.

Results and discussions

The main pathological processes diagnosed were represented by the presence of foreign bodies, inflammatory disorders and neoplasic disease, most of them evolving simultaneously.

The most exciting part of endoscopy is the removal of foreign bodies, a very colorful pathology, where a wide area of objects can be observed the challenging task being to pull them out.

In figure 1 can be observed the aspect of foreign bodies presence, with the foreign body in the center of the image and the forceps trying to retrieve it. Some irritations can be seen on the surface of mucosa, a severe hyperemia being observed near pylorus.

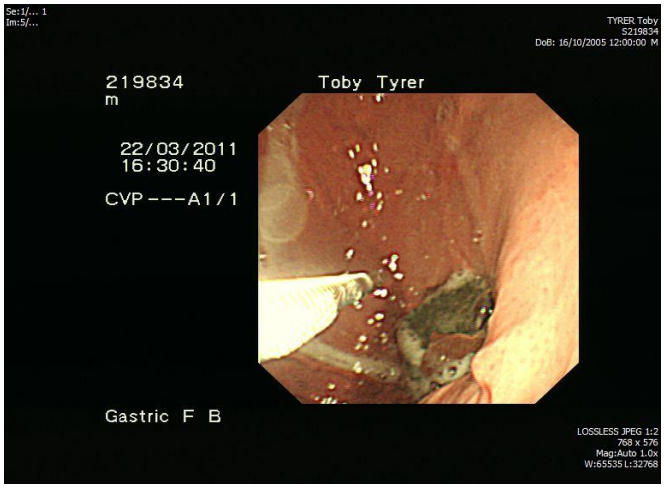

Fig. 1. Foreign body, represented by a piece of wood

The irritative action of the wood piece produces small lesion near pylorus and is responsible for the expressed symptomatology: chronic vomiting, inappetence and weight loss.

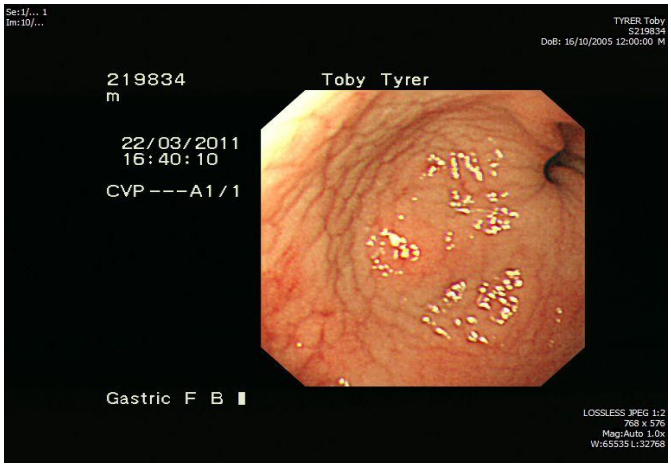

Fig. 2. Hyperemia and ulcerative areas – pyloric region

In figure 2 can be seen a small ulcerative area having the same cause represented by the presence of foreign body. The presence of foreign bodies has been diagnosed in 19 patients, surprisingly large number and also surprisingly by the nature of foreign body (battery, toys, etc.).

The lesions produced by inflammatory processes are very common diagnosed when taking endoscopies, inflammation developing primary or secondary to neoplasia or presence of foreign bodies. Lesions characteristic to inflammatory disease has been discovered in 59 cases.

A severe case of inflammation can be seen in figure 3 where a diffuse hyperemia covers the whole mucosa with small red points throughout the fundus of the stomach. The lesions are well defined, the diagnosis of gastritis being easy to state. In the same image can be observed pathological aspect of mucosa, with loss of rugal folds and oedema.

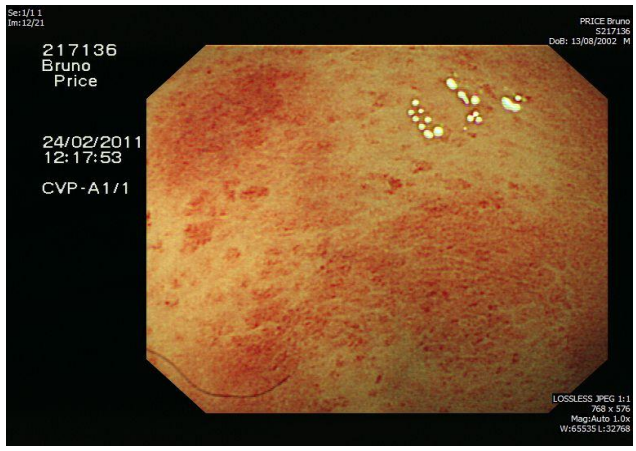

Fig. 3. Gastritis - Severe hyperemia

A particular aspect of inflammation can be present in the small bowel, in duodenum, where the lesions of villi are consequent to inflammatory disorders.

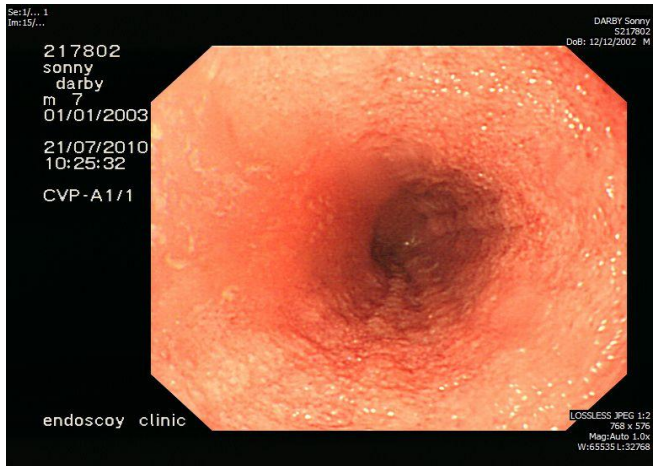

Fig. 4. Lymphangiectasia

In figure 4 the villi appears visible, shiny, glove fingers like, this aspect being well defined when inserting a large amount of water through the water channel.

The presence of neoplasia can be distinguished both in stomach and colon, in the first case observing the aspect of gastric carcinoma, diagnosed after histopathological analyses and in the second patient, the aspect of colonic multiple benign polyps.

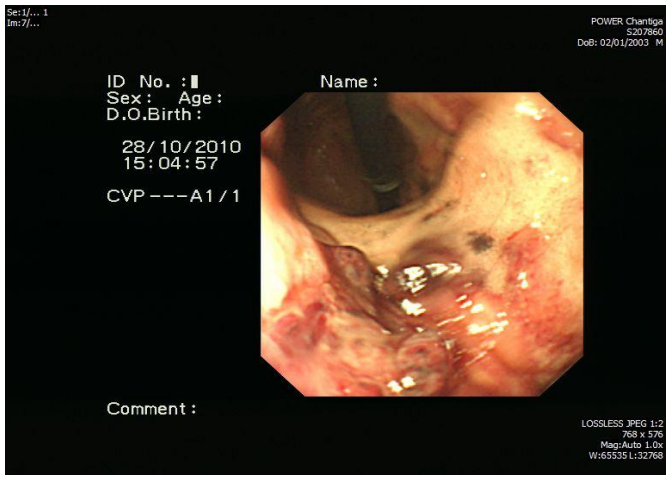

Fig. 5. Gastric carcinoma – Hyperemia and oedema of gastric mucosa

In figure 5 the endoscopic aspect of gastric carcinoma is revealed with ulcerative areas, generalized hyperemia, oedema of the mucosa and also necrotic lesions at the same level.

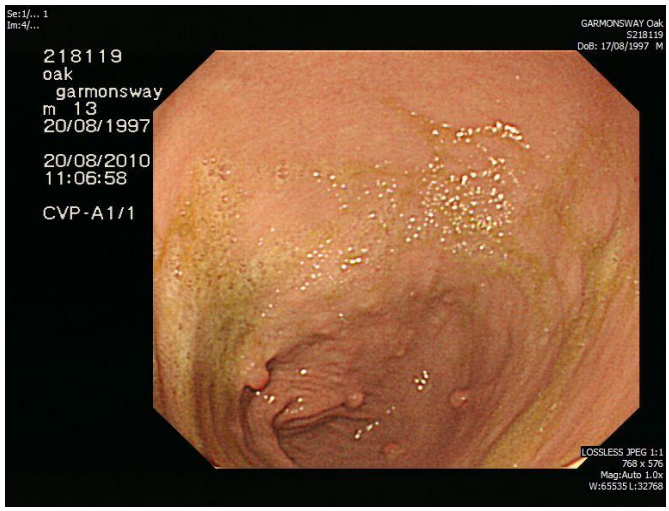

Fig. 6. Multiple benign polyps

In figure 6 can be seen the presence of polyps in colon, these being involved in the appearance of carcinoma. The polyps are well defined structures, tied to mucosa, circular in shape and can produce large hemorrhagic lesions if unproperly removed.

The neoplasic processes have been diagnosed in 6 cases, 3 with carcino and 3 with lymphoma.

After this study, can be stated that endoscopy provides enough information to state a diagnosis and also provides therapeutic means to cease the symptomatology of different cases. The use of endoscopic investigation is the fulfillment of other imaging methods such as ultrasonography or radiology where just part of data can be gathered.

All these three imaging methods ease the diagnosis of gastrointestinal disease and also make use of every possible information to assess this kind of patients.

Conclusions

1. The endoscopic examination is a complementary diagnostic method that is being used as a final stage of diagnosis when the gastrointestinal tract observation is implied;
2. The main purpose of endoscopy is to assess the lesions within gastric and intestinal lumen, to gather sample for further analysis and to interpret all the obtained data in order to state a diagnosis;
3. The investigation was made on a number of 72 canine patients presented with chronic gastrointestinal disease non-responsive to treatment;
4. Out of 72 patients, 19 presented foreign bodies, 59 were diagnosed with inflammatory disease and 6 with neoplasia, many of these evolving simultaneously.

Bibliography

1. Leib M.S., 1990 - *Colonoscopy. In: Tams TR (ed.): Small Animal Endoscopy*, St Louis: C.V. Mosby, 211-244;
2. Matz M.E., 2004 - *Endoscopic and cytological procedures for evaluation of the gastrointestinal tract. In: Ettinger SJ, Feldman EC (eds): Textbook of Veterinary Internal Medicine: Diseases of the Dog and Cat, 6th ed.,* Philadelphia, Elsevier Saunders, 374-377;
3. Mălăncuș R.N**.**, Gh. Solcan, Cristina Tofan (Malancus), 2010 - *The use of ultrasonography in gastrointestinal disease in dogs*, Lucr. Stiintifice USAMV Iasi, seria Medicina Veterinara vol 53, pag. 772-777.
4. Tams T.R., 1999 - *Small Animal Endoscopy. 2nd ed.,* Mosby, St. Louis.

PRODUCTION OF ANTIBODIES AGAINST β-LACTAMASE ENZYMES ISOLATED FROM ANTIBIOTICS RESISTANT *ESCHERICHIA COLI*

A. A. ALHUMIANY
Department of Medical Microbiology, Faculty of Applied Medical Sciences,
Taif University, Turaba, KSA. alhumiany@hotmail.com

Abstract
Antibiotic resistant bacteria considered as a dangerous threat for both human and animal life and may be result in evolving of emergent pathogens specifically in β-lactams antibiotics resistant E. coli. The current research aims to isolate and purify β-lactamases and to produce antibodies against β-lactamases resistant E. coli to be used as a prophylactic agents against the resistant isolates of E. coli infection. Twelve E. coli isolates were tested for antibiotic resistance. Two E. coli β-lactam antibiotics resistant isolates were inoculated and β-lactamases production was enhanced by addition of ampicillin antibiotic. The produced enzymes were isolated and purified as confirmed by electrophoresis. Four groups of rabbits were injected with these enzymes. Standard enzyme and a control groups were run parallel with the tested groups, then serum was separated and characterized. The produced serum was confirmed to be against β-lactamases as indicated by immunoblot assay. The produced anti β-lactamases serum was used as a trial to solve the antibiotic resistance in vitro by minimum inhibitory concentration test (MIC), dropping in MIC was noticed after using the serum.
Key words: Antibiotic resistance. Escherichia- coli. β-lactamases. MIC test. Sensitivity test

Introduction

Antibiotic rresistance may be defined as the ability of a microorganism to resist the action of antimicrobial agents at concentrations achievable in the body after normal dosage. Resistance to antibiotics may be evolved due to misuse in clinical treatment [1, 2]. In recent years, resistance to β-lactam antibiotics, such as the widely-used cephalosporins and penicillins, has become a major challenge for disease therapy, particularly in common hospital-acquired infections. In the search for the mechanisms behind this increasingly prevalent form of resistance, microbiologists have identified a new type of β-lactamase enzyme, called inhibitor-resistant TEMs (IRTs), which can withstand the effects of β-lactamase inhibitor compounds [3-5]. β-lactamases are the major defence used by bacteria to overcome the effects of penicillins, cephalosporins and related β-lactam antibiotics [6, 7]. β-lactamase production is mediated by genes carried on either plasmid or on the chromosome and more than one type may be produced by the same species at the same or at different times [8]. There are four main types or classes of β-lactamase enzymes. Class A β-lactamases (30 KD) have a conserved serine at the active site and is believed to utilize an acyl-enzyme reaction pathway. Class B β-lactamases are zinc-dependent enzymes and its molecular weight 25 KD. Class C enzymes are serine enzymes that have a molecular weight of about 39 KD. Class D which includes OXA β-lactamases (25 KD) were long recognized as a less common but also plasmid-mediated β-lactamase variety that could hydrolyze oxacillin [9-11]. There is no apparent sequence homology with either class A or C β-lactamases. The metal binding involves three histidines and a single cysteine residue, shown to be absent in the other β-lactamases [8, 9], and are inhibited by the same inhibitors as the class A enzymes, but they have a substrate specificity and amino acid sequence very different from that of class A enzymes [11-13]. The purpose of this study is to isolate and purify β-lactamases enzymes from antibiotics resistant *E. coli* and to develop antibodies against these enzymes.

Material and methods

Bacterial strains: Twelve strains of *E. coli* previously isolated from human (faeces and urine samples), clinical specimens from persons suffering from diarrhoea and urinary tract infection were identified by IMVC test and by PCR, were generously provided by the department of medical microbiology, Faculty of applied medical sciences, Taif University, KSA.

Chemicals: Ammonium sulphates extra pure (Hi Media laboratories), dialysis bag, phosphate buffer saline (PBS), sephadex G. (Sigma Aldrich, St. Louis, MO), gel filtration column (Sigma Aldrich, St. Louis, MO), Sodium dodecyl sulphate solution (SDS 10%), ammonium persulphate solution (APS 10% W/V), electrode buffer solution (2x), loading buffer (2x), staining solution of Commassie blue.

Animals, Media and antibiotic discs: Twelve New Zealand white female rabbits weighing approximately 1.5 kg were kept under the standard hygienic conditions. The rabbits were grouped into four groups each group contain three rabbits. The first group of rabbits was injected with standard TEM Sigma Aldrich, St. Louis, MO), second group was injected with purified extract from H1 third group was injected by purified extract from H2 and the fourth group served as negative control**.**

Media and antibiotic discs: Mueller-Hinton agar medium and commercial antimicrobial discs were from Oxoid, Wade Road Basingstoke, Hampshire, RG24, 8PW UK. The antibiotics tested at concentration of microgram (μg) and they include: amoxicillin (25 μg), cefotaxime (30 μg), gentamicin (10 μg), enrofloxacin (10 μg), oxacillin (1 μg), amoxicillin/clavulanic acid (30 μg) and ampecillin/sulbactam (30 μg), trimethoprim/ sulfamethazole (25μg) and 0.5 McFarland turbidity standard.

Methods

Production and purification of β-lactamases E. Coli:

Two isolates (H1 and H2) of *E. Coli* (contain TEM, OXA and SHV [14] were isolated from patients and used for production of β-lactamases. β-lactamases were isolated and purified as described previously [15-18]. The isolates were inoculated into 1.2 ml of tryptic soya broth containing 100 mg/L ampicillin, incubated at 37°C for 48 hour (hr) and 20% of ammonium sulphate solution was added for precipitation of beta-lactamases. The solution was kept at 4°C for 48 hr and then centrifuged at 8,000 rpm for 30 minute to pellet the bacterial cells. After centrifugation, the pellet was removed and the supernatant was harvested, then ammonium sulphate saturation of the supernatant was raised to 50% according to equation which was described by [19]. Then, solution was pelleted at 48,000 rpm for 10 minutes and the supernatant was discarded. The pellet was re-suspended in 10 ml PBS (pH 7.2) and dialysed against same buffer for 72 hr at 4°C with steering at 50 rpm. Dialysed buffer was changed every 24 hr. After dialysis, the extracted solution was purified by gel filtration chromatography with sephadex G75. The purification was confirmed by running 10 μl of the purified mixture in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to determine the molecular weight.

Polyclonal antibody production against β-lactamases (TEM, SHV and OXA)

Polyclonal antibodies were prepared against β-lactamases in New Zealand rabbits ***(20)***. The first group of rabbits was injected with standard TEM (Sigma Aldrich, St. Louis, MO), second group was injected with purified extract from H1, third group was injected by purified extract from H2 and the fourth group served as negative control. The rabbits in each group were

initially inoculated with equal amount (1 ml) of complete Freund's adjuvant (CFA) and the purified concentrated β-lactamases (500 μg) in multiple sites intramuscular (i.m) and subcutaneously (s.c) except the control group which received equal amount of CFA and PBS in multiple sites i.m and s.c. Three booster inoculations of equal amounts of incomplete Freund's adjuvant (ICFA) and the purified β-lactamases (500 μg) were inoculated i.m and s.c at one week intervals. A final poster dose of the purified β-lactamases was injected intravenously to the first three groups. Antibody production was monitored before each inoculation and after bleeding by double immunodiffusion [21]. Two weeks after the last injection, the rabbits were bled and the sera harvested after centrifugation at 3500 rpm for 15 min at 4ºC. Serum was aliquoted and then stored at -20ºC.

Western blot analysis or detection of anti-β-lactamases specific polyclonal antibodies

Western blot analysis was done as described previously [22]. Briefly, the protein level of the purified and the standard (β-lactamases was quantitated by spectrophotometer and approximately 50 μg of the protein was resolved in 10% SDS-PAGE. Subsequently, the resolved proteins were transferred onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH). A 5 ml of 5% non fat dry milk was added to each membrane followed by incubation at room temperature for 2 hr on rotor. After incubation, 5 ml of diluted (1:300) rabbit polyclonal antibodies against β-lactamases was loaded over each nitrocellulose membrane, and then incubated for 45 minutes at room temperature with rotor. The membrane washed 3 times each for 5 minutes in PBS with shaking for 5 minutes in each wash.. After washing, alkaline phosphatase conjugated goat anti-rabbit (Sigma, St. Louis, MO) at dilution of 1:6000 were used to detect the antigen-antibody complexes. The sizes of the proteins on SDS-PAGE were estimated by using Amersham molecular weight markers, 10-100 KD, (Amersham).

Results

Sensitivity test was done to the twelve *E. coli* isolates and the result revealed that out of 12 *E. coli* isolates: 4 isolates were sensitive to enrofoxacin (33.3%), 5 isolates were resistant to cefotaxime (41.6%), 6 isolates were sensitive to cefotaxime (50%), and 1 isolate was intermediate sensitive (8.3%). In the meantime, out of the twelve isolates, 11 isolates were resistant to trimethoprim/ sulfamethazole (91.6%), and 1 isolate was sensitive (8.3%). It has been also found that 11 isolates of the twelve were resistant to amoxicillin/clavulanic acid by 91.6%, while 1 isolate was sensitive (8.3%). Regarding the gentamicin, 3 isolates were sensitive to gentamycin (25%), while 9 isolates were resistant (75%). Interestingly, all isolates were 100% resistant to amoxicillin, oxacillin and to ampicillin/sulbactam (Table 1).

Minimum inhibitrory concentration test also was done to the twelve *E. coli* isolates to amoxicillin/clavulanic acid (μg/ml) and the results revealed that out of 12 *E. coli* isolates one isolate (H3) was sensitive (8.3%). Eleven isolates from twelve showed high MIC value (91.7%). E. coli (code No H4) was resistant (32μg/ml), sample H2,H5 and H9 also was resistant (64 μg/ml), samples H1,H6 and H11 was resistant (128 μg/ml), also samples with code number H12, H10 and H7 were resistant (256 μg/ml) and finally one sample showed high MIC (512 μg/ml). (Table 2)

Table 1: Antibiogram of 12 *E. coli* isolates

Strain	EF	CTX	SXT	AMC	G	AX	OX	AS
H1	-	26	-	-	-	-	-	-
	R	S	R	R	R	R	R	R
H2	-	28	-	-	18	-	-	-
	R	S	R	R	S	R	R	R
H3	-	-	-	20	-	-	-	-
	R	R	R	S	R	R	R	R
H4	20	24	-	-	18	-	-	-
	S	S	R	R	S	R	R	R
H5	22	-	-	-	-	-	-	-
	S	R	R	R	R	R	R	R
H6	-	-	-	-	-	-	-	-
	R	R	R	R	R	R	R	R
H7	-	26	22					
	R	S	S	R	R	R	R	R
H8	22	22	-	-	-	-	-	-
	S	S	R	R	R	R	R	R
H9	-	-	-	-	-	-	-	-
	R	R	R	R	R	R	R	R
H10	20	24	-	-	17	-	-	-
	S	S	R	R	S	R	R	R
H11	-	18	-	-	-	-	-	-
	R	I.M	R	R	R	R	R	R
H12	-	-	-	-	-	-	-	-
	R	R	R	R	R	R	R	R

H= Human, R= resistant, I.M= intermediate, S= sensitive, Ef= enrofoxacin,
CTX= cefotaxime, SXT= trimethoprim/sulfamethazole,
AMC= amoxicillin/clavulanic acid, G= gentamycin, AX=amoxicillin,
OX= oxacillin and AS= ampicillin/sulbactam

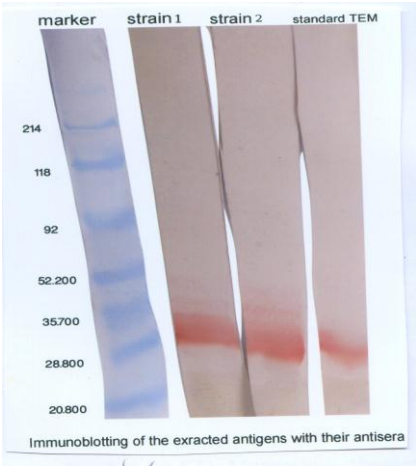

Fig. 1. SDS–PAGE of TEM and SHV β-lactamases extracted from *E. coli* strain H1

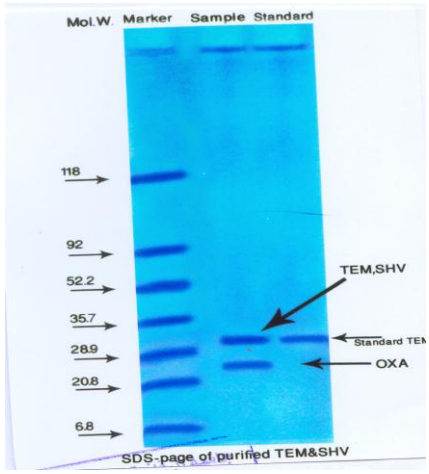

Fig. 2. Immunoblot neutralization between β-lactamases and antiserum

Table 2. Minimum Inhibitrory Concentration of (MIC) of 12 E-coli isolates to Amoxicillin/Clavulanic acid (µg/ml).

Sample code	2	4	8	16	32	64	128	256	512
H1	R	R	R	R	R	R	S	S	S
H2	R	R	R	R	R	S	S	S	S
H3	R	S	S	S	S	S	S	S	S
H4	R	R	R	R	S	S	S	S	S
H5	R	R	R	R	R	S	S	S	S
H6	R	R	R	R	R	R	S	S	S
H7	R	R	R	R	R	R	R	S	S
H8	R	R	R	R	R	R	R	R	S
H9	R	R	R	R	R	S	S	S	S
H10	R	R	R	R	R	R	R	S	S
H11	R	R	R	R	R	R	S	S	S
H12	R	R	R	R	R	R	R	S	S

Discussion

Antibiotic resistant is highly dangerous problem that threats both animal and human health, *E. coli* is a common inhabitant of human and animal gut and it is considered an indicator of fecal contamination in food. β-lactams antibiotics are widely used in human and veterinary medicine**.** Concerning SDS-PAGE results for determination of the molecular weights of the purified β-lactamase TEM, SHV and OXA (Fig. 1). strain H1 secret soluble extracellular TEM and SHV β-lactamase give one band at 30 KD, similar study indicated that SDS-PAGE of purified TEM-1 and SHV-1 from *E. coli* strain give one band at 30 KD [17]. Meanwhile, other study [21] found that the molecular weight of TEM and SHV ranged

around 25 KD on SDS- PAGE electrophoresis. This difference may be due to the use of sepharose 4B which yield <70% recovery of β-lactamase. Concerning the immunoblotting (Fig. 2), the obtained results revealed that one band formed at molecular weight of 30 KD between β-lactamase antigen with its specific antiserum indicating that the produced antibodies are β-lactamase specific. These results agreed with those reported that one bands appear at size of 30 KD after SDS-PAGE electrophoresis [22].

Concerning the antimicrobial supportability patterns, cefotaxime was the effective drug among the tested antichemotherapeutics. It inhibited 50% of all tested in isolates, then enrofoxacin which only inhibited 33.3% of all tested isolates, then gentamicin, which inhibited 25% of all tested isolates. Meanwhile, amoxicillin/ clavulanate and trimethoprim/ sulfamethazole, inhibited 8.3% of all tested isolates. In contrast, amoxicillin, oxacillin and ampicillin/ sulbactam had no effect. Similar results were reported [7] which showed that *E. coli* isolates were susceptible to cefotaxime. Comparable results were also reported [4] in another study which found that a total number of 72 isolates of *E. coli* were susceptible to cefotaxime (55%) and lower percentage (45%) was recorded [15].

Concerning the results of minimum inhibitrory concentration test of the twelve E. *coli* isolates to amoxicillin/clavulanic acid (μg/ml). our results revealed that out of 12 *E. coli* isolates one isolate (H3) was sensitive (8.3%) and the value was 4μg/ml while eleven isolates from twelve showed high MIC value (91.7%) and the values were begun from 32μg/ml Similar results were obtained by [15] who found that the MIC of *E. coli* against amoxicillin/clavulanic acid was ≤ 4 μg/ml. in conclusion, β lactamase (30 KD) produced by β lactam resistant bacteria could produce anti β lactamase in immunized rabbits. Anti β lactamase can neutralize β lactams in MIC tests resulting in reduction in CFU of multi- drug resistant E- coli.

References

1. Nandivada LS, Amyes SG, Plasmid-mediated β-lactam resistance in pathogenic Gram negative bacteria isolated in South India. J Antimicrob Chemother 1990, 26: 279-290.
2. Chaibi, E B, Sirot D, Paul G, Labia R. Inhibitor resistant TEM β-lactamases phenotype, genetic and biochemical characteristics. J. Antimicrob. Chemother., 199943: 447-458.
3. Essack SY, Hall LC, Pillay DG, McFadyen ML, Livermore DM. Complexity and diversity of Klebsiella pneumonia strains with extended- spectrum β-lactamases isolated in 1994 and 1996 at a teaching hospital in Durban, South Africa. Antimicrob Agents Chemother, 2001,45 (1): 88-95
4. Joseph MV, Patricia A, Bradford C, Urbanm G. Surveillance and detection of inhibitor-resistant β-lactamases in clinical isolates of Escherichia coli. Columbia Undergraduate Science J, 2007, 12(14), 112-114.
5. Livermore D M. β-lactamases in laboratory and clinical resistance. Clin Microbiol Rev 1995, 8 (4): 557-584.
6. Jacoby GA. Extrachromosomal resistance in Gram negative organisms: the evolution of β-lactamase. Trends Microbiol 1994, (10): 357-360.
7. Li Y, Jian HL, Gong ZH, Yu-S P, Yong JW. Molecular characterization of extended-spectrum β-lactamase-producing *Escherichia coli* isolates from chickens in Henan Province, China J Med Microbiol, 2009,58 (13): 1449-1453.
8. Hindler JA, Howard BJ, Keiser JF, Antimicrobial Agents and Antimicrobial Susceptibility Testing. In: Clinical and Pathogenic Microbiology. 1994, 2nd edition. St. Louis. Mosby.
9. Paterson L, Hujer KM, Hujer AM. Extended-spectrum β-lactamases in Klebsiella pneumoniae bloodstream isolates from seven countries dominance and widespread prevalence of SHV- and CTX-M-type β-lactamases. Antimicrob Agents Chemother 2003, 47: 3554-3560.

10. Bush, K.; Jacoby, G.A. and Medeiros, A.A. A functional classification scheme for β-lactamases and its correlation with molecular structure." Antimicrob Agents Chemother 1995,39: 1211-1233.
11. Dever LA, Dermody TS, Mechanisms of Bacterial Resistance to Antibiotics. Archives of Internal Medicine, 1991, 151: 886-895.
12. Canto C, Haung W, Palzkill T. Selection and characterization of amino acid substitution at residues 237-240 of TEM-I beta lactamase with altered substrate specificity. J Bio Chem 1996, 271(13): 22538-22554.
13. Sougakoff W, Jarlier V. Comparative potency of mecillinam and other β-lactam antibiotics against Escherichia coli strains producing different β-lactamases. J. Antimicrob. Chemother., 2000, 46 Suppl. S1:9-14.
14. Reguera J, Baquero F, Perez-Diaz J, Martinez J. Factors determining resistance to β-lactam combined with β-lactamase inhibitors in *Escherichia coli*. J Antimicrob Chemother 1991, 27: 569–575.
15. Silva J, Aguilar C, Becerra Z, Lopez-Antunano F Garcia R, Extended spectrum β-lactamases in clinical isolates of Enterobacteria in Mexico. Microbial Drug Resistance, 1999, 5 (3): 189-193.
16. Georgi G, Valax P, Ostermerier M, Horowitz PM. Folding and aggregation of TEM β-lactamase: Analogies with the formation of inclusion bodies in *Escherichia coli*. Protein Sci 1994, 3, 1953–1960.
17. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 1970, 227 (5259): 680–685.
18. John S, Thompson Charles D, Severson Nancy A S, John EK. Immunological Distinction of Mycobacterial Beta-Lactamases. Infect Immun 1972, 5(4): 542-546.
19. Onon SO. Purification and parial characterization of the exotoxin of corynebacterium ovis. Biochem J 1979, 177, 181-186
20. Weiland E, Theil HJ, Hess G, Weiland F. Development of monoclonal neutralizing antibodies against bovine viral diarrhea virus after pre-treatment of mice with normal bovine cells and cyclophosphamide. J Virol Methods 1989, 241: 237–243.
21. Louis A, Eriquez M, Richard FD. Purification by Affinity Chromatography and Properties of a β-Lactamase Isolated from Neisseria gonorrhoeae. Antimicrob Agents Chemother, 1979, 15(2): 229 -234.
22. Towbin H, Stacchelin T, Gordan J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets, procedures and some applications. Proc. Nati. Acd. Sci. USA. 1979, 79: 4350-4354.

EFFICIECY OF DESINFECTANTS USED FOR DECONTAMINATION HALLS FOR SELLING MEAT AND MEAT PRODUCTS

Ruslan ANTOCI, Nicolae STARCIUC, Victor USATENCO, Aurel CIUCLEA, Natalia OSADCI, Tatiana GOLBAN
UAS Chișinău, Molodova

Abstract
Maintain sanitary epidemiological status of the hall for selling meat and meat products is one of the most important tasks aimed at preventing and minimizing the risk of infections and food poisoning. 2/3 of health events, such as food poisoning, is caused by consumption of food, mainly of animal origin, contaminated at different stages of processing, storage and carrying. Therefore, the purpose of investigations was to establish the level and composition of the microbial load of the halls for the marketing of carcasses and meat products from the central square of mun. Chisinau after the rehabilitation measures.
Key words: disinfection, sample, microorganisms, decontamination

Introduction

Rapid advancement of food technology, the preparation and packaging, in order to provide safety and quality of animal food supplies continue to maintain food contamination by contaminants that are introduced natural or accidental or improper handling or storage of food. Quality and food safety is based on the efforts of all those involved in the complex chain including agricultural production, processing, transportation, preparation and consumption. According to the European Union and World Health Organization - food safety is everyone's responsibility, from their origin until they reach the table.

To maintain the quality and safety of the food chain recalled the procedures necessary to ensure that foods are integrated and monitoring procedures to ensure carrying end operations smoothly.

Food safety policy in the EU consider the full range of food for consumption by animals or humans. It provides extensive regulations and emphasizes the responsibility of manufacturers and suppliers in terms of their participation in providing quality food supply.

Food processing industry is based on modern systems to ensure quality and safety of products they put on the market. The three main systems in use are: Good Manufacturing Practices - GMP, Risk Analysis and Critical Control Points - HACCP and Quality Assurance Standards - QAS.

These quality management systems, include the relationship with suppliers (farmers and sellers of raw materials), transportation agencies, product vendors to ensure quality assurance procedures at every level.

The consumer is the end point of the food chain. A food that was in good condition at time of purchase must be treated carefully to avoid contamination with microorganisms or their products. Here lies an important role in improving the level of the hall for storage and sale of meat and meat products.

Materials and methods

Investigations were performed in the agricultural central square of mun. Chisinau and at the Department of Epidemiology of the Faculty of Veterinary Medicine, ASUM. For disinfection performance of the hall was used Ecocid S product, manufactured by KRKA.

Samples were collected from: meals, refrigerators, floor, steps, scales. For insemination was used nutrient arificial media as: peptone agar and broth, Endo media, Levin media, bismuth sulfate agar.

Results and discussion

Investigations were made in the hall no. 3 of the agricultural central square of Chisinau. Samples were collected on the hall table, refrigerator, floor, scales, etc.(fig. 1,2,3), which were subsequently shipped to the laboratory for bacteriological and bacterioscopic examinations.

Fig.1

Fig. 2

Fig.3

Disinfection of the hall was done in three stages: first stage was the dry mechanical cleaning (removal of mechanical materials deposited on surfaces), the second step - cleaning hydromechanic (wash surfaces with soapy water and detergents), the third stage - stage chemical (application of disinfectant solution on the surfaces under mechanical cleaning and sanitation).

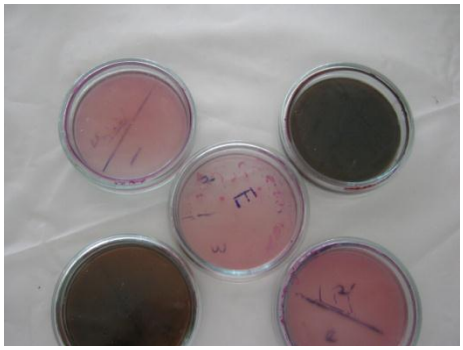

Fig. 4

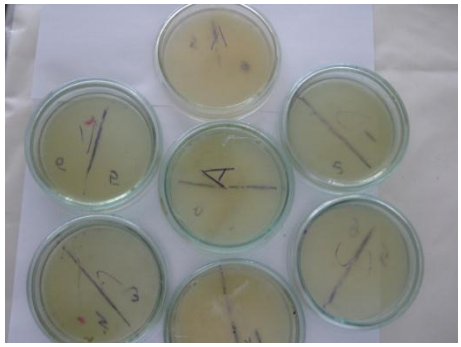

Fig. 5

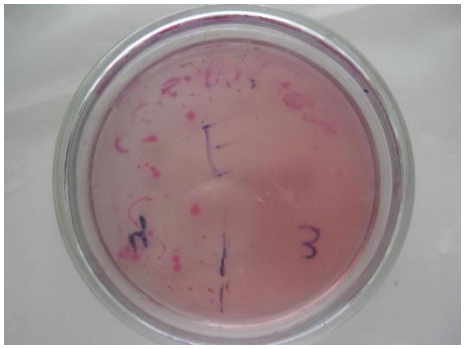

Fig. 6

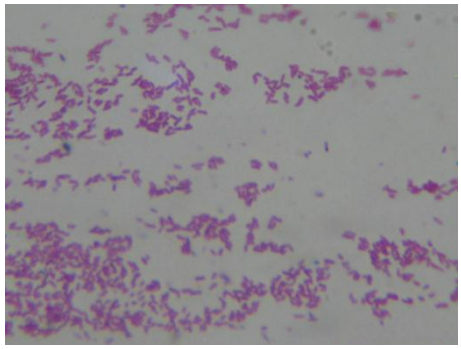

Fig. 7

In figures 4 and 5 are broths which sowings were made with material samples taken, and the figure 6 colonies of microorganisms that have developed at the Endo media after 24 hours incubation in the thermostat at +37 ^{O}C. After that from the grown colonies were prepared smears that were painted by Gram method. In fig. 7 are presented microorganisms (E. coli) that have grown on Endo which was colescted from the scales.

Conclusions

1. Health security of meat and meat products is the responsibility of everyone involved along the food chain and decontamination and control mechanism to ensure minimum risk their contamination with pathogenic microorganisms.
2. Zero risk of food security it is rarity, it should be cliar that the best legislation and best control systems can not protect us entirely. It is important to know well and respect the basic principles of veterinary food security.

References

1. Haesebrouck,F., Vancraeynest, B., Catry, P., Butaye, A. MRSA from animals: a public treat. Vlaams Diergeneeskundig, Tijdschrift, v. 75, 2009, p. 254-261.
2. Helle Ericsson Unnerstad, Torkel Ekman. Veterinary microbiology. 2008.
3. Leonard F.C., B.K. Markey, - Meticillin-resistant Staphylococcus aureus in animals: a review: Vet.J., v. 175, 2008, p. 27-36.
4. Mihaiu Marian, Sorin Daniel Dan, Ovidiu Rotatru. Control of animal health product. Ed. Risoprint. 2009.

5. Răpuntean Gh., Rapuntean S. Special Veterinary Bacteriology . Ed. Academic Press, Cluj-Napoca, 2005.
6. Roberts M.C., 1996 – Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility and distribution, FEMS Microbiol. Rev., 19 (1996), p. 1-24
7. Schwarz S. and Elisabeth Chaslus-Dancla, 2001 – Use of antimicrobial in veterinary medicine and mechanisms of resistance. Vet. Res., 32 , 2001, p. 201-225.
8. Strommenger B., C. Kehrenberg, C. Kettlitz, C. Cuny, J. Verspohl, W. Witte, S. Schwarz, – Molecular characterization of methicillin-resistant Staphylococcus aureus strains from pet animals and their relationship to human isolates: J.Antimicrob.Chemother., v. 57, 2006, p. 461-465.
9. ***European Food Safety authority, 2008 - Report from the Task Force on Zoonoses Data Collection including guidance for harmonized monitoring and reporting of antimicrobial resistance in commensally Escherichia coli and Enterococcus spp. From food animals, The EFSA Journal, 2008, 141, p. 1-44
10. ***World Health Organization, 2007 – Critically important antimicrobials of human medicine, Report of the second WHO expert meeting, 29- 31 May 2007
11. ***World Organization For Animals Health, 2007 – List of antimicrobial of veterinary importance, General Session of OIE (Resolution No. XXVII), May 2007
12. ***http://www.wikipedia.org
13. ***http://www.ziarullibertatea.ro

CHEMICAL CHARACTERISTICS AND BIOLOGICAL EFFECTS OF UNCONVENTIONAL FEED LOCAL PIGS IN THE TRADITIONAL CARE SYSTEM IN NORTH MINAHASA DISTRICT NORTH SULAWESI PROVINCE

Betty BAGAU, Hendronoto Arnoldus W. LENGKEY[2], Meity R. IMBAR[1], Fenny R. WOLAYAN[1]
[1]Sam Ratulangi University, Manado, Indonesia; [2]Padjadjaran University, Bandung, Indonesia, betty_bagau@yahoo.com

Abstract
This study aimed to identify the type of feed local pigs is traditionally reared in North Minahasa regency, North Sulawesi Province. This study obtained preliminary data, the ownership of cattle generally range below 5 tails with most of the maintenance system is extensive, semi-intensive and thus less controlled feeding pattern. The study was conducted in 11 villages which are determined by sampling puposive and has obtaineddata that can recommend the type of feed local pigs are typically used in animal husbandry and of variations in feed ingredients used taken 4 (four) types of materials thatuse the top position. The fourth composition of feed ingredients has been analyzed chemically by proximate analysis to determine the quality or content of nutrients. The results of this study can be concluded that rice bran, coconut pulp, wallet and plant tubers taro/taro forest (mixture of stems and leaves) is a type of feed raw materials most commonly used in the maintenance of local pigs in North Minahasa Regency. The second type of feed raw material used is largely determined by the types of crops are cultivated on the location and maintenance.

Keywords : local pig, semi-intensive, feeding pattern, feed ingredients

Introduction

Commodity pigs it is possible to thrive in North Sulawesi because it is caused by the Minahasa people of North Sulawesi in particular, Manado, Bitung, is largely a consumer product pigs. In 1998 the pig population in the province amounted to 3.89% of the total pig population in Indonesia.

Today the pig farmers in the area of North Sulawesi in general have superior offspring to maintain the nation's pigs-Landrace cross Yorkshire, Duroc, Poland China, which is derived from the project help the President (BANPRES). Also in North Sulawesi are the descendants of local pig species *Sus Selebensis* maintained to a lot of rural areas or in villages that although ideally the genetic quality has not been achieved but a lot of traditionally farmed by farmers in villages with a small amount of possession but not with the purpose komesial as family savings. Many factors influence the success of the pig business, among the environmental factors are easily addressed both quantitatively and qualitatively is the factor of food or feeding a day-to-day is handled directly by the farmer.

So far so maintenance effort models can still survive even contribute economically viable for farmers, because there is a purpose to maintain a family of pigs as the savings that can be sold at any time in addition to fulfilling the animal protein nutrition (meeting the needs of the early days of holidays, weddings , salvation, etc.). On the maintenance of local pigs or pig village administration is not the main concentrations of even the first year of studies found a breeder who does not use or provide concentrates in the maintenance of livestock due to only utilize the available natural food in the maintenance area is used as a main dish of pigs is therefore it is necessary to identify the characteristics of the feed in terms of type and quality associated with livestock will need nutrients and how they affect the growth of pigs

when compared to using commercial rations or concentrates. Feed characteristics can be measured or judged by the content of the nutrients contained in such materials that can be measured by laboratory analysis techniques Nutrients are protein, fat, carbohydrates, minerals, vitamins, and energy value. Feed ingredients identified in this study were very varied and identified 10 types of feed ingredients and which occupied the top four places are the Pacific Islands Taro tuber (purse), rice bran, coconut pulp, stems and leaves of Talas/taro forest.

Material and methods

Study to inventory the types of feed used in livestock feeding on local pigs have been carried out by the method of survey, in several villages in North Minahasa Regency that according to preliminary observations have a number of livestock ownership. The village election conducted purposive sampling as well as samples of breeder and this is because the unavailability of data at district and village level statistics on the number of breeders and the number of livestock ownership.

Data is collected by direct observation and interview techniques by using a list of questions that have been provided. The final results of data collection are presented in tabular form of data and variables that were analyzed quantitatively (Singarimbun, and Effendi, 1995).

The data collected are: Number of Livestock ownership (supporting data, age and weight of cattle (data support), number of feed and the feeding means (supporting data) and feed type (primary data).

Types of feed were analyzed chemically identified by proximate analysis to determine the content of the nutrients contained in such materials.

Results and discussion

1.General State of Study Sites

Geographically North Minahasa regency lies between latitude $01^0$18'30"-$01^0$53'00" S longitude $124^0$44'00"-$15^0$11'00" E, total area is 937.65 km^2 or 6.14% of the province of North Sulawesi, Indonesia.

The border area is in the east by Bitung City, west of the City adjacent to Manado, on the north bordering the District Sangihe, Talaud district, North Sulawesi and Maluku Sea,and on the south by the Minahasa regency, the territory is divided into 10 subdistricts and 125 villages. In 2004, the population of North Minahasa District totaled 174,852 inhabitants.

Commodity Profile

Table 1. Commodity Profile in North Minahasa Regency

No	Sector/Commodity	Seed/No	Description
1.	Primary-Plantation: Coconut	Leading	Production Last Year (2006): 49,060.00 Tons
2.	Primary-Plantation: Cloves	Leading	Production Last Year (2006): 402.00 Tons
3.	Secondary-Industry: Integrated Oil Industry	Leading	And availability of raw materials in the area (For Kom. Secondary Tertiary) Coconut (44,014.00 tons)
4.	Secondary-Industry: Coconut Oil	Leading	And availability of raw materials in the area (For Kom. Secondary Tertiary) Coconut (44,014.00 tons)

5.	Secondary-Plantation: Coffee	Not Featured	Production Last Year (2006): 3.00 Ton
6.	Primary-Plantation: Cashew	Not Featured	Production Last Year (2006): 18.00 Ton
7.	Primary-Plantation: Pepper	Not Featured	Production Last Year (2006): 40.00 Ton

Data source : *Statistics of Indonesia 2006-2008 Estates Directorate General of Estate Crops Ministry of Agriculture Jakarta 2007*

Livelihood

North Minahasa Regency that most of the region is coastal, the main livelihood of the population are farmers. In coastal areas, based on the results of the study-RLKT RTL-Likupang Wori Bay Area in 2004, the role of agriculture in providing employment is dominant, which is about 78.07%. From these percentages, approximately 53.49% and 24.58% are farmers are fishermen. The main crops cultivated are coconut, cloves, fruits and pulses. (RTLRLKT Coastal North Minahasa, 2004).

Village Survey

Of the 10 districts in North Minahasa district, the survey determined that 11 villages Mentahage Island; Wori; Kima Bajo; Langsa; Darunu; Pontoh; Kema; Likupang; Serey; Wusa and Winetin. The location of these villages are far from the central district.

2. System Maintenance and Number of Livestock Ownership

The number of local pigs scattered in North Minahasa district not included in the scope of the study, identifying data is the average number of livestock ownership in the 10 villages which are rural purposes. Data obtained from a breeder that, on average ownership of pig between 1-5, is almost evenly 1-2.

System maintenance is generally semi-intensive, livestock and tied in the back yard beside the house and there are also some cages, but some are released to the location of farm / forest foraging alone in the daytime and nighttime grounded and bonded.

Fig. 1. Pigs Livestock Maintenance System (removable, tied up and caged)

3. Feed Types Identified

The selection of the type of feed that familiarity will be described, based on the use bymost farmers in feeding the pigs are kept coconut pulp, rice bran and taro tubers Islands (purse).

Table 2. Type of feed (3 ranked highest) Uses in Each Study Site

No.	Village	Type of feed
1.	Wori	1. Taro tuber (purse) 2. Coconut pulp 3. Coconut
2.	Island Mentahage	1. Forest tuber crops (a mixture of stems, and leaves). 2. Coconut pulp 3. Kitchen waste (vegetables, fish waste)
3.	Kima Bajo	1. Taro tuber (purse) 2. Coconut pulp 3. Banana skin
4.	Langsa	1. Plant a forest tuber (mixture of stems and leaves). 2. Coconut pulp 3. Rice bran
5.	Darunu	1. Coconut pulp 2. Forest tuber crops (a mixture of stems, and leaves). 3. Heart banana
6.	Pontoh	1. Taro tuber (purse) 2. Cassava 3. Coconut pulp
7.	Kema	1. Rice bran 2. Fish waste 3. Cassava
8.	Likupang	1. Coconut pulp 2. Fish waste 3. Rice bran
9.	Serey	1. Rice bran 2. Fish waste 3. Cassava
10.	Wusa	1. Rice bran 2. Beans and corn stover 3. Ccoconut pulp
11.	Winetin	1. Coconut pulp 2. Taro tuber (purse) 3. Rice bran

Sources: *Survey research Juni-July 2009*

Table 3. The content of nutrients of feed types identified

No.	Nutrient	Coconut pulp	Rice bran	Hump purse (*Xanthosoma sagitifolium*)	Stems and leaves of taro (*Colocasia esculenta* L) Scott.) sp
1.	Water (%)	71.98	8.63	86.63	85.80
2.	Ash (%)	0.56	13.75	8.56	12.09
3.	Crude protein (%)	7.16	5.43	8.48	16.20
4.	Coarse fibers	18.98	21.09	14.05	18.10
5.	Crude fat (%)	24.15	4.03	1.55	8.24
6.	BETN (%)	49.15	55.7	67.36	45.37
7.	GE (kcal/kg)	4955	3603	4083	3219
8.	Calcium (%)	0.003	0.023	0.36	0.22
9.	Phosphorous (%)	0.0008	0.21	0.38	0.11

Sources: *Laboratory of Nutrition and Food Chemistry Faculty of Animal Husbandry Padjadjaran University in Bandung, 2009*

a. Coconut pulp

North Sulawesi region dubbed the "nyiur melambai" considering the number of coconut trees in this area. The remaining coconut pulp is the result of extortion coconut meat coconut milk to be taken. Old fruit pulp is the material source of vegetable oil (oil content 35%). Nutrient composition of an old coconut meat is protein 3.4%, fat 34.7%, carbohydrate 14%, calcium 0.021% and 0.021% phosphorus, 46.9% water. The procedure of making coconut oil is an old fruit peeled and then cut open and the meat is removed from the shell. And shredded coconut meat is ground manually or using machinery. Crushed fruit and add water with a ratio of 1:2. Furthermore, the extract pressed by machine or manually presses and then filtered to obtain coconut milk (Elfianus, 2008).

Fig. 2. Coconut pulp Fresh / Cooked

b. Rice bran

Rice has a composition, 70-72% endosperm, 20% rice bran, rice bran 7-8.5% and 2-3% of embryos (Ju and Vali, 2005). Using rice bran in Indonesia to date is as animal feed. This is because the nutrient content of rice bran is high enough.

Fig. 3. Rice bran

In this study the data obtained using rice bran as feed local pigs are classified as coarse bran. The use of rice bran as livestock feed local pigs in almost all villages surveyed due to the rice plant is one of the leading commodities in North Minahasa regency.

c. Pacific Islands Taro tuber (purse)

Belitung taro *Xanthosoma sagitifolium* with the scientific name of this family include chronic Areacea and is a plant that has a stem or stem tuber is actually false petiole. Tubers are used as food by boiling or frying. In the western part of the African continent, in North Sumatra, South Sumatra, East Kalimantan, North Sulawesi and West Nusa Tenggara have been cultivated by farmers on a regular basis. Belitung taro planting using spacing of 50 x 50 cm and 100 x 100 cm. While the cultivation of irregular covering areas of Aceh, Central Kalimantan, Bengkulu, West Kalimantan and East Nusa Tenggara. In general, farmers cultivated plants in the yard around the house and gardens. The average yield per clump ranged from 0.25 to 20 kg.

Fig. 4. Purse plants *(Xanthosoma sagitifolium)*

Fig. 5. Hump purse

d. Stem and Leaf Taro *(Colocasia esculenta* (L) Scott.) Sp

Taro *(Colocasia esculenta* (L) Scott.) is divided into two varieties, namely varieties of *C. esculenta* varieties *var.esculenta* and Talas *C. esculenta var. antiquorum. C. esculentava esculenta,* the tuber is single bulbs of medium size or large (depending on the variety). Varieties found in the study site are a kind of Talas / taro tubers in the absence of forests. This type of taro grown in the soil slightly moist has a stem and green leaves are smooth and shiny.

Fig. 6. *Colocasia esculenta* (L) Scott

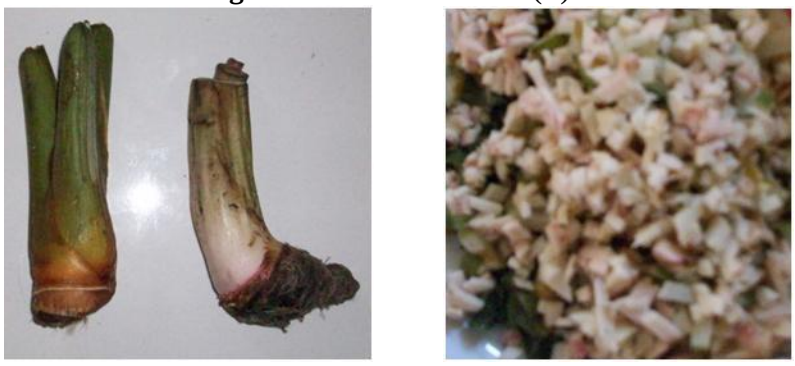

Fig. 7. Talas plant stems / tuber forest

Conclusion

Based on the research results can be concluded that:

1. Coconut pulp, rice bran, and plant bulbs purse taro / taro forest is a type of feed raw materials most commonly used in the maintenance of village pigs in North Minahasa district of North Sulawesi Province.
2. This type of feed raw materials are used is largely determined by the types of crops are cultivated on the location and maintenance.

References

1. AOAC, 1984. *Official Methods of Analysis of the Association of Official Chemist. AOAC*. Inc, Arlington, Virginia.
2. Goniwala E, 2008. Teknik Pengolahan V*irgin Coconut Oil* menggunakan ragi tape. buletin teknik pertanian vol. 13 no. 2.
3. Kalangi, J.K.J., 2004. Profil Peternakan Babi di Kema Kabupaten Minahasa. Jurnal Zootek. Volume 19 ; 175–180. ISSN 0852–2626. Fakultas Peternakan Universitas Sam Ratulangi Manado.
4. Laboratorium Nutrisi Ternak dan Kimia Makanan, 2009. Fakultas Peternakan Universitas Padjadjaran. Bandung
5. Mahmud Z., Ferry Y., 2005. Prospek pengolahan hasil samping buah kelapa. Pusat Penelitian dan Pengembangan Perkebunan, Prespektif volume 4 nomor 2.
6. Najoan, A., 2000. Evaluasi Kualitas Beberapa Jenis Bahan Pakan Ternak Babi di Daerah Sulawesi Utara Melalui Analisis Proksimat dengan pembanding Data Nash dan NRC. Laporan Penelitian. Fakultas Peternakan UNSRAT. Manado.
7. Parakkasi, A., 1990. Ilmu Nutrisi dan Makanan Ternak Monogastrik. Penerbit Universitas Indonesia, Jakarta.
8. Pulungan, I. 1985. Perencanaan Pengembangan Peternakan. Fakultas Peternakan, Institut Pertanian Bogor.
9. Sihombing, D.T.H. 1997. Ilmu Ternak Babi. Gajah Mada University Press, Yogyakarta. Hal. 200– 240.
10. Singarimbun, M., dan S. Effendi, 1995. *Metode Penelitian Survai*. LP3ES, Jakarta.
11. Sosroamidjojo, M.S., dan Soeradji, 1983. Peternakan Umum. CV. Yasaguna, Jakarta.
12. Steel R. G. D. dan J. H. Torrie. 1993. *Prinsip Statistik*. Penerbit PT. Gramedia Pusat Utama, Jakarta.

PERSPECTIVES REGARDING THE APPLICATION OF LENTIVIRAL VECTORS IN VETERINARY SPECIFIC PROPHYLAXIS

Ana BEJANARIU*, Luanda LUDU*, Gh. SAVUŢA[*]
* Universitatea de Ştiinţe Agricole şi Medicină Veterinară Iaşi

Abstract
The use of viral vectors in vaccinology provides an effective mean to elicit antigen-specific cellular immune responses. Several viral vectors have proven efficacious in inducing immune responses after direct injection in vivo. Among them lentiviral vectors are very attractive delivery systems, as they are able to efficiently transduce into and express foreign genes in a wide variety of mammalian cells. Lentiviral vectors achieve high transduction efficiency irrespective of the proliferative status of the target cells, thus circumventing one of the main limitations of oncovirus-derived retroviral vectors in which transduction is restricted to dividing cells. This advantage is reflected in a number of successful preclinical tests in various animal models of human diseases and will undoubtedly translate into their exploitation for the use in veterinary medicine. Most studies have focused on the induction of cellular immune responses in the field of protective cellular immunity against viruses esspecially against human imunodefincy virus. Research conducted by our team in collaboration with specialists in the field resulted in the elaboration of an efficient vaccine for veterinary use, capable to protect against West Nile virus infection in horses. One of our future research concern is related to the development of a vaccine against influenza A virus, which due to the proprieties of the lentiviral vectors should be able to protect against multiple influenza A subtypes assuring a long term cellular immunity. One of the problems regarding these vectors is the reluctance of scientific public opinion regarding their use. Based on these considerations we intend to clarify some aspects concerning the principle and methods of production, applications and optimization of lentiviral vectors.

Key words: viral vectors, lentiviral vectors, veterinary prophylaxis

The use of viral vectors

Viruses attack their hosts and introduce their genetic material into the host cell as part of their replication cycle. This genetic material contains basic 'instructions' of how to produce more copies of these viruses, hijacking the body's normal production machinery to serve the needs of the virus. The host cell will carry out these instructions and produce additional copies of the virus, leading to more and more cells becoming infected. Some types of viruses actually physically insert their genes into the host's genome. This incorporates the genes of that virus among the genes of the host cell for the life span of that cell. Viruses like this could be used as vehicles to carry 'good' genes into a human or animal cell. Firstly, the scientists would remove the genes in the virus that cause disease. Then they would replace those genes with genes encoding the desired effect (for instance, insulin production in the case of diabetics). This procedure must be done in such a way that the genes which allow the virus to insert its genome into its host's genome and leave it intact (10).

Many gene therapy clinical trials rely on retroviruses or adenoviruses to deliver the desired gene. Other viruses used as vectors include adeno-associated viruses, lentiviruses, pox viruses, alphaviruses, and herpes viruses. These viruses differ in how well they transfer genes to the cells they recognize and are able to infect, and whether they alter the cell'sDNApermanently or temporarily.

Gene therapy is most commonly associated with genetic deficiencies. But the spectrum of potential applications of gene therapy goes well beyond that:

- Genetic deficiency
- Viral infections (Example: human immunodeficiency virus)
- Autoimmunity (example: rheumatoid arthritis)
- Cancer
- Diseases in which several genes and the environment interact, such as diabetes, coronary artery disease (10).

Why to choose the lentiviral vectors?

Lentiviruses are distinct members of the Retroviridae family of viruses. Lentiviral vectors have been constructed from several types of lentiviruses, but the most commonly used is the human immunodeficiency virus or HIV, a virus whose molecular biology has been extensively studied. Lentiviral vectors have the unique advantage of being able to stably transduce dividing and non dividing cells. The lentivectors were identified in 1996 and this discovery quickly led to a wide range of applications in preclinical therapeutic gene transfer. (5)

Lentiviral vectors are viral-based gene delivery systems that can stably deliver genes or RNAi into primary cells or cell lines with up to 100% efficeincy. LVs binds to targeted cells using an envelope protein which allows to release of the LV RNA containing the gene or gene sliceing sequence into the cell. The LV's RNA is then converted into DNA using an enzyme called reverse transctiptase by a process called reverse transcription. The DNA pre-integration complex then enters the nucleus and integrates into the target cell's chromosomal DNA. (4)

Lentiviral vectors are the only genetic vector system that affords both high and stable gene delivery. Other vectors, such as adeno-associated viral vectors can persist in non-dividing tissues, but are cured from cells once they divide. Lentiviral vectors integrate their payload sequence ino the chromosome of tranduced cells so that it is copied along with the chromosomal DNA when the cell divides. The propreties of lentiviral vectors make them high suitable for efficient and stable gene delivery into cell lines and primary cells.

In 2012, many lentiviral vectors derived from HIV are available but also from other animal lentiviruses like feline (FIV), equine (EIAV) and simian(SIV) viruses.This class of vehicles has reached a certain maturity, and the results of early clinical trials based on the use of lentiviral transfer of a therapeutic gene are encouraging. (5)

The use of lentiviral vectors for gene therapy approaches is mainly motivated by the fact that HIV-1 and all members of the subfamily of lentiviruses have this unique property among retroviruses to infect non-mitotic cells.

Indeed, through lentiviruses technique it has been developed a strategy of nuclear import due to the DNA Flap, a structure created during reverse transcription. This stage of translocation through the nuclear pore represents the ability of a lentivirus to infect quiescent cells (1). The addition of Flap in a central position in the lentiviral vectors is accompanied by an increase from two to ten orders of magnitude of the transduction rate, compared to other viral vectors that are not so efficient from a transductional point of view. That's why this sequence is used for it benefits on the construction of lentiviral vectors. (2)

Many obstacles must be overcome in the development of recombinant lentiviruses. Firstly it was considered that the HIV vectors are not safe because they are derived from a virus that is infectious to human kind. On the other hand, developing vectors from a virus whose molecular biology and pathogenesis are well understood and for which multiple drug

therapies are available, than from viruses whose pathogenicity in humans is uncertain may be a real advantage, especially in our days when all resources are focalized on HIV fundamental research.

Pseudotyping lentiviruses with different envelope proteins can increase their stability and their tropism, that is to say, the affinity of the virus to a cell type, tissue or an organ donated. This also improves gene transfer and minimizes toxicity. In summary, the ideal lentivector must occur easily (with aVSV.G envelope), do not spread into the target cell and not to recombine with other vector that may be found into the cells.

Lentiviral vector production

The 293T cell line is used for the production of lentivectors. The 293 T cells are human embryonic kidney cells. 293T cell line is an important variant of 293 cell line that incorporates the SV40 Large T-antigen that allows for episomal replication of transfected plasmids containing the SV40 origin of replication (7).

Three plasmids ar co-transfected:

The first one is a vector plasmid, which contains the transgene expression cassette. The second one is a packaging plasmid. And the third one is a plasmid containing an envelope.

The vector plasmid contains cis-acting sequences (LTRs, psi encapsidation signal, cPPT/CTS) necessary for the vector to transduce target cells and integrate the transgene into the host cell genome. This plasmid requires the Ψ sequence for efficient encapsidation in vector particles and does not contain nor envelop protein, neither Gag nor Pol ones. The expression is driven by the U3 promoter.

In the packaging plasmid are found the elements required for vector packaging such as genes of structural proteins (Gag and Pol), non structural HIV-1 genes (Tat and Rev), and the enzymes that generate vector particles. The human cytomegalovirus promoter (ieCMV), which is responsible for the expression of viral proteins during translation, is also contained in this packaging plasmid. The packaging signals and their adjacent signals are removed.

The third plasmid contains VSV_G. The use of the VSV-G protein instead of the HIV-1 envellope allows an increase of target cell tropism (8).

Security optimization of lentiviral vectors

The suppression of auxiliary gene

Genes like vif, vpr, vpu and nef of HIV that are not essential to viral replication in vitro yet are very important in terms of virulence and pathogenesis in vivo were excluded from the production of lentiviral vectors derivev from lentiviruses. Moreover, the absence of homology between the three sites of recombination required to form a replicative virus makes it impossible to detect recombinant virus production in a replicative lentiviral vectors (8).

"Self-inactivated" vectors

The deletion of HIV promoter and of U3 region situated in the 3'LTR U3 overcomes to the formation of "*self-inactivated*" vectors. In fact, the deletion of the 3'LTR U3 in 5'LTR is copied during retrotranscrition process producing LTR fragments from which U3 region is entirely deprived of its promoter and regulatory sequences. Thus the only possible transcription is induced by the internal promoter (regulating expression of the transgene), located downstream of the encapsidation sequence.Vector'sRNA is therefore completely deprived of the encapsidation signal of the wild virus.

Insertional mutagenesis

Currently all oncogenic events described in gene transfer protocols using integrative vectors seem to be correlated with the presence of enhancer regions of retroviral vectors. These enhancers are able, after vector integration to perturb cellular proto-oncogenes expression even away from the site of integration. Other such mechanisms can also disrupt cell physiology. Through insertional mutagenesis the occurrence of these specific events is prevented.

Effectiveness amelioration

Insertion of Flap sequence

Adding Flap in a central position inside lentiviral vectors allows the stimulation of gene transfer complementary to nuclear import of vectorial genome at the same level as wild virus (3).

Pseudotyping proteins

One of the first stages of developing lentiviral vectors derived from HIV was the natural replacement of the virus envelope with vesicular stomatitis virus envelope protein (VSV-G), thus increasing its stability and thus the vector tropism (7).

DNA vaccines in veterinary use

DNA vaccines represent a new frontier in vaccine technology. One important application of this technology is in the veterinary field. Infectious diseases represents a continuous threat to companion animals in many places of the world, especially those with limited access to veterinary care. Viral, bacterial and parasitic plague a large swathe of animals, from pediatric animals from pet stores in developed countries to feral animals roaming the streets in underdeveloped areas.

In addition to targeting pathogenic agents, DNA vaccines are also being used as cancer vaccines for companion animals. These vaccines incorporate plasmids encoding tumor antigens that induce the formation of antibodies that are expected to target tumor cells in the vaccinated animals, leading on the regression of tumors. DNA vaccines are, overall, an exciting prospect in the field of companion animal oncology.

While DNA vaccines represent an interesting possibility for targeting infectious disease and cancer in companion animals, their potential use in livestock is even greater. Livestock production in the USA alone is significant in scale: the USA is the world's largest beef producer, second largest beef export and second largest pork producer. Most livestock operations incorporate large number of animals, where herd immunity is of greater importance than the health of individual animals. Herd immunity involves conferring protection to a large population of animals by vaccinating a significant percentage of the whole group. Herd immunity prevents the rapid spread and decreases the persistence of a disease. This is especially important for diseases with longer incubation period, where infected animals cannot readily be identified and can transmit disease to other members of a herd. While a large number of commercial vaccines are available for the wide span of livestock diseases, DNA vaccines possess an inherent advantage in that vaccinated animals can be distinguished from infected animals, since they have the potential to be less expensive to produce, they could be of value in large-scale animal operation. Furthermore, many commercial vaccines come in the form of cocktails, offering protection against several

diseases in one vaccine. DNA vaccines are particularly able to provide such an option, as several plasmids encoding different genes can be incorporated into a DNA vaccine (9).

Theravectys company and the partnership with USAMV Iasi

A researcher from Pasteur Institute working on the laboratory of Molecular Virology and Vectorology (VMV), Dr. Pierre Charneau and his team has firstly identified the Flap structure. Based on this discovery, P. Charneau decided todevelop a start-up company called TheraVectys in 2005, company that has as main aim to develop prophylactic and therapeutic vaccines. TheraVectys already developed an anti-HIV therapeutic vaccine.For reasons of safety and efficacy the company spent all the last years in order to optimize their HIV vaccine candidate, particularly regarding the choice of promoter, antigen, to improve the regulatory sequences etc.This vaccine resulted in immune responses in mice and rats, with an absence of toxicity (at doses up to 100 times higher than the potential for injected dose in humans). In addition, other studies have strengthened the arguments for biodistribution method for vaccine safety.The use ofthese vectors has many advantages, because they are able to efficiently transduce dendritic cells(DC) and to activate them. These cells play a key role in the immune response because once transduced and activated, they will induce the development of a specificcellular response against their antigen. Furthermore, the transgene integration and expression inthe cellular genome of any target cells will allow the effector cells to recognize and eliminatethe transduced cells populations. All these characteristics confer to their vaccine candidate avery important advantage, therefore their vaccine will make the subject of a futur clinical trial in the next few months.

The perspectives of TheraVectys are not reduced only to the development of an anti-HIV vaccine. Different diseases and conditions have been considered to produce other prophylactic and therapeutic vaccines, among them: West Nile, malaria, hepatitis C,hepatitis B, melanoma and influenza. All stages of lentiviral vector optimization for anti-HIV therapy have served to facilitate the development of these new projects.

Collaboration between TheraVectys and USAMV Iasi has been established few years ago in order to develop a lentiviral vector used in the vaccination against West Nile virus (6). Promising results have been obtained and horses have been vaccinated in Romania.

The project with the title „The fight against genetically recombinated influenza viruses: test of new lentiviral vector based vaccines“ with the acronim RO-FLU, is included in the general objective of the Fourth program – Partnership in prioritar fields, hinting the increase of competitive activities of research and developement through the partenership in the specific objective Veterinary Public Health, diagnosis, surveillence, and control of animal diseases. The partenership between USAMV Iasi and reference laboratories of diagnosis and research from Romania and foreing countries (TheraVectys Pasteur Institute, Paris) is meant to has an final result the developement of an anti-influenza vaccine efficient in mice, swine, poultry and human.

Developing this collaborative project team many fundamental benefits, should result:

- bringing together researchers working in the field of influenza viruses and novel vaccine vectors should result in the development of innovative vaccine strategies and a technological transfer in Iasi
- effort in the generation of shared tools and resources such as experimental animal models, as well as standardized and reproducible assay to evaluate vaccine candidates

to develop and testing a vaccine that will recognize and protect against any influenza A pathogen.

Currently, production of high titers oflentiviral vectors is a time consuming, multi-step procedure with low reproducibility. To solve these problems, InvivoGen has developed LENTI-Smart™,a novel method to generate high titers of lentiviral vectors, simply, rapidly and efficiently. Depending on the specific needs, LENTI-Smart™ kits are availablefor the production of either integrating or non-integrating lentiviral (NIL) vectors. Integrating lentiviruses are best for stable transgene expression.NIL vectors are particularly useful for transient transgene expression in gene therapy protocols and stem cell modifications, where the risk of insertionalmutagenesis is a safety concern.

Conclusions

The knowledge of lentiviral vectors in Romania should be an advantage for the researchers and pharmaceutical companies. The perspectives offered on the veterinary field may be considered as a basic benefit in future human vaccination or treatment of different conditions. From a practical point of view we should seriously consider that potentially clinical trials are promoted only after preclinical trials. If one of our main concernswas the reluctance of romanian scientific opinion regarding the use of lentiviral vectors the arguments that strongly support their use are strengthened by their practical application and related to the development of a vaccine against West Nile virus and influenza A viruses, which due to the proprieties of the lentiviral vectors will be able to protect against multiple influenza A subtypes assuring a proper immunogenicity and the capacity to protect against a high virulent influenza challenge.

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References

1. Nathalie J. Arhel, Sylvie Souquere-Besse, Pierre Charneau. 2006.Wild-type and central DNA flap defective HIV-1 lentiviral vector genomes: intracellular visualization at ultrastructural resolution levels, Retrovirology 3:38, pag. 1-7
2. Nathalie J. ArheL, Sandie Munier şi col., (2006) Nuclear Import Defect of Human Immunodeficiency Virus Type 1 DNA Flap Mutants Is Not Dependent on the Viral Strain or Target Cell Type, Journal of Virology, vol. 80, nr. 20, pag. 10262–1026
3. Nathalie J Arhel, Sylvie Souquere şi col., (2007) HIV-1 DNA Flap formation promotes uncoating of the pre-integration complex at the nuclear pore,The EMBO Journal 26, pag. 3025–3037
4. Coates, Gene Therapy to Improve Pancreatic Islet Transplantation for Type 1 Diabetes Mellitus, pp.274-284 (11)
5. Boro Dropulic. 2006. Lentiviral Vectors: not just for Gene Therapy, Biotechnology, page 70
6. Boro Dropulic. 2011. Lentiviral Vectors: Their Molecular Design, Safety and Use in Laboratory and Preclinical Research, Human Gene Therapy 22:649-657 (June 2011)

7. Iglesias MC, Frenkiel MP, Mollier K, Souque P, Despres P, Charneau P. 2006. A single immunization with a minute dose of a lentiviral vector-based vaccine is highly effective at eliciting protective humoral immunity against West Nile virus, J Gene Med.2006 Mar;8(3):265-74.
8. Nils Loewen, Eric M. Poeschla, (2005) Lentiviral Vectors, Adv Biochem Engin/Biotechnol 99, Springer-Verlag Berlin Heidelberg, pag. 169–191
9. Barbara Mitta, Markus Rimann, Martin Fussenegger, (2005) Detailed design and comparative analysis of protocols for optimized production of high-performance HIV-1-derived lentiviral particles, Metabolic Engineering 7 pag. 426–436
10. Laurel Redding and David B. Weiner. 2009. DNA vaccines in veterinary use, Expert Rev. Vaccines B(9), 1251-1276
11. www.genetherapynet.com

DETECTION AND SEROTYPING OF *LISTERIA MONOCYTOGENES* IN MEAT AND MEAT PRODUCTS

C. CARP-CĂRARE, A. VLAD-SABIE, V. FLORIȘTEAN
University of Agricultural Sciences and Veterinary Medicine Iaşi
Faculty of Veterinary Medicine

Abstract

The aim of this study was to detect and serotype Listeria monocytogenes in meat and meat products, during the 2011 period. The meat samples were collected from slaughterhouses, from different stages of the technological flow. The detection of Listeria monocytogenes has performed by ISO 11290- 1-A1/2004 standard method and confirmed by real-time PCR. Numerous subtyping strategies have been developed to characterize Listeria monocytogenes isolates. Serotyping, the traditional method for the subtype characterization of L. monocytogenes is not very discriminatory, but it is a universal method which gives important information on differentiation. The confirmation of Listeria monocytogenes isolated strains was performed by a real-time PCR assay, using TaqMan Pathogen Detection Kits (Applied Biosystems). The assay use specific primers and a probe for Listeria monocytogenes and the detection performed by fluorescence curves analysis. The assay has also, an internal control, detected by a different probe. In this study on a total number of 155 meat samples, 15 (9,67%) gave positive results for L. monocytogenes by conventional method and only 12 (7,74%) samples were confirmed by real-time PCR. These results indicates a high efficiency of the real-time PCR method, compared with classical methods witch can provide fals positive results. Three different of Listeria monocytogenes serotypes were found in meat: 1/2b, 1/2c and 4b.

Key words: detection, serotyping, Listeria monocytogenes, meat

Introduction

Listeria monocytogenes is a food-borne pathogen that has the potential to cause human listeriosis, a severe illness that may lead to death (Makino et al., 2005; Vanegas et al., 2009). Numerous sporadic cases and outbreaks of listeriosis have been linked to a wide variety of foods of animal and vegetable origin. *L. monocytogenes* infections are particularly dangerous to certain risk groups, including, pregnant women, the elderly, newborns and immunocompromised patients. Manifestations of listeriosis include meningoencephalitis, septicemia, abortion and a high fatality rate 30% (Liu, 2006; McLauchlin et al., 2004).

Products such as raw milk, soft cheese produced from raw milk, raw meat products and salads are frequently implicated in foodborne with *L. monocytogenes*. In this context, hygiene weak points during the slaughtering and milking processe are the main critical points for *Listeria* contamination (Jemmi R., 2006).

A variety of conventional and rapid methods are available for the detection and identification of *L. monocytogenes* in food samples and specimens from animal listeriosis. Conventional methods remain the 'gold standard' with which other methods are compared. They are usually very sensitive, but they are time-consuming and laborious, requiring prolonged incubation (1-2 days) and selective enrichment to reduce the growth of background flora, and biochemical identification.

PCR and more recently real-time PCR technologies have become powerful diagnostic tools for the analysis of microorganisms in food and can potentially fulfil the requirements of the industry (Jantzen, 2006).

Materials and methods

Culture enrichment of food samples

The study was performed on 155 samples, randomly collected from different sources. Each sample was placed in an individual sterile polyethylene bag and transported to laboratory. The samples were first processed by classical microbiological methods, and verify by real-time PCR.

Culture enrichment of food samples was performed by Food Microbiology Laboratory (Iasi Veterinary Medicine Faculty), according to the ISO 11290. Food samples (25 g/25 ml) were added to 225 ml of half-Fraser broth and homogenized in a sterile stomacher bags for 1 minute. For snail meat, we used samples of 10 g, collected from foot region. After 24 hours of incubation at 30ºC, 0,1 ml were used for enrichment step, in 10 ml of Fraser broth and incubated 24 hours at 37ºC. The selective medium used was Palcam and Ottaviani-Agosti Agar and examined for typical *Listeria* colonies.

Serological tests of Listeria monocytogenes

To confirm the serotype within serogroups, the isolates were serotyped using commercial Listeria antisera (Denka Seiken, Japan), in accordance with the manufacturer's instructions. The principle of measurement is based on mixing the reagent with *L. monocytogenes* strain and the antigen antibody reaction occurs to produce agglutination.

DNA extraction

The extraction of bacterial DNA was performed, the average enrichment with the PrepMan Ultra (Applied Biosystems, Foster City, USA). One ml of enriched culture was transferred in the 2-ml microcentrifuge tubes. The samples were centrifuged for 3 minutes at room temperature in a microcentrifuge at 14000 rpm speed, to pellet bacteria and residual food or other debris, and then the supernatant was trowed away. 100 µl PrepMan reagent was transferred into the samples tubes. The tubes were closed and mixed for resuspend the pellet, then placed in a heat block set at 100ºC for 10 minutes, the cooling was done at room temperature for 2 minute. We centrifuged the tubes at 14000 rpm for 3 minutes and we transferred the supernatant in other tubes. We used 2,5 µl DNA solution for a PCR reaction.

Real-time PCR conditions

For real-time PCR reaction, we used TaqMan® *Listeria monocytogenes* Detection Kit (Applied Biosystems, Foster City, USA). The kit use: specific primers and probe for *Listeria monocytogenes*, TaqMan Environmental Master Mix (Taq-polimerase and buffer), IPC (internal positive control) detected by a different fluorophore, and NC (negative control).

Reactions and data analysis were performed in the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, USA). Amplification reaction (25 µl) contains: 2,5 µl DNA sample, 12,5 µl TaqMan Environmental Master Mix 2X, 0,5 µl of each F and R primers (100 µM), 0,25 µl TaqMan probe (50 µM), 8,5 µl RNase/DNase-free water. The PCR conditions were: 10 minutes at 95ºC for enzyme activation and DNA denaturation, followed by 40 cycles: 95ºC 15 sec. and 60ºC 1 min.

Results and discussions

Detection of Listeria monocytogenes by classical methods and real-time PCR

In our study, we tested a number of 155 meat samples by classical microbiological method -ISO 11290- 1-A1/2004. The positive samples of *Listeria monocytogenes* were confirmed by real-time PCR and serotyped.

Fifteen samples were positive for *L. monocytogenes* by classical microbiological methods, but only twelve were confirmed by real-time PCR (table 1).

Table 1. Detection of *Listeria monocytogenes* by ISO standard method and real-time PCR method

Meat samples	ISO 11290- 1-A1/2004 standard method		Total number of samples	Real-time PCR	
	Positive *L. monocytogenes* samples	Negative *L. monocytogenes* samples	155	Positive *L. monocytogenes* samples	Negative *L. monocytogenes* samples
Raw sheep meat	4	21	25	3	22
Raw poultry meat	1	19	20	1	19
Raw pork meat	2	21	23	2	21
Raw pork sausages	0	10	10	0	10
Raw bovine meat	2	18	20	2	18
Snail meat (foot region)	6	24	30	4	26
Raw fish	0	10	10	0	10
Smoked fish	0	17	17	0	17

We isolated 15 strains from fresh meat (4 from sheep meat, 2 from bovine, 2 from pork meat, 1 from poultry meat and 6 from snail meat), salad by standard method, but 3 strains were negative by real-time PCR (1 from sheep meat and 2 from snail meat). The explanation for the false positive results by classical method, can be association of other bacteria, included other *Listeria spp.*, which inhibited the growth of *Listeria monocytogenes*. These sublethally injured bacteria require special culture conditions for damage repair, before being able to be detected in culture.

Listeria spp. are shed in the feces of asymptomatic animal carriers. Therefore, contamination of meat is normally due to faecal contamination during the slaughtering process (T. Jemmi et al., 2006).

Cross-contamination, which can occur within the environment of food-processing equipment, is considered to be a possible source of *Listeria* contamination in processed food. *Listeria monocytogenes* is able to attach to and survive on various working contact surfaces (Romanova et al., 2002). One reason may be its ability to form biofilms (Boruki et al., 2003).

The conventional serotyping slide agglutination technique has been uses with great success in diagnostic and epidemiological investigations but is not routinely used because of the cost factor associated with the requirement of purchasing the whole spectrum of type specific antisera.

Presence of *Listeria monocytogenes* in fresh snails samples indicates a primary contamination from the soil, water, or plants (Kirkan, 2006).

Serological determination of Listeria monocytogenes strains isolated from meat

Serological determination was realized only by confirmed samples. Three serotypes were found in meat isolates. Nine strains (75%) isolated from raw meat (sheep, poultry, pork, bovine and snails meat) belonged to serotype 1/2c. Two strains (16,66%) isolated from snail

meat belonged to serotype 1/2b and 1 strain (8,34%) also isolated from snails meat belonged to serotype 4b. The results are showed in table 2.

Other studies have shown that *L. monocytogenes* strains isolated from meat processing environments are frequently of serotypes 1/2a, 1/2/b and 1/2c. This may be associated with enhanced capacity to *L. monocytogenes* serotype 1/2c to attach the stainless steel surfaces and form biofilms, in food-processing environments. Most human clinical isolates belong to three serotypes 1/2a, 1/2b and 4b (Lunden et al., 2000). Serovar 4b is the most frequently involved in causing disease, especially in pregnant women (Răpuntean, 2005).

Table 2. *Listeria monocytogenes* serotypes isolated from meat samples

Meat samples	**Positive *L. monocytogenes* samples**	**1/2b**	**1/2c**	**4b**
Raw sheep meat	3	-	3	-
Raw poultry meat	1	-	1	-
Raw pork meat	2	-	2	-
Raw bovine meat	2	-	2	-
Snail meat	4	2	1	1
Total (%)	12	2 (16,66%)	9 (75%)	1 (8,34%)

The current reference methods for the detection of *L. monocytogenes* allow the recovery of this pathogen from a variety of foods with relative ease. The introduction of chromogenic media efficiently improved the isolation of *L. monocytogenes*. Food producers and distributors have great interest in more rapid methods, which has helped to bring about the desired changes in the available technology. (Jantzen et. al., 2006).

Commercial kits based on real-time PCR and other molecular techniques are available for the identification of *L. monocytogenes* in foods are: BAX Detection System (DuPont-Qualicon), The LightCycler® *Listeria monocytogenes* Detection Kit (Roche/Biotecon), or Probelia (Bio-Rad).

Conclusions

1. Fifteen samples were isolated from meat samples by classical microbiological method, but only 12 were confirmed by real-time PCR.
2. The real-time PCR kits are very rapid, sensitive and accurate compared with traditional methods wich takes 4-5 days.
3. The meat contamination could be attributed to the practices on the sanitary management of the animals, or slaughtering processes.
4. Three serotypes were found in meat: 1/2b, 1/2c and 4b.
5. The 4b serotype is most frequently isolated from human listeriosis.

Acknowledgments

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References

1. Borucki, M.K.; Peppin, J.D.; White, D.; Loge, F.; Call, D.R. (2003). Variation in biofilm formation among strains of *Listeria monocytogenes.* Appl. Environ. Microbiol. 69:7336-7342.
2. Janzten, M.M.; Navas, J.; Corujo, A.; Moreno, R.; López, V.; Martínez-Suárez, J.V. (2006). Specific detection of *Listeria monocytogenes* in foods using commercial methods: from chromogenic media to real-time PCR. Spanish Journal of Agricultural Research. 4:235-247.
3. Jemmi, T.; Stephan, R.(2006). *Listeria monocytogenes*: food-borne pathogen and hygiene indicator. Rev. Sci. Tech. Off. int. Epiz. 25:571-580.
4. Kirkan, Ş.; Göksoy, E.; Kaya O. (2006). Detection of *Listeria monocytogenes* by using PCR in Helix pomatia. Turk. J. Vet. Anim. 30:375-380.
5. Liu, D. (2006). Identification, subtyping and virulence determination of *Listeria monocytogenes,* an important foodborne pathogen. J. Med. Microbiol.. 55:645–659.
6. Lundén, J.; Miettinen, M.K.; Autio, T.J.; Korkeala, H.J. (2000). Persistent *Listeria monocytogenes* strains show enhanced adherence to food contact surfaces after short contact time. J. of Food Prot. 63:1204 –1207.
7. Makino, S.I.; Kawamoto, K.; Takeshi, K.; Okada, Y.; Yamasaki, M.; Yamamoto, S.; Igimi, S. (2005). An outbreak of food-borne listeriosis due to cheese in Japan, during 2001. Int. J. Food Microbiol. 104:189–196.
8. McLauchlin, J.; Mitchell, R.T.; Smerdon, W.J.; Jewell, K. (2004). *Listeria monocytogenes* and listeriosis: A review of hazard characterisation for use in microbiological risk assessment of foods. Int. J. Food Microbiol. 92:15–33.
9. Răpuntean Gh., Răpuntean S. (2005). Bacteriologie veterinară specială. Ed. AcademicPres. 38-46
10. Romanova, N.; Favrin, S.; Griffiths, M.W. (2002). Sensitivity of *Listeria monocytogenes* to sanitizers used in the meat processing industry. Appl. Environ. Microbiol. 68:6405–6409.
11. Vanegas, M.C.; Vasquez, E.; Martinez, A.J.; Rueda, A. (2009). Detection of *Listeria monocytogenes* in raw whole milk for human consumption in Colombia by real-time PCR. Food Control, 20:430-432.

A CLINICAL STUDY OF GOATS CONTAGIOUS ECTHYMA IN ROMANIAN FARMS

Tiberiu CONSTANTIN, Stelian BĂRĂITĂREANU
University of Agronomic Sciences and Veterinary Medicine – Faculty of Veterinary Medicine, Bucharest, Romania; doruvet@yahoo.com

Abstract
Contagious ecthyma, also called contagious pustular dermatitis is a viral dermatitis of goats, sheep, and wild ruminants worldwide. Contagious ecthyma induces acute pustular lesions that progress to scabs in the skin of the lips, around the outside and inside of the mouth, face, ears, vulva, lets, scrotum, teats, and feet, usually around the coronet and in the interdigital region. Usually, contagious ecthyma would be estimated to last between four to six weeks and restore to health spontaneously, without any form of intervention. The severe and extensive forms of contagious ecthyma are rarely and usually described in young goats. In this study two goat flocks with 503 animals (84 kits) were clinically evaluated to identify and characterize contagious ecthyma lesions, in the period October 2010 - March 2012. The results confirmed the presence of contagious ecthyma cases in both flocks in a proportion of 2.38% (12/503). Lesions were located in the skin of lips (100%), muzzle (91.66%), ears (66.66) and feet (50%). One kid had generalized contagious ecthyma.
Keywords: sore mouth, scabby mouth, goat orf infection, contagious pustular dermatitis

Contagious ecthyma (Gk, *ek*, out, *thyein*, to rush) is a common infectious and contagious disease that can affect sheep, goat, wild ruminants (steenbok, alpacas, chamois, thar, reindeer, musk ox, mountain goat, bighorn sheep, dall sheep) as well as human beings and canides [8, 11].

In the end of the 19th century, for first time, in Germany is described contagious ecthyma in small ruminants [2, 13]. In Romania, close to Bucharest, Paul Riegler identified in 1935 a similar disease in sheep and goat herds [14]. Today, contagious ecthyma has been found worldwide, especially in countries that raise sheep and goats [13]. The young animals (4 - 6 months) are most affected, registering the worst forms, goats are more sensitive than sheep, and the improved goat breeds are more susceptible (Boer or Boer crosses, more rarely Saanen) [11, 12]. Most vulnerable year periods are seasons with extreme temperatures (late summer and winter), when the immune system of the animals can be affected by sudden changes of temperature [2, 9, 13].

After 8-10 days of incubation [9], the goats with contagious ecthyma develop papillomatous lesions around the mouth and nostrils. The infection may also affect ears, inside of the mouth, lower legs and udder of nursing ewes. Usually, contagious ecthyma would be estimated to last between four to six weeks and recovery occurring without treatment. Nevertheless, the lesions can develop into extensive disease, with secondary bacterial infection, and sometimes fatality [6, 9, 14, 15].

Diagnosis is based on the herd history and the lesion characteristics, but the accurate diagnosis is based on immunologic tests and viral isolation [9].

The economical losses are due to the decreased of dairy goat production, and lower growth and mortality in young goats [4]. In young goats, morbidity can approach 100% and mortality up to 20%. [7, 10, 13].

The first objective of the goat contagious ecthyma study was the evaluation of the new clinical cases present in period October 2010 - March 2012, in goat flocks located in

different counties. The second objective was the analyses of the dominant pathological aspects associated with the contagious ecthyma in young goats.

Materials and method

The research was conducted in two flocks with 503 goats, located in two Romanian counties (Braila and Giurgiu). The goat breeds have been Banat White, France Alpine, Charpatian, and their cross-breeds. Goats were raised in a mixed management system (range pastures and shaded pens). All goats were not vaccinated against orf infection.

The method used in this research consist in physical exam of animals that were evaluated for specific contagious ecthyma lesions (multifocal areas of papules, blisters, pustules, crusts or papillomatous proliferations) around of the mouth (lips, muzzle), ears, feet (interdigital region), teats and vulva/scrotum.

Results and discussion

All adult goats (419/503) were without papule, vesicle, pustule or crusted lesion on the skin. In both flocks were identified 84 young goats, with age between two and16 weeks. The lesions described in contagious ecthyma were observed in young goats at a rate of 14.28% (12/84).

The most common location of contagious ecthyma lesions has been around the mouth (fig 1). The lesions in the skin of lips have been in all clinical cases (12/12), and the muzzle lesion in 91.66% (11/12). Progressive evolution of the contagious ecthyma was previously described in several experimental infections. Lesions on the lips appear about 4-7 days after infection, initially patches with a diameter of 0.5 mm, often go unnoticed are observed. The lesions have been on the soft skin areas or crossing of mucous membranes and skin (lips, commissural, on the wings of the nose). Macules fast moving up to the state of the crust over a period of 1-2 weeks (vesicle, with 1-2 mm diameter, passes the pimple in about 2 days) [9, 11, 13].

In 66.66% (8/12) of clinical cases were observed lesions in the skin of ears (fig 2). The ear lesions progressed through the same sequence of stages: papule, vesicle, pustule and crusted lesion. In sheep lesions were associated with ear tagging [1].

Foot lesions in young goat were described in 50% (6/12) of cases (fig 3). Clinical signs identified were similar to those described in other papers. The foot lesions affect the coronary band and soft tissue of interphalangeal area. The hair was sintered, wet, and it falls off with cutaneous layers (fig. 4). Foot lesions can evolve independently, but often are accompanied with other skin lesions. A new epithelium is forming in two weeks.

In other clinical studies, foot lesions have been described as the most common location observed due to overlapping injuries and co-infection (e.g., *Fusobacterium necrophorum, Dichelobacter nodosus*) that masks the characteristic appearance [11, 13].

The lesions inside of the mouth were observed in only one kid, classified as generalized contagious ecthyma. Lesions were located in soft gingival tissue, tongue, cheeks, hard palate, typically up to the pharynx, esophagus, ruminal pillars, sometimes abomasums and intestines. The lesions were ulcerative, red-brown and swollen. Mandibular and retropharyngeal lymph nodes were sensitive.

Table 1. Location of lesions observed in twelve clinical cases of contagious ecthyma in two goat flocks with 503 animals, located in Braila and Giurgiu counties

Location	Clinical cases	
	%	No.
Lips	100	12/12
Muzzle	91.66	11/12
Ears	66.66	8/12
Feet	50	6/12
Other locations (generalised form)	8.33	1/12

In generalized contagious ecthyma, the trachea and lungs infection have been rare reported. Animals have difficulty grasping, chewing, swallowing and rumination [3, 11, 13]. The young animals with mucous membrane lesions usually die because of starvation. Fungus as *Candida albicans* can grafts on the ulcerated mucosa [7]. Also, in severe forms of disease, scabs confluence and they harden and the yellow colour changes into brown. Brown scabs can persist for three weeks, and are itchy, painful, ihor, and make prehension and mastication difficult. Nostrils can be obstructed. Favorable areas for various pyogenic bacteria or larvae will appear when the scabs are removed [11, 13]. In the U.S.A., until fly *Cochliomyia hominivorax* was eradicated, its larvae produced increased mortality due to necrotic tissue development [8]. In more severe cases, the tissues are getting away from the deep scales remaining bleeding areas [5, 11, 13].

Fig. 1. Kid with severe, hard and dry proliferative contagious ecthyma lesions in the skin of lips and muzzle

Fig. 2. Kid with papillomatous proliferative contagious ecthyma lesions in the skin of ear

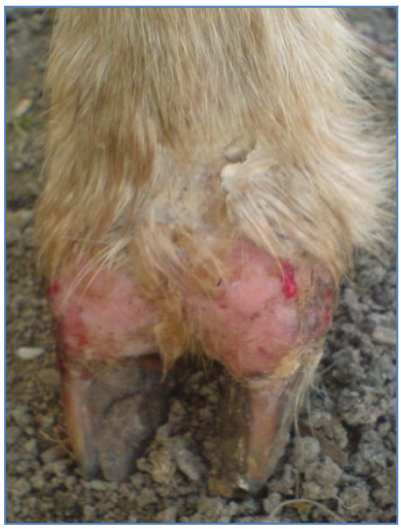

Fig. 3. Contagious ecthyma foot lesions in young goat. Denudated skin lesions with moist and ulcerative lesions in the coronet

Fig. 4. Hard, dry and crusty contagious ecthyma foot lesions in young goat

Conclusion

This study indicates that in period October 2010 - March 2012, in two flocks located in Braila and Giurgiu counties, contagious ecthyma has been a proportion of 2.38% (12/503). All lesions were observed in young goats (12/84), and clinical disease weren't observed in adult goats (0/419). Both farms haven't vaccination program for goat orf infection, and this low proportion of the clinical cases suggest that the contagious ecthyma is endemic in both flocks. Lesions were located in the skin of lips (100%), muzzle (91.66%), ears (66.66) and feet (50%). The lesions inside of the mouth were observed in only one kid with generalized contagious ecthyma.

Bibliography

1. Allworth, M.B., Hughes, K.L., Studdert, M.J., Contagious pustular dermatitis (orf) of sheep affecting ear following ear tagging. Aust. Vet. J., 1987; 64:61–62.
2. Bassioukas, K., Orfanidou, A., Stergiopoulou, C.H., Hatzis, J., Orf. Clinical and epidemiological study. Aust. J. Dermatol., 1993; 34:119–123.
3. De la Concha-Bermejillo, A., Guo, J., Zhang, Z., Waldron, D., Severe persistent orf in young goats, J. Vet. Diagn. Invest., 2003; 15:423–431.
4. Guisasola, O.J.I., Ectima contagioso (ovino), RACVE Conferencia, Sección: Patología Médica, Madrid, 29 nov 2006.
5. Guo, J., Zhang, Z., Edwards, J.F., Ermel, R.W., Taylor, C., De la Concha-Bermejillo, A., Characterization of a North American orf virus isolated from a goat with persistent, proliferative dermatitis, Virus Res., 2003; 93:169–179.
6. Hawkins, C.D., Ellis, T.M., Davies, M.K., Peet, R.L., Parkinson, J., An unusual outbreak of contagious ovine ecthyma. Aust. Vet. J., 1991; 68:210–211.
7. Hopkins M. F., Warren, G., Soremouth in sheep, Sheep Extension Program, Univ. of Tennessee, [http://animalscience.ag.utk.edu] (accesed: April 03, 2012).

8. Merck Veterinary Manual. Contagious ecthyma (orf, contagious pustular dermatitis, sore mouth). Whitehouse Station, NJ: Merck & Co., Inc. 2006. [http://www.merckvetmanual.com] (accesed: April 03, 2012).
9. Radostits, O.M., Gay, C.C., Hinchcliff, K.W., Constable, P.D., Veterinary Medicine, A Textbook of the Diseases of Cattle, Horses, Sheep, Pigs and Goats. 10th Edn., W.B. Sounders, London, New York, Oxford. 2008.
10. Reindl, N.J., Wolff, P.L., Bjork, C. L., Female fecundity, neonatal mortality and the impact of contagious ecthyma on a captive herd of muskoxen, Paper presented at The First Arctic Ungulate Conference, Nuuk, Greenland, 3-8 September, 1993.
11. Schoenian, S., Soremouth (orf) in sheep and goats. Small Ruminant Info Sheet. 2010. [http://www.sheepandgoat.com/articles/soremouth.html] (accesed April 03, 2012).
12. Spickler, A.R., Contagious ecthyma. Last Updated: April 14, 2007, [http://www.cfsph.iastate.edu/DiseaseInfo/factsheets.php] (accesed April 03, 2012).
13. Tórtora, L.J. Ectima contagioso de ovinos y caprinos, Ciencia veterinaria. Vol.4 www.fmvz.unam.mx/fmvz/cienciavet/revistas/CVvol4/CVv4c9.pdf., 1987.
14. Vasiu, C., Viroze specific ovinelor si caprinelor. in Viroze la animale, Ed. Dokia, Cluj-Napoca, 1998; 177-182.
15. Yeruham I., Perl, S., Abraham, A., Orf infection in four sheep flocks. Vet J., 2000; 160:74–76.

MICROBICIDAL ACTION OF SOME POLYPHENOLS ON *PROTOTHECA* ISOLATES FROM BOVINE MASTITIS

Cosmina BOUARI, Pompei BOLF, Gabi BORZA, Nicodim FIŢ, George NADĂŞ, Flore CHIRILA, Adrian GAL, Cornel CATOI
University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca,
Faculty of Veterinary Medicine, Manastur street, 3-5 no., code 400372, Romania
cosminacuc@yahoo.com

Abstract
Bovine mastitis caused by Prototheca spp. can assume high significance because of economic losses and the potential risk to public health. In this study we investigated the in vitro inhibitory activity of some polyphenols such as: carvacrol, α pinene, β pinene, catechin, epigallocatechin gallate (EGCg) comparative with antifungal agent – amphotericin B against twelve clinically isolates of Prototheca zopfii by the international guidelines M27A2. All isolates exhibited a good susceptibility to carvacrol (MIC 2-4 µg/ml) and catechin (MIC 4-8 µg/ml); they were less susceptible to other antifungal agents, such as amphotericin B. These activities of carvacrol and catechin were approximately 3-fold, respectively 2-fold higher than those of amphotericin B. This result indicates these polyphenols can inhibit pathogenic Prototheca species. Therefore, we suggest that carvacrol and catechin may be effectively used solely or combined as a possible agent for antifungal therapy in protothecosis.
Keywords: *Prototheca*, carvacrol, catechin, bovine mastitis

Introduction

Algae of the genus *Prototheca*, are the only known plant-like organisms that cause infectious diseases in humans and animals (Marques 2006, Moller 2007). The genus *Prototheca* consists of microscopical, unicellular, achlorophyllic algae with asexual reproduction by autosporulation with variable numbers of sporangiospores (DiPersio 2001, Malinowski 2002). They are resistant and ubiquitous and can be isolated from a great variety of environmental sources (Melville et al., 1999; Bexiga et al., 2003; Zhao et al., 2004). Only 2 species are pathogenic, *Prototheca wickerhamii* and *Prototheca zopfii* (Pore, 1998; Malinowski et al., 2002; Melville et al.,2002). *P. wickerhamii* is generally associated with human pathology presenting essentially cutaneous or subcutaneous lesions and also more rarely generalized infections (Zaitz et al., 2006; Hightower and Messina, 2007; Lass-Florl and Mayr, 2007; Narita et al., 2008). On the other hand, *P. zopfii* has been associated with animal infections and mainly bovine mastitis (Moller 2007).

Cattle udder infections caused by Prototheca spp. strains have been linked to the constant contact of the mammary gland with water sources on the dairy. All stages of lactation appear to be equally susceptible to new infections including dry cows. Mammary gland infections caused by *Prototheca* strains are rarely observed with clinical signs. Non-clinical outbreaks are frequently characterized by the secretion of milk characterized by a normal or only slightly increased number of somatic cell count. In some cases a reduced milk production is sometime observed in cows with subclinical infections. Due to the determinal impact of *Prototheca* infections on milk quality and quantity and to the lack of response to most antibiotic treatment, the only control method to date has been the elimination of the infected animals (Costa et al., 1997; Buzzini et al., 2008).

There are many studies regarding the *in vitro* efficiency of some antimicrobial compounds, but the development of resistance towards these agents such as: amphotericina

B, Ketoconazole, Fluconazole, is an important scientific interest in the discovery of an other new class of compounds.

In this way our study aimed to investigate and to compare the *in vitro* efficiency of some polyphenols with antimicrobial activity.

Materials and methods

Research performed for testing the polyphenols, with possible application in protothecosis therapy were conducted during February-April 2012, within Microbiology Laboratory, Faculty of Veterinary Medicine Cluj-Napoca. A total of 12 *P. zopfii* isolates collected from cows mastitic milk samples, and 1 *P. wickerhamii* referent strain (RE-4608014ATCC16529), from American Type Collection, were used for the study. The isolates were identified on the basis of morphological, cultural aspects and on biochemical features (assimilation of glucose, galactose, glycerol, sucrose and trehalose, and growth at 28 and 37ºC).

In vitro susceptibility testing were performed using both classical diffusimetric and broth microdilution methods, following the CLSI (formerly NCCLS – The National Committee for Clinical Laboratory Standards) guidelines based on documents M27-A2 for yeasts.

Five natural compounds: carvacrol, αpinen, βpinen, catechin, EGCg - acquired as standardized products (Carl roth, Germany) – and one antifungal drug (Amphotericin B) (Janssen Research Foundation, Bersee, Belgium) – were investigated.

• Carvacrol, or cymophenol, $C_6H_3CH_3(OH)(C_3H_7)$, is a monoterpenoid phenol, obtained from S*atureja hortensis* (savory) essential oil,and it is new due to the ability to inhibit the growth of several bacteria strains e.g. *E. coli*, *Bacillus cereus*, *Pseudomonas aeruginosa* (Du et al., 2008, Cox and Markham, 2007).

• Pinene ($C_{10}H_{16}$) is a bicyclic monoterpene chemical compound.There are two structural isomers of pinene found in nature: α-pinene and β-pinene, both forms are important constituents of pine resin.

• Catechins are natural phenols antioxidant, the major component of green tea extract, have various antioxidant, antimicrobicidal, anticarcinogenic effect and reduction in atherosclerotic plaques (Chyu et al., 2004, Mittal et al, 2004).

Algae were inoculated into glucose-agar and after 48 hours growth, colonies were directly suspended in saline solution so that turbidity matched the turbidity corresponding of 2 tube McFarland standard, which is 600x106 CFU/ml. Kirby Bauer diffusimetric method wes performed by using sterile glucose agar plates with seven wells. In each well wes distributed 50 µl for each products tested. The plates were incubating in aerobiotic condition at 37º for 48 hours and the results were appreciated according the inhibition diameter area.

For broth microdilution method tests were perfomed in sterile U-bottom 96-well plates. Ten different dilutions of each natural compound tested in broth supplemented with 2% glucose were prepared. The positive growth control wells without polyphenols (antimicrobial agent) and the negative control wells (without *Prototheca* suspension) were also prepared. The plates were incubated for 48 h, at 37ºC, under aerobiotic conditions. Tests were performed in duplicate. Microplates were read visualy.

Result and discussion

Tests performed in order to establish the effect of polyphenols upon *Prototheca* in vitro growth showed different aspects according from the plants from which polyphenol was obtained, to the species tested and also according to the concentration used.

Table 1 summarizes the *in vitro* susceptibility profile of *Prototheca* isolates to natural products comparative with synthetic drug (amphotericin B).

All strains tested were susceptible to carvacrol and catechin. More than that a strong potentiatig effect was revealed between two compounds. Epigalo catechin gallat (EGCg) and Amphotericin B proved to be efficient at a higher MIC (10-14 µg/ml). All *Prototheca zopfii* strains were shown to be *in vitro* resistant to αpinen and βpinen (fig. 1, 2).

A graphic representation of the average of diameters of inhibition area for each natural product tested compared to synthetic drug is depicted in the fig. no 3.

Although there are many reports regarding the effectiveness of some plant extracts (*Maleleuca alternifolia, Citrus bergamota*) in wich concern the assessement of antifungal effect of carvacrol and catechin upon *Prototheca in vitro* growth we can say that the study undertaken have global priority us there are no similar studie. Our research realised in order to found a new antimycotic compound,with possible application in bovine mastitis protothecosis therapy, allow us to assert that the results obtained encourages the used of products such as: carvacrol and catechine.

Table 1. *In vitro* susceptibility profile of two *Prototheca species* to natural products tested

Isolates tested		Polyphenols					
		Carvacrol	αpinen	βpinen	EGCg	Catechin	Amphotericin B
P. zopfii	1	42	-	-	20	20	20
	3	44	-	-	-	22	30
	4	46	-	-	-	24	-
	5	48	15	-	-	40	-
	8	45	-	-	-	45	-
	10	44	-	-	18	45	-
	12	42	-	-	-	28	12
	17	44	12	-	15	22	-
	19	46	-	-	-	45	-
	20	44	-	-	12	40	20
	22	48	-	-	-	20	-
	28/9	47	-	-	12	22	30
Average of inhibition area (mm in diameter)		45	-	-	6	31	10
P. wickerhamii		48	-	-	-	40	32

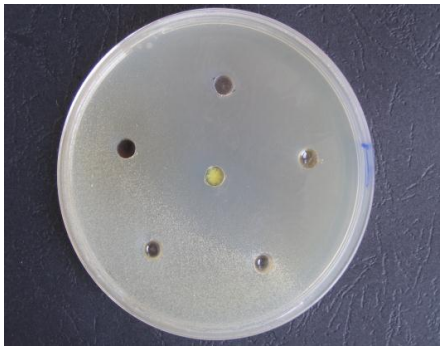

Fig.1. P. zopfii (5 isolates)-the efficiency of polyphenols compared with Amphotericin B

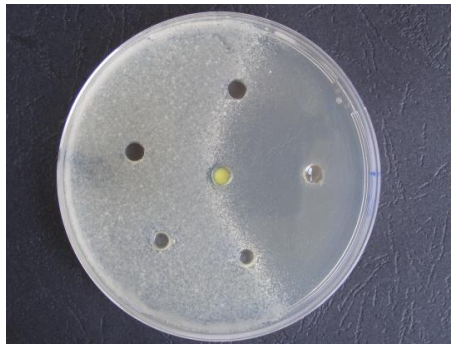

Fig.2. P. zopfii (4 isolates)-potentiating area between carvacrol and catechin; resistance to

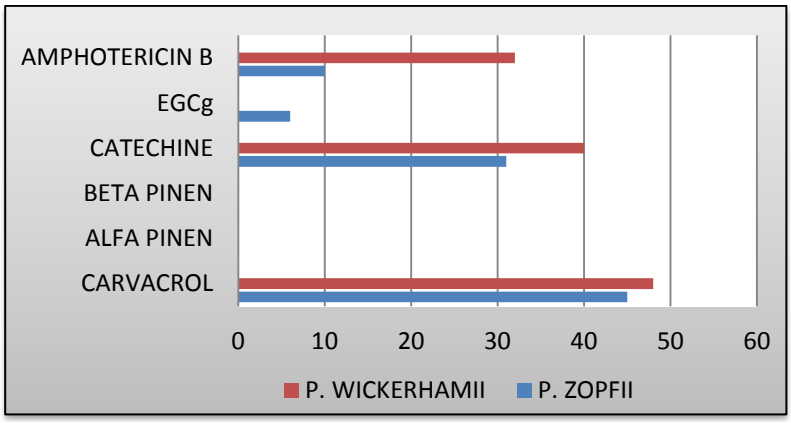

Fig. 3 Average efficiency of polyphenols tested compared with Amphotericin B for both *Prototheca* species

Conclusions

1. All *P. zopfii* tested isolates and *P. wickerhamii* referent strain were shown to be susceptible to carvacrol and catechine and resistant to α,β pinene.
2. *P. wickerhami* prouved to be suscentible to Amphoteicin B, but the efficiency wes lower than these of polyphenols.
3. The results obtained encourage further research towards determining the degree of correlation *in vitro - in vivo* and to determine the appropriate dose for the commencement of *in vivo* experiemtal studies.

Acknowledgments

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References

1. Bexiga, R., L. Cavaco, and C. L. Vilela. 2003. Isolation of *Protothecazopfii* from bovine milk. Rev. Port. Cienc. Vet. 98:33–37..
2. Buzzini P, Turchetti B, Facelli R, et al 2004 First large-scale isolation of Prototheca zopfii from milk produced by dairy herds in Italy. Mycopathologia;158:427-30.
3. Chyu KY; Babbidge, SM; Zhao, X; Dandillaya, R; Rietveld, AG; Yano, J; Dimayuga, P; Cercek, B et al (2004). "Differential effects of green tea-derived catechin on developing versus established atherosclerosis in apolipoprotein E-null mice". *Circulation* 109 (20): 2448–53.
4. Costa, E.O., Melville, P.A., Ribeiro, A.R., Watanabe, E.T., Parolari, M.C., 1997. Epidemiologic study of environmental sources in a Prototheca zopfii outbreak of bovine mastitis. Mycopathologia 137, 33–36.
5. Cox SD, Markham JL (2007). "Susceptibility and intrinsic tolerance of *Pseudomonas aeruginosa* to selected plant volatile compounds". J. Appl. Microbiol. 103 (4): 930–6
6. DiPersio, J. R. 2001*. Prototheca and protothecosis.* Clin. Microbiol. Newsl. 23:115-*120*
7. Du WX, Olsen CE, Avena-Bustillos RJ, McHugh TH, Levin CE, Friedman M (2008). "Storage Stability and Antibacterial Activity against *Escherichia coli* O157:H7 of Carvacrol in Edible Apple Films Made by Two Different Casting Methods". *J. Agric. Food Chem.* 56 (9): 3082–8.
8. Hightower, K.D., Messina, J.L., 2007. Cutaneous protothecosis: a case report and review of the literature. Cutis 80, 129–131.
9. Lass-Florl, C., Mayr, A., 2007. Human protothecosis. Clinical Microbiology Reviews 20, 230–242.
10. Marques, S., E. Silva, J. Carvalheira, and G. Thompson. 2006*. In vitro antimicrobial susceptibility of Prototheca wickerhamii and Prototheca zopfii isolated from bovine mastitis.* J. Dairy Sci. 89:4202-*4204*
11. Malinowski, E., H. Lassa, and A. Klossowska. 2002*. Isolation of Prototheca zopfii from inflamed secretion of udders.* Bull. Vet. Inst. Pulawy 46:295-*299*
12. Marques S, E. Silva, J. Carvalheira, and G. Thompson. 2006. In Vitro Antimicrobial Susceptibility of *Prototheca wickerhamii* and *Prototheca zopfii* isolated from Bovine Mastitis, J. Dairy Sci. 89:4202–4204.
13. Melville, P. A., E. T. Watanabe, N. R. Benites, A. R. Ribeiro, J. A.Silva, J. F. Garino, and E. O. Costa. 1999. Evaluation of the susceptibility of Prototheca zopfii to milk pasteurization. Mycopathologia 146:79–82.
14. Melville, P. A., N. R. Benites, I. L. Sinhorini, and E. O. Costa. 2002.Susceptibility and features of the ultrastructure of *Prototheca zopfii* following exposure to copper sulphate, silver nitrate and chlorexidine. Mycopathologia 156:1–7.
15. Möller, A., U. Truyen, and U. Roesler. 2007. Prototheca zopfii genotype 2—the causative agent of bovine protothecal mastitis? Vet. Microbiol. 120:370-374.
16. Mittal A, Pate MS, Wylie RC, Tollefsbol TO, Katiyar SK (2004). "EGCG down-regulates telomerase in human breast carcinoma MCF-7 cells, leading to suppression of cell viability and induction of apoptosis". *Int. J. Oncol.* 24 (3): 703–10.
17. Moroni, P., 2008. Large-scale screening of the in vitro susceptibility of Prototheca zopfii towards polyene antibiotics. Medical Mycology 46, 511–514.
18. Narita, M., Muder, R.R., Cacciarelli, T.V., Singh, N., 2008. Protothecosis after liver transplantation. Liver Transplantation 14, 1211–1215.
19. National Committee For Clinical Laboratory Standards, Reference method for broth dilution testing of yeasts, Approved standard M27-A2. 2-nd ed. NCCLS, Wayne, Pa. (2002).
20. Pore, R. S. 1998. *Prototheca* and *Chlorella.* Pages 631–643 in Topley& Wilson's Microbiology and Microbial Infections. Vol. 4. 9th ed. L. Ajello and R. J. Hay, ed. Arnold Publications, London, UK.
21. Zaitz, C., Godoy, A.M., Colucci, F.M., de Sousa, V.M., Ruiz, L.R., Masada, A.S., Nobre, M.V., Muller, H., Muramatu, L.H., Arrigada, G.L., Heins-Vaccari, E.M., Martins, J.E.,2006. Cutaneous protothecosis: report of a third Brazilian case. International Journal of Dermatology 45, 124–126.
22. Zhao, J., W. Liu, G. Lv, Y. Shen, and S. Wu. 2004. Protothecosis successfully treated with amikacin combined with tetracyclines. Mycoses 47:156–158.

FROM THE HISTORY OF THE ROMANIAN SCIENTIFIC SOCIETIES OF VETERINARY MEDICINE

D. CURCĂ*, Ioana Cristina ANDRONIE V. ANDRONIE****
*Faculty of Veterinary Medicine Bucharest, Romania
**University Spiru Haret, Bucharest, Romania,
curca_fiziopat@yahoo.com

Abstract

The union of the young veterinary surgeons who decided to establish the Scientific Medical Society of Romania, at the Veterinary School of Bucharest. The "Statute of the Scientific Medical Society from Romania" drawn in the first meeting of 15 May 1871, were voted in the general assembly of August 14-16, 1871, chaired by Mauriciu Colben. The Scientific Medical Society didn't have its own journal upon establishment. Therefore, in 1879, Alexandru Locusteanu, Mihai Măgureanu, Gheorghe Perşu and Panait Constantinescu, publish the ***Veterinary Surgeon****. Succeeding to dismiss some difficulties, 33 veterinary surgeons met on 10-12 May 1882 in Bucharest for the First Congress of Veterinary Medicine, the first of this kind in Romania. The topics of the congress, set in 1881, included the following subjects: sanitary police, animal husbandry, public hygiene, pathology, miscellanea. Once the Society of Veterinary Medicine was relaunched, the* ***Journal of Veterinary Medicine****, Animal Husbandry, Hygiene and Rural Economy also appeared. It has been established in 1888 too, by I. Şt. Furtună, and had an Annex, the Bulletin of the Society of Veterinary Medicine. These meetings debated the important problems of the veterinary pathology confronting that period: the foot and mouth disease, of horses glanders, tuberculosis, the bovine gastro-entero-nephritis (babesiosis) etc. The Society existed until July 21, 1949, when, after this last meeting, it was discontinued abusively by the communist regime. Because the centenary of the Romanian Society of Veterinary Medicine was closing, a group of teaching staff from the Faculty of Veterinary Medicine of Bucharest started in the spring of 1970, the activities necessary to resume the Romanian Society of Veterinary Medicine: it was re-established on May 15, 1971, 22 years after its discontinuation and one hundred years after its foundation.*

Keywords: history, scientific societies, Romanian journals

In the Romanian Principalities, because no schools of veterinary medicine existed yet at that time, the authorities employed numerous "masters in surgery and veterinary medicine" who had graduated at Vienna, Pesta, Paris, Lyon etc., and who, after 1831, activated in different army units, in agreement with the "Military rules".

After several failed attempts to establish a school of veterinary medicine, in 1853 the first courses of veterinary medicine started in Bucharest, taught, as the documents show, by the master in surgery Vasile (Wolfgangus) Lucaci (Fig. 1.), the future proto-veterinarian of Wallachia. The courses entitled "Lectures of veterinary medicine, particularly about the infectious diseases of the animals" were not taught, however, in an actual veterinary school, but in a school teaching mainly human medicine, including in the school established by Nicolae Crețulescu (Fig. 2.) which functioned between 1841-1846, within Colțea Hospital.

After the establishment of the "Medical human and veterinary education" in 1853, by Carol Davila (Fig. 3.), the veterinary medicine education was performed until 1859 at Mihai Vodă Monastery, in whose cells the Army Hospital has been functioning even since 11831. The first School of Small Surgery functioned here too (1853-1859), which subsequently became the School of Medicine and Pharmacy (5, 14, 17, 22), whose professor of "veterinary medicine" was Vasile (Wolfgangus) Lucaci; human medicine was taught by Professor dr. Iacob Felix, Professor dr. Iuliu Teodori and Professor dr. L. Fialla.

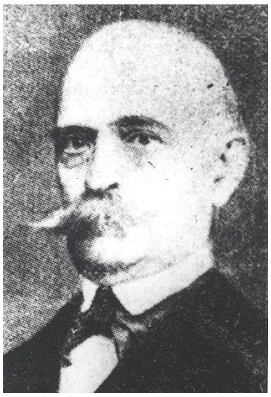


Fig.1. State protoveterinarian Vasile (Wolfgangus) Lucaci (1806 – 1890)

Fig. 2. Professor Dr. Nicolae Crețulescu (1812-1900)

Fig. 3. Professor General Doctor Carol Davila (1828-1884)

During the early days of the veterinary school (1853), after the establishment of the Veterinary School (1856) and until 1887 (Fig. 4.), the teaching activity was characterized by material difficulties and repeated changes of the regulations of operation. Most of the didactic staff consisted of military doctors, employees of army units, who were departing for military assignments and left aside the teaching activity (9, 19, 20, 21).

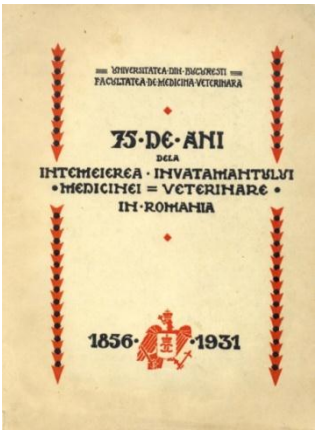


Fig. 4. 75 years anniversary of founding the Veterinary Medical Education in Bucharest-Romania

Fig. 5. Central pavilion of the veterinary campus, on Splaiul Independence 105

Among 1885-1887, the building of the Higher School of Veterinary Medicine was constructed at Splaiul Independenţei nr. 105 (Fig 5), the building plans being developed by architect Nicolae Cerkez. During period of construction the head of the School was Dr. Ion Popescu (1885-1890).

At that time, the head veterinarian of the Romanian army was Mauriciu Kolben, from 1871 until his retirement in 1893. In this position, M. Kolben organised the military veterinarian service and struggled to send abroad veterinary doctors for training, particularly in animal husbandry and in the diagnosis and treatment of the contagious diseases in farm animals.

On August 27, 1873, the Ministry of Education announced a competition for the open positions of professor at the School of Veterinary Medicine. Louis Vincent was among the candidates for a position at the department of anatomy, physiology, zoology and surgery. In the same spirit, Dr. Mihail Măgureanu the veterinarian of Bucharest volunteered to teach pro bono "meat inspection". After the ministry approved, the courses started in 1880 (see Medicul veterinar, 1880, I, p. 99).

In 1874, General E. Florescu, Ministry of War, established a commission consisting of several military officers among whom Professor Mauriciu Colben, the head veterinarian of the Romanian army, whose task was to purchase Thoroughbred Arabian horses from Arabia. In August 1874 they bought 10 stallions, which were sent to Nucet – Dâmboviţa stud, established at that time for this very purpose.

In the first half of the 19th century, in the Romanian Principalities there was a feeling of necessity to establish a scientific forum which to debate the problems of the veterinary medicine profession. This need was particularly felt by the first graduates of the Veterinary School of Bucharest and it was supported by the current created by the human doctors and naturalists from Iasi who had established a Medical Circle of lecture (January 11, 1830), which later became the Society of Doctors and Naturalists from Iasi, headed by doctors Iacob Cihac and Mihai Zotta. A few veterinarians were among the active members of the society (10, 11, and 12). The members of the Society of Doctors and Naturalists from Iasi also published a periodic publication, initially called „Foaia Societăţii", which thereafter became „**Buletinul Societăţii de medici şi naturalişti**" din Iaşi (Bulletin of the Society of Doctors and Naturalists from Iasi); later it changed again its title, becoming "Revista medico-chirurgicală" (Medical-surgical review) which is regularly published up to the present time (Fig. 6.).

One of the major events which preceded the union of the Romanian principalities Walachia and Moldova (done by Alexandru Ioan Cuza in January 1859) was the union of the young veterinary surgeons who decided to establish the Scientific Medical Society of Romania, at the Veterinary School of Bucharest. The documents showing this event are found in file 40/1871 in the State Archives, documents of the Ministry of the Interior, Medical Service. The purpose of the society was to elevate the scientific prestige of the profession of veterinary surgeon.

These documents show that on 15 May 1871, 14 veterinarians drew the draft of a project regarding the "Statute and Regulation of the Society" which, together with a "Statement" were forwarded for approval by the Ministry of Cults and Public Education.

Fig. 6. The evolution of journal named "Society of Physicians and Naturalists of Iassy", from 1830 until now

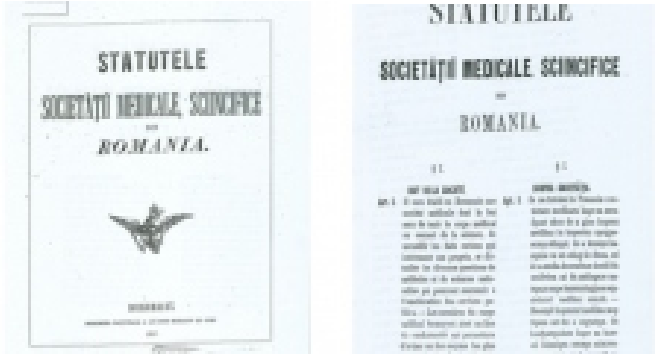


Fig. 7. Statutes of Scientific Medical Society of Romania, published in 1857

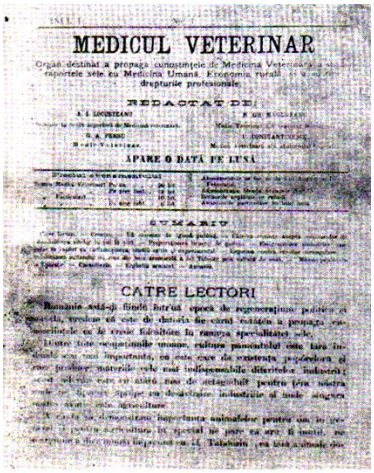
The "Statute of the Scientific Medical Society from Romania" (Fig. 7.), is a very interesting linguistic document, because it is typed on two columns: in the left column the text was types in perfect French, while in the right column the text was typed in a Romanian language of transition, in which not just the words were sometimes approximate, but the text too had both Latin and Cyrillic letters (1, 11, 16).

The "Declaration" specifies that the members of the Society of Veterinary Medicine aimed to "work jointly to perfect the different branches of the science trying to solve all matters" of interest: animal hygiene, reproduction and education of the domesticated animals, animal diseases, epidemics, contagious diseases and forensic medicine".

At the same time, the veterinary doctors pledged "always to consider the general interests and the dignity of their profession" and to "make disappear the obstacles they were confronted so far" so that the "science of veterinary surgeons develops freely", hoping that the "emulation among the veterinarians will bring useful and profitable developments for all".

This "Statement" was signed by: Professor Mauriciu Colben, I. Popescu, I. Ioanin, I. Popovici, I. Georgescu, I. Constantinescu, M. Similache, Andronescu, G. Persu, Gh. Putzurianu, Th. Drăgănescu, D. Preotescu and two more signatures that cannot be deciphered (one for sure belonged to Louis Vincent).The "Statute of the Scientific Medical Society from Romania" drawn in the first meeting of 15 May 1871, were voted in the general assembly of August 14-16, 1871, chaired by Mauriciu Kolben, assisted by Ion Popescu, with secretaries I. Georgescu and a signature that cannot be deciphered, treasurer Dumitru Preotescu, with elected members Panait Constantinescu and Louis Vincent.

The Scientific Medical Society didn't have its own journal upon establishment. Therefore, in 1879, Alexandru Locusteanu, Mihai Măgureanu, Gheorghe Perşu and Panait Constantinescu, publish the „Medicul veterinar" (Veterinary Surgeon) (Fig. 8.) which, unfortunately, cease to appear after exactly 13 issues (Gomoiu V. – History of the Romanian medical press p. 134, Bucharest, 1936).



MEDICUL VETERINAR

Organ destinat a propaga cunostintele de Medicina Veterinara si raporturile sale cu Medicina Umana, Economia rurala si drepturile profesionale.

REDACTAT DE:

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SUMARIU

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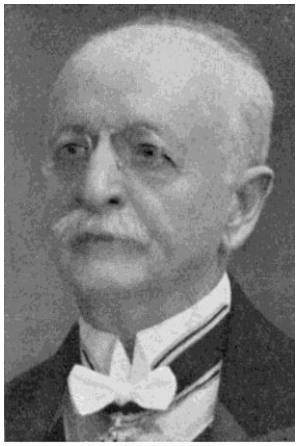


Ion St. Furtuna

Fig. 8. First review of veterinary medical profession entitled "VETERINARIAN" - the first issue appeared in August 1879 and the last number (13), appears in August 1880

Upon initiative of I. Şt. Furtună, by the time he was first year student of the Veterinary School, the students established, as of October 19, 1880, a scientific society, the "Society of veterinary medicine students" located on the premises of the "Higher School of Veterinary Medicine". It intended to have a "library" and a "journal" publishing scientific papers. The journal's title was Progresul veterinar" and the board of editors was headed by student I. Şt. Furtună, until October 18, 1883, when he obtained the license of veterinary surgeon. In 1888, the society voted a new statute responding to the requirements of that time.

At the meeting of 15 February 1893 the "Society of veterinary medicine students" voted the publishing of a journal, as proposed upon the establishment of the society, but the name was „Clinica veterinară" (Fig. 9). Much later, in February 1933, started to be published the Journal of the "Society of veterinary medicine students" (Fig.10).

The purpose of the journal was to publish the clinical observations, the results of the experiments conducted by the students in the laboratories of the Higher School of Veterinary Medicine and to publish translations of papers which the students need.

In "Clinica Veterinară" journal of the veterinary students, the inaugural paper of professor Paul Riegler was "Bacteriological research in glanders diagnosis, 1893". His first publication was "A case of tuberculosis in parrots" and the coincidence was that his last paper was also on fowl tuberculosis, "A serious case of epizootic tuberculosis in a pheasant farm".

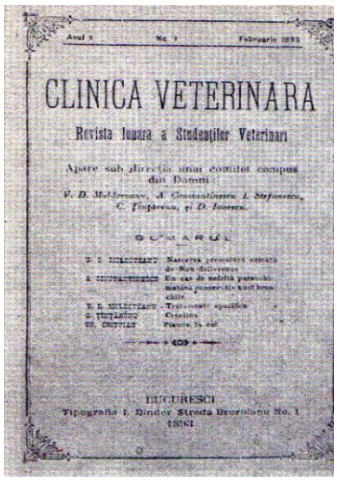


Fig. 9. Veterinary Students Magazine named: Veterinary Clinic, published in February 1893

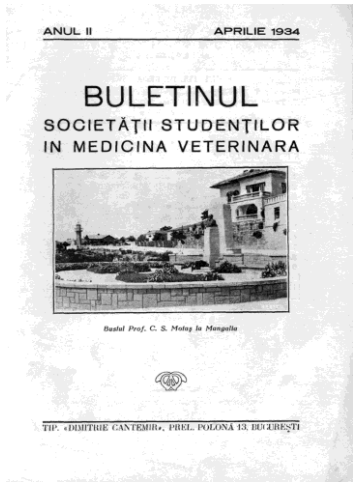


Fig. 10. Veterinary Medicine Students Society Magazine, published in February 1933

The increase of the number of veterinary surgeons who graduated the Veterinary School of Bucharest and the decrease of animal export from the value of 28 million lei in 1877, to just 4 million lei in 1882, prompted the specialists in this field to meet and discuss the problems which caused this alarming decrease and to determine measures for rehabilitation.

Succeeding to dismiss some difficulties, 33 veterinary surgeons met on 10-12 May 1882 in Bucharest for the First Congress of Veterinary Medicine (Fig. 11.), the first of this kind in Romania. The topics of the congress, set in 1881, included the following subjects: sanitary police, animal husbandry, public hygiene, pathology, miscellanea. Professor Al. Locusteanu presented a report on tuberculosis in humans and animals, proposing to assign this disease to the field of the sanitary-veterinary police. Another report concerned the gastro-entero-nephritis (Babesiosis) and it was presented by the veterinary surgeon of Dolj County, the future Professor Constantin Vasilescu.

From 6 to 8 October 1884, the "First Congress of the human doctors, veterinary surgeons and pharmacists" took in Bucharest, and Louis Vincent was one of the secretaries. The idea of this meeting of the sanitary specialists was born at Turnu-Măgurele in 1877, being proposed by Iacob Dimitrie Felix, the commander of the army hospitals from that area and by Carol Davila, head of the sanitary service of the Romanian army, whose assistant was the veterinary surgeon Mihail Măgureanu (alias Mihail Ghiuţă), who helped him to organize the Romanian troops for the assault on the fort from the right bank of the Danube. Among the participants in the Congress there also were 29 veterinary surgeons: Antonescu Grig from Galaţi, Botez G. from Buzău; Cătescu Iuliu from Călăraşi; Kolben M. from Bucharest; Constantinescu Panait from Bucharest; Constantinescu Pandele from Bucharest; Calcianu Christian from Bucharest; Diaconescu P. from Ploieşti; Drăghescu Gr. from Bârlad; Fomescu C. from Constanţa; Georgescu I. from Constanţa; Gavrilescu C. from Bucharest; Locusteanu Al. Din Bucharest; Lentz I. from Ungheni; Măgureanu M. from Bucharest; Mihăilescu N.

from Bucharest; Moscu C. from Galaţi; Nagy I. from Brăila; Petrescu Eugen from Fălticeni; Popescu G. from Târgovişte; Popescu I. from Bucharest; Sergescu C. from Constanţa; Similof I. from Galaţi; Starcovici C. from Bucharest; Vasilescu C. from Craiova and Vincent Louis from Bucharest; Sălcianu G., Basilescu C. and Petrescu L also attended the second day of the works.

Primul Congres National al medicilor veterinari 1882 -randul doi, pe scaune, de la stanga la dreapta: D. Curteanu, Panait Constantinescu, Pandele Constantinescu, M. Colben, G. Persu, A. Locusteanu, M. Magureanu, C. Fometescu

Fig. 11. Participants on the first Congress of Veterinary diplomats in Romania, which took place on 10-12th May, 1882, where they discussed issues of veterinary police, public hygiene, veterinary and animal pathology

The Romanian Society of Medical Sciences appointed a commission which to prepare and work out the Regulation of the Congress of the Romanian sanitary corps. The provisional secretary of the commission was Louis Vincent (Universul, nr. 28, of 21 September 1884, p. 2). The Regulation stipulated three sections: medical, veterinary and pharmaceutical; each section was to hold separate meetings, and all sections were to meet in general meetings.

In the general meeting of October 6, 1884, Professor I. Felix presented the report "About disinfection". After the general meeting from the first day of the Congress, the meeting of the veterinary section took place in the same afternoon, chaired by Professor M. Kolben elected honorary President; Professor Al. Locusteanu, executive President; M. Măgureanu and Louis Vincent, Vice-presidents; Professors Gavrilescu C. and N. Mihăilescu, were appointed secretaries. During the works of the section, Professor Al. Locusteanu presented the report: „Disinfection of the people and animals in case of cattle plague" (18, 21).

In the meeting of the medical section from October 7, 1884, the president of the section, Professor C. Severeanu, announced that the report of Professor Al. Locusteanu

planned "to be delivered during the general meeting", will be delivered within this section attended by veterinary surgeons too. Another report, "Relation between human tuberculosis and animal tuberculosis and hygienic measures to limit it" described information based on findings of experimental compared medicine and of own research. The etiological agent of the disease had been recently isolated and grown by R. Koch, in 1882, followed by a study on the etiology of tuberculosis in humans and animals (1884). On the third day, on October 8, 1884, within the veterinary section, the topic of discussion concerned the gastro-entero-nephritis, because in 1878 this disease killed over 50,000 cattle. The first one to speak was Louis Vincent, who showed the importance of diagnosing and controlling the disease and proposes a program to study systematically this disease.

On the evening of October 8, 1884, the final day of the Congress, the 130 participants attended the banquet organized at Boulevard Hotel, with sentimental "toasts" of "fraternisation" between the human and veterinary doctors in the field on the compared pathology (Universul of 11 October, 1884, p. 2). This spirit of collaboration didn't die with the end of the Congress.

Given the necessity of discussions to clarify the different scientific matters in the field of veterinary medicine, increasing in number and complexity, during the meeting of November 6, 1888, following the call by Gh. Ionescu Brăila, D. Curteanu and Louis Vincent, addressed to all the veterinary surgeons all across Romania, 38 responded and 21 actually participated in the meeting which revived the activity of the Romanian Society of Veterinary Medicine. The meeting took place on the premises of the Higher School of Veterinary Medicine. This call was prompted by the rather obscure activity of the Romanian Society of Veterinary Medicine at that time.

In the name of the steering committee, Louis Vincent showed that the large number of veterinary surgeons attending the meeting is a sign of approval of the purpose proposed by the steering committee and passed to the establishment of the provisional office. M. Măgureanu is elected as provisional President, while N. Focşa is elected as secretary. The meeting elected a commission consisting of Al. Locusteanu, Şt. I. Furtună, Paul Oceanu and D. Curteanu, who drew up a project of statute of the society of veterinary medicine of Bucharest, written and approved within the same meeting of November 6, 1888 (Bulletin of the Romanian Society of veterinary Medicine, 1888-1889, p. 3-7). During the same meeting, Louis Vincent is elected secretary general, and subsequently, Vice-president of the Romanian Society of Veterinary Medicine.

Once the Society of veterinary Medicine was "relaunched", the Journal of Veterinary Medicine, Animal Husbandry, Hygiene and Rural Economy also appeared (Fig. 12). It has been established in 1888 too, by I. Şt. Furtună, and had an Annex, the Bulletin of the Society. The Bulletins of the Society, reporting the regular debates, show that Louis Vincent was one of the most active animators of the debates and there was almost no meeting in which he would not speak, playing a decisive role in drawing the conclusions. These meetings debated the important problems of the veterinary pathology confronting that period: the foot and mouth disease of horses, glanders, tuberculosis, the bovine gastro-entero-nephritis (babeiosis) etc. Although he was quite young, Louis Vincent, who's many sided competence was notorious, was appointed in 1892 in the commission for the development of the third edition of the Romanian pharmacopeia, where he works intensely. This edition was published in 1893.

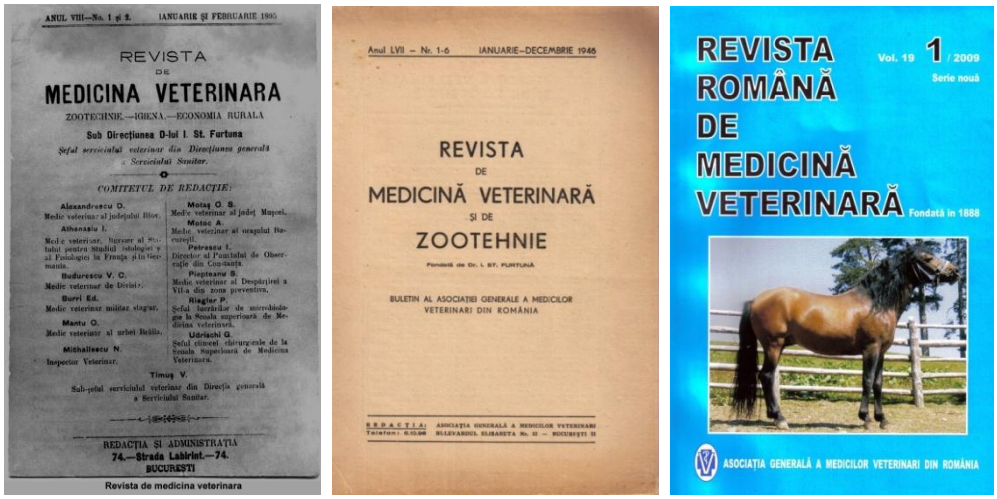
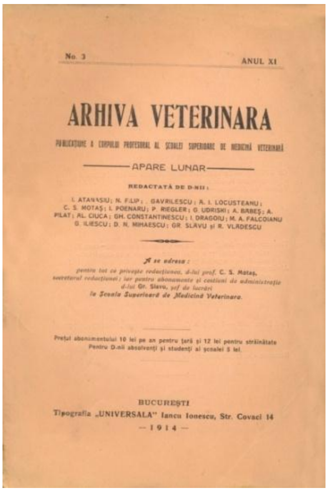


Fig. 12. Journal of Veterinary Medicine, Livestock, Rural hygiene and Economics, under direction of I. Șt. Furtună, head Veterinary service of the General Medical Service. The magazine is published in April of 1888, in city Focşani

The first number of the Bulletin of the Romanian Society of Veterinary Medicine appeared in December 1888 and included the proceedings of the meeting of November 6, 1888, when the Romanian Society of Veterinary Medicine was established and when it statute was drawn up. The meeting of January 4, 1889 discussed and voted the Regulation of the Romanian Society of Veterinary Medicine (Bulletin of the Romanian Society of Veterinary Medicine, 1888-1889, p. 33-55). The society existed until July 21, 1949, when, after this last meeting, it was discontinued abusively by the communist regime.

As long as it functioned, judging by the content of the proceedings and of the research published in its Bulletin included in the Veterinarian Archives (Fig. 13), the journal of the Didactic Corps of the Faculty of Veterinary Medicine, the Society of Veterinary Medicine brought a substantial scientific contribution to the development of the veterinary medicine in Romania, thus accomplishing the goal of its establishment on May 15, 1871.

Professor Paul Riegler published studies and notes on the tuberculosis and para-tuberculous bacilli, on glanders-action of the bacilli; on glanders toxins; on serum therapy; on the experimental transmission in cattle; on the bactericidal coal and on vaccinations; on the symptomatic coal; on experimental transmission and treatment of dourine; on agalactia in the Romanian sheep; as well as other important papers which were published in the Veterinary Archives, journal which he established in 1904 and which he headed for decades. He has been in the board of editors of the Journal of Veterinary Medicine and he published in 1896, 1897, and 1898 the "Bulletin and Memories of the Romanian Society of Veterinary Medicine".



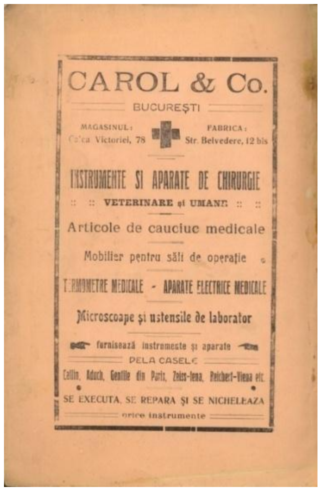


Fig. 13. "Veterinary Archives" review appeared on first in March 1904, - cover 4: ads for instruments and equipment for veterinary and human surgery, laboratory microscopes and laboratory instruments

In 1929, the Scientific Society of the Military Veterinary Corps was established and it had 244 members in 1933, assigned to seven circles: a central one in Bucharest and six branches in Bârlad, Iaşi, Chişinău, Sibiu, Timişoara and Focşani. The meetings of these circles were also often attended by civil veterinarians from those towns.

Once the Scientific Society of the Military Veterinary Corps had been established in 1929, the Military Veterinary Journal also appeared (Fig. 14 a and b.), scientific publication of the Military Veterinary Corps, under the direction of the general veterinarian Vintilă Rădulescu (1879-1937), head of the army Veterinarian service, an illustrious personality of the entire Romanian Military Veterinary Corps (Fig. 15.).

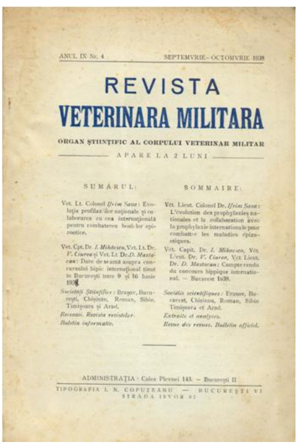


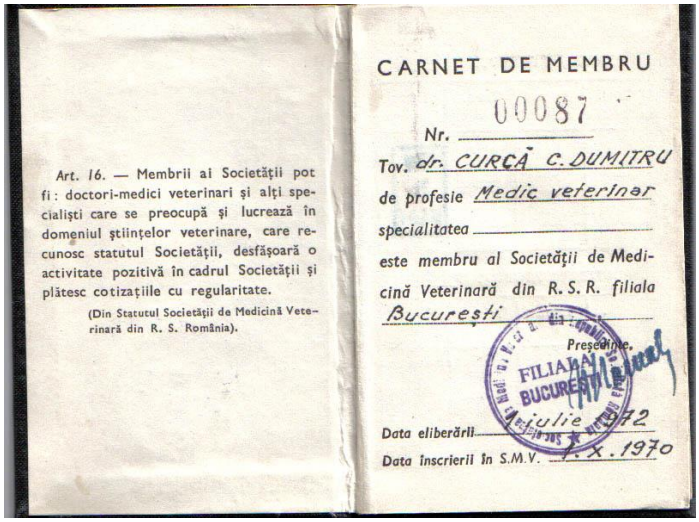
Fig. 14. a, b Military Veterinary journal

Fig.15. General Vintilă Rădulescu, veterinarian, vet army chief

The collaboration in the field of compared pathology was resumed at a higher intensity in 1964, after the establishment of the Society of Compared Pathology, within the Union of the Societies of Medical Sciences; however, the society ceased to function after a period. After 1990, this activity was resumed within the Institute of Compared Medicine, approaching various fields of interest.

Nevertheless, the spirit of the scientific activity within the Society didn't vanish; the veterinary surgeons working within the Faculties of Veterinary Medicine, of the related research institutes (I.N.Z., I.P.I.A.,"Pasteur" Institute, etc.), continued their activity within the Scientific Circles from the Faculties of: Bucharest (1949-1971), Arad (1949-1957), Iaşi (1961-1971), Cluj (1962-1971) and Timişoara (1962 – 1971).

Because the centenary of the Romanian Society of Veterinary Medicine was closing, a group of teaching staff from the Faculty of Veterinary Medicine of Bucharest started in the spring of 1970, the activities necessary to resume the Romanian Society of Veterinary Medicine: it was re-established on May 15, 1971, 22 years after its discontinuation and one hundred years after its foundation. This is the merit of the veterinary medicine profession from Romania, of the veterinary surgeons acting as teaching staff in the Faculties of Veterinary Medicine and of the specialists from the Research Institutes with medical-veterinary profile who, by their work and support for the steering group, by their perseverance and ability, managed to convince the decision-makers of that time to approve the re-establishment of the Romanian Society of Veterinary Medicine, as well as its statute (Fig. 16.).



Art. 16. — Membrii ai Societăţii pot fi: doctori-medici veterinari şi alţi specialişti care se preocupă şi lucrează în domeniul ştiinţelor veterinare, care recunosc statutul Societăţii, desfăşoară o activitate pozitivă în cadrul Societăţii şi plătesc cotizaţiile cu regularitate.

(Din Statutul Societăţii de Medicină Veterinară din R. S. România).

CARNET DE MEMBRU

Nr. 00087

Tov. dr. CURCĂ C. DUMITRU

de profesie Medic veterinar

specialitatea

este membru al Societăţii de Medicină Veterinară din R. S. R. filiala Bucureşti

Preşedinte.

FILIALA BUCUREŞTI

Data eliberării 1 iulie 1972

Data înscrierii în S.M.V. 1.X.1970

Fig. 16. Memberships card with number 00087 - the Society of Veterinary Medicine, with an enrollment date in the Company 1. X. 1970 (Prof. Dr. Doc. Şt. Neculai Stamatin signature, the presidents of Bucharest branch)

A meritorious contribution to the activity of the Romanian Society of Veterinary Medicine came from the military veterinarians, recruited among the graduates of the Veterinary School of Bucharest, some of them being: Dumitru Preotescu, Ion Popescu, Panait

Constantinescu, Gheorghe Perşu, Constantin Gavrilescu, Pandele Constantinescu, Nicolae Străulescu, Gheorghe Udriski (5, 6, 8, 10). Several military veterinarians also had important publishing activity, either by writing scientific articles, or by issuing books that were very appreciated at that time. Of these, many scientific articles being published in the Military Veterinary Journal, we cite:

- The veterinary captain Nicolae Moga published the brochure: "The Golubatz fly", edited in Bucharest, in 1891, which actually is his PhD thesis for the title of veterinary surgeon, from 1890; another brochure was "Horse and cattle breeding in Romania and sown pastures", published in 1904 la Brăila; "Bee farming", published un 1905, at Brăila;

- The veterinary general Grigore Hortopan (1880-1957), is the author of a Veterinary Encyclopaedia with the subtitle "Rearing and diseases of the domesticated animals", published in Bucharest, with more than 900 pages (Fig. 17.). The aim of this encyclopedia was to gather the knowledge on "breeds, breeding, rearing, maintenance and healing the diseases of animals", based on a substantial literature and approaching the whole field, insisting on the anatomy, physiology and pathology of the domesticated animals. It aimed to answer some of the stringent needs of that time concerning the improvement of animal productions by improving the animal breeds (in cattle, pigs, sheep, poultry etc.);

- The veterinary general Petre Stavrescu published two papers, each with more than 600 pages: "Science of horse rearing in modern conception", published in 1930, at Bucharest, and "Hypology" (27), published in 1900 (Fig. 18.), Bucharest, the latter one receiving the Adamache award of Romania Academy.

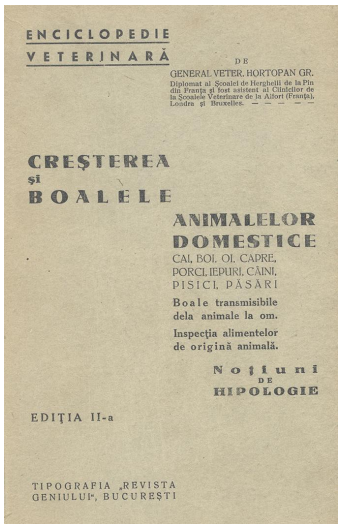


Fig. 17. Growth and animal disease, by veterinary General Gregory Hortopan, Second Edition

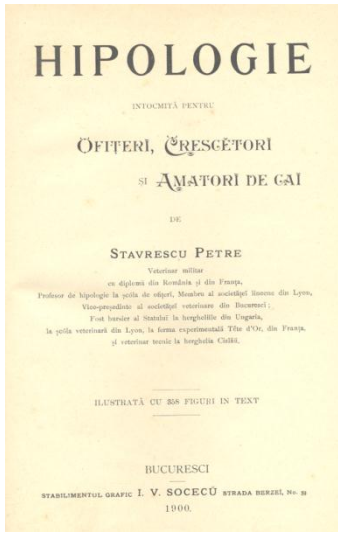


Fig. 18. Hippology for officers, breeders and fans of horses, by veterinary General Petre Stavrescu

The professors from the Faculty of Veterinary Medicine participated actively in the life of other scientific and professional societies too. Thus, Paul Riegler was founding member and several times President of the Society of Biology, Vice-president of the

Academy of Medicine, of the Romanian Royal Society of Medicine History, member of Société de Pathologie comparée from Paris, Vice-president of the General Association of the Veterinary Surgeons, etc.

Of the scientific societies which supported the progress of biology we may mention" the "Society of sciences from Bucharest" (1897), from which the "Romanian Society of Naturalists" split in 1899, with its publications edited starting from 1901; the „Society of Biology", established in 1907 by V. Babeş, Paul Riegler, I. Cantacuzino, Gh. Marinescu, I. Athanasiu and D. Voinov, at Bucharest, under the name of „Biological Association" and which published starting with 1911 the „Annals of biology". At the same time, Cantacuzino also was the founder of the "Journal of Medical Sciences", "Annales de Biologie" and "Archives Roumaines de pathologie experimentale et de microbiologie".

Bibliografie

1. *** 75 de ani de la întemeierea învăţământului medicinei veterinare în România, 1856-1931, Tipografia Cultura, Bucureşti, 1931
2. *** Alma Mater Veterinaria Bucurescensis la a 140-a aniversare, Ed. All, Bucureşti, 2001
3. *** Personalităţi din trecutul medicinei veterinare, Vol. I, Societatea de Medicină Veterinară, Bucureşti, 1987
4. Bălan St., Mihăilescu M. - Istoria ştiinţei şi tehnicii în România, Ed. Academiei Române, Bucureşti, 1985
5. Curcă D. - First observations on monodactylism (sindactylism) in swine made by Professor C.N. Vasilescu between 1890-1894, 31th International Congress on the History of Veterinary Medicine, 6-10 September, 2000, Brno, Czech Republic, Book of abstracts, p.64-65
6. Curcă D. - The Romanian Scientist-Prof. Victor Babeş, Historia Medicinae Veterinariae, 27, 5-6, 2002, p.333-347
7. Curcă D. - Formarea primelor şcoli de agricultură şi a celor de medicină veterinară din România, Simpozion Facultatea de Medicină Veterinaară, Rezumat p.11-13, Bucureşti, 11 oct. 2002
8. Curcă D., Ioana Cristina Andronie, Andronie V. - Romanian priorities in control and eradication of epizootic diseases in veterinary medicine, The 32nd Congress on the History of Veterinary Medicine, 15-19 August, 2001, Oslo, Norway, Abstracts, p.20-21
9. Curcă D., Ioana Cristina Andronie, Andronie V. - The establishment of the first Agricultural Schools and Veterinary Medicine Schools in Romania, 33rd International Congress on the History of Veterinary Medicine, Lutherstadt Wittenberg, Germany, 21-24 august 2002, Abstracts, p.23, Proceedings: History of Veterinary Medicine and Agriculture, p. 117-127.
10. Curcă D. - Romanian priorities in control and eradication of epizootic diseases in veterinary medicine, Lucrări ştiinţifice U.Ş.A.M.V., Seria C, vol. XLIV-XLV, 2001-2002, p.127 - 140
11. Diaconescu Mircea (coordonator) – Oameni şi fapte din istoria medicinii militare româneşti, Editura Pro Transilvania, Bucureşti, 2005, vol. 1 şi vol. 2.
12. Druţu Ch.D. - Istoricul învăţământului agricol în România, Bucureşti, 1906
13. Georgescu, Bogdan, Gabriel Predoi, Nicolae Cornilă. – Profesorul Constantin Gavrilescu (1865 – 1941) – Rev. Rom. Med. Vet. 1/2006, p. 141.
14. Gheorghe Florian, Mihai Popescu, Conf. Dr. Ion Rotaru – Prezenţe militare în ştiinţa şi cultura românească. – Editura Militară, Bucureşti, 1982, p. 121 - 122
15. Giurescu C.C., Giurescu D. - Istoria Românilor din cele mai vechi timpuri până azi, Ed. Albatros, Bucureşti, 1971
16. Hortopan Gr. - Enciclopedie veterinară. Creşterea şi boalele animalelor domestice. Noţiuni de hipologie, Editia a II-a, Tipografia „Revista Geniului", Bucureşti, 1912
17. Iftimovici R., Istoria medicinei, Ed. All, Bucureşti, 1994
18. Ioan D., Marinescu N. - Istoricul învăţământului sanitar militar în România, Tiografia „Ion C. Văcărescu", Bucureşti, 1935
19. Iorga N. - Viaţa şi domnia lui Barbu Dimitrie Ştirbei domn al Ţerii Româneşti (1848-1856), Vălenii de Munte, 1910

20. Iorga N. - Istoria învăţământului românesc, Bucureşti, 1928
21. Iorga N. - Istoria Românilor, vol. VIII, Bucureşti, 1938
22. Pascu Şt. şi col. - Istoria militară a poporului român, vol. V, Centrul de Studii şi Cercetări de Istorie şi Teorie Militară, Editura Militară, Bucureşti, 1988
23. Pascu Şt. şi col. - Istoria militară a poporului român, vol. VI, Centrul de Studii şi Cercetări de Istorie şi Teorie Militară, Editura Militară, Bucureşti, 1989
24. Picu Valeria Maria - Învăţământul agricol din Bucureşti, Ed. Ceres, Bucureşti, 2002
25. Simionescu C., Moroşanu N. - Pagini din trecutul medicinii veterinare româneşti, Ed. Ceres, Bucureşti, 1984
26. Stancu I.- Reprezentanţi de seamă ai medicinei veterinare româneşti (1856-2001), Ed. Coral Sanivet, Bucureşti, 2002
27. Stavrescu P. - Hipologie întocmită pentru ofiţeri, crescători şi amatori de cai, Stabilimentul grafic I.V. Socecŭ, Bucuresci, 1900
28. Urechia V.A. - Istoria şcoalelor de la 1800-1864, vol. I-IV, Bucureşti, 1892-1901
29. *** Istoria Militară a poporului român. Volumul V, Editura militară, Bucureşti, 1988

EPIDEMIOLOGICAL, CLINICAL AND PATHOLOGICAL INVESTIGATIONS FROM AN FELINE INFECTIOUS CORYZA OUTBREAK

Gabriela DARABAN, Oana TĂNASE, Carmen SOLCAN, Elena VELESCU
"Ion Ionescu de la Brad" University of Agricultural Sciences and Veterinary Medicine of Iasi
Faculty of Veterinary Medicine; gabbriella81@yahoo.com

Abstract
The importance of the previous respiratory tract disease is given by the very high incidence, high transmissibility and also by the fact that 80-90% from infectious respiratory diseases of the cats have the causative agent the feline calicivirus or feline herpesvirus type 1.In our country, specialized literature hasn't reported data on scientific research regarding feline calicivirus infection, named also feline infectious coryza. This aspect and also the great importance of the disease in cats, are the reasons of this investigations, while the practical aim of this research paper is to describe the epidemiological, clinical and pathological features from an feline infectious coryza outbreak. The study group consisted in three Persian kittens which came in March 2012 at consultation in a private veterinary practice from Iassy. The kittens, males aged two months and 15 days, are from a unvaccinated mother and were imported from Russia. Applying the basic semiological methods were observed clinical manifestations specific to feline calicivirus infection, with different severity from case to case. After different periods of time, the three kittens died. After necropsy were observed different lesions. The clinical and pathological manifestations specific to feline calicivirus infection are severe in kittens without previous immunity. Feline calicivirus infection produces losses by morbidity and mortality, especially in young, and also by the therapy and specific prevention expenses.

Keywords: calicivirus, clinical and pathological manifestations, kitten, previous respiratory tract disease.

Introduction

The importance of the previous respiratory tract disease is given by the very high incidence, high transmissibility and also by the fact that 80-90% from infectious respiratory diseases of the cats have the causative agent the feline calicivirus or feline herpesvirus type 1.

In our country, specialized literature hasn't reported data on scientific research regarding feline calicivirus infection, named also feline infectious coryza.

This aspect and also the great importance of the disease in cats, are the reasons of this investigations, while the practical aim of this research paper is to describe the epidemiological, clinical and pathological features from an feline infectious coryza outbreak.

Material and methods

The study group consisted in three Persian kittens which came in March 2012 at consultation in a private veterinary practice from Iassy. The kittens, males aged two months and 15 days, are from a unvaccinated mother and were imported from Russia.

Based on the epidemiological, clinical and pathological investigations, a presumptive diagnosis has been established.

The necropsy was performed shortly after the death of the animal. Gross lesions were noted and samples were collected for the histological investigations. From the organs with gross lesions, the most characteristic areas were sampled for investigations.

The execution of the histological permanent preparations involved the following steps:

1. Fixation of the samples: in formalin solution 10% for two days, while the solution were changed several times.
2. Washing of the pieces - one hour with tap water, indicated with water jet; in alcohol 70º for 45 minutes; in alcohol 80º for 45 minutes; in alcohol 96º for 45 minutes; in absolute alcohol 100º for one hour, three times consecutively; in xylen for one hour, twice over.
3. Followed by paraffin including samples. After solidifications, the samples were sectioned using the SLEE CUT 6062 microtome, obtaining the 5 µm sections.
4. The colorations of the sections was performed by using the trichrome method HEA (Hematoxylin – Eosin – Aniline blue).

Results and discussions

Applying the basic semiological methods were observed clinical manifestations specific to feline calicivirus infection, with different severity from case to case.

A day after they were brought to Romania, the three Persian kittens had these clinical signs: fever, cortical inhibition, anorexia, rhinitis with serous and sero-mucous secretions, ocular secretions, diarrhea and further were observed ulcerative oral lesions, on the dorsal side of the tongue (Figures 1 - 3).

Fig. 1. Cortical inhibition

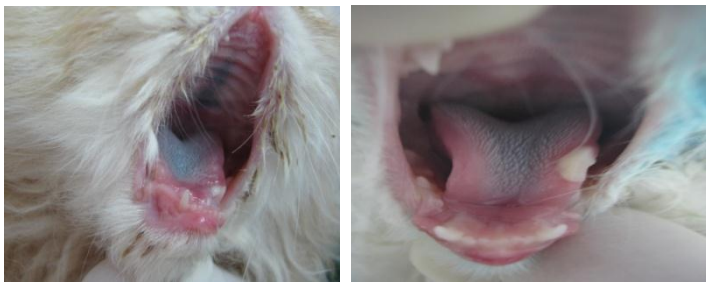


Fig. 2. Ulcerative oral lesion

Although therapy has been started, the three kittens died after different periods of time.

Six days after consultation, the white kitten, the one with most severe clinical signs, died. Next day the red kitten died. The last kitten, cream-colored, presented serous ocular secretions in large amount, especially on the right eye, also loss of appetite and clinical

manifestations of nervous system. Two weeks after consultation, the cream-colored kitten, feed up then with syringe with milk, start to eat milk alone and wet food. Ocular secretions and clinical manifestations of nervous system disappeared, general condition of the kitten has improved only for 3 - 4 days. Loss of appetite, ocular secretions, clinical manifestations of nervous system, diarrhea and dehydration appeared again, ending with death.

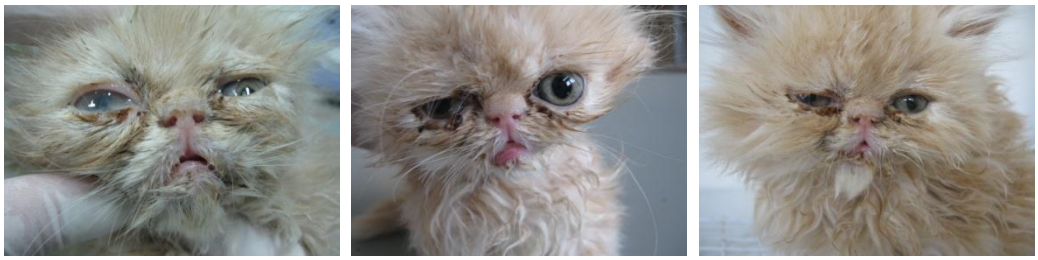


Fig. 3. Kerato-conjunctivitis and serous rhinitis

The therapy measures involved: the mucilage rice and mint tea administration for the diarrhea; ophthalmic unguents with kanamycin 2×/day; oral washes with methylene blue 2×/day; administration of Duphalyte 10-15ml/24h, Glucose 10-15ml/24h, Vitamin C 1ml/24h, Enteroguard, Spectam, Clamoxyl.

After necropsy were observed different lesions. The gross lesions are presented in figures 4 – 6 and the microscopic lesions are presented in figures 7 - 8.

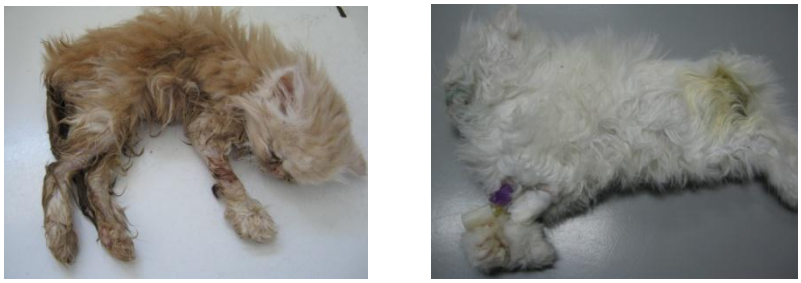


Fig. 4. Dehydrated, thin body

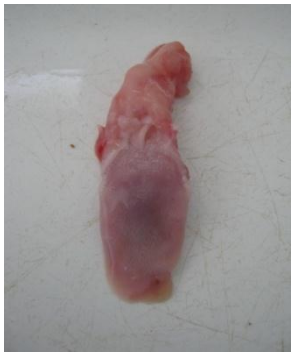


Fig. 5. Superficial ulceration on the antero-dorsal side of the tongue

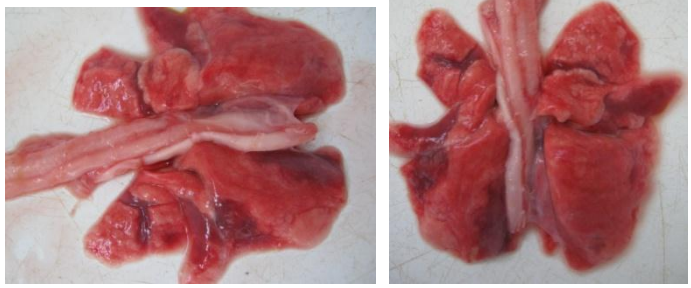


Fig. 6. Interstitial pneumonia

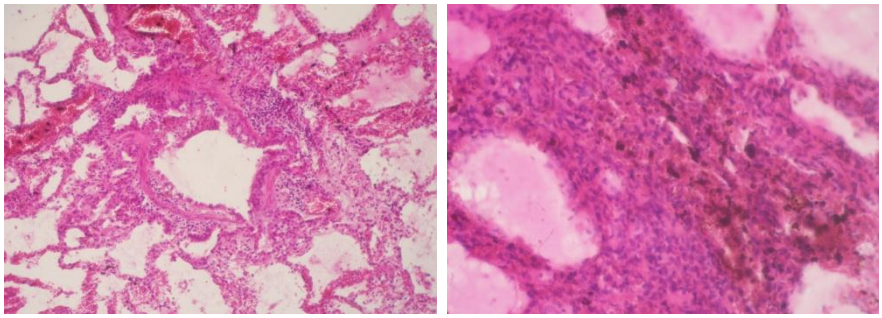


Fig. 7. Microscopic lesions (cat lung) - Celullar infiltration with lymphocytes, plasmatic cells and macrophages; HEAx150

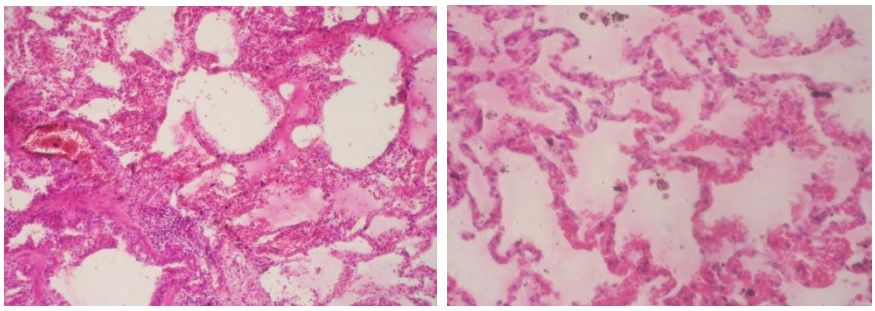


Fig. 8. Microscopic lesions (cat lung) - Hyperplazia of the alveolar coating; HEAx150

Conclusions

1. The clinical and pathological manifestations specific to feline calicivirus infection are severe in kittens without previous immunity.
2. Feline calicivirus infection produces losses by morbidity and mortality, especially in young, and also by the therapy and specific prevention expenses.
3. Illegal transportation of cats outside the country, no matter the purpose, plays a very important role in the epidemiology of the feline calicivirus infection.

References

1. Dawson Susan, Radford A. şi Gaskell Rosalind, 2004 - *Clinical update on feline respiratory pathogens,* Companion Animal Practice, pag. 320-323, available on-line at http://inpractice.bvapublications.com/.
2. Gaskell Rosalind şi Knowles J., 1989 – *Feline respiratory disease,* In Practice, pag. 23-26, available on-line at http://inpractice.bvapublications.com/.
3. Moga-Mânzat R., 2005 – *Boli virotice şi prionice ale animalelor,* Editura Brumar, Timişoara.
4. Velescu Elena, 2004 - *Patologia bolilor infecţioase la animale, Ediţia a II – a,* Editura „Terra Nostra", Iaşi.
5. Radford A.D. şi colab., 2007 – *Feline calicivirus,* Veterinary Research 38, pag. 319-335, available on-line at http://publications.edpsciences.org/.

OBSERVATIONS REGARDING CASES OF FELINE CALICIVIRUS INFECTION IN INDOOR CATS

Gabriela DARABAN, Carmen SOLCAN, Oana TĂNASE, Simona DIMITRIU, Andrei BĂISAN, Elena VELESCU
"Ion Ionescu de la Brad" University of Agricultural Sciences and Veterinary Medicine of Iasi
Faculty of Veterinary Medicine; gabbriella81@yahoo.com

Abstract
Because in Romania the research of feline calivirus infection is limited, and also due the importance of the disease, the aim of this scientific paper is to describe the clinical manifestations of feline calivirus infection and correlate between different predisposing factors and the frequency of the disease in indoor cats from Iassy. It is important to understand the association of the external and internal risk factors with the previous respiratory tract infections, so to diagnose in time and to improve the procedures for the decrease of the disease incidence. For the investigation, the study group consisted in cats presents in Internal and Infectious Diseases Clinics from the Faculty of Veterinary Medicine of Iassy, during April 2010 - April 2012. From the total of 160 examined cats, 28 indoor cats presented clinical signs specific to feline calicivirus infection. For each case, from which biological samples were collected for laboratory tests, a clinical chart was maintained. The data demonstrate that the high sensibility is present in cats aged from one month to three years old, and the females are more susceptible than males. The clinical manifestations specific to feline calicivirus infection are moderate in indoor cats. For the indoor cats, who live in small number and in hygienic conditions, the prevalence of feline calicivirus infection is low. In the species, receptivity depend first on age and sex factors and after that on breed factor.

Keywords: calicivirus, indoor cat, previous respiratory tract disease, risk factors.

Introduction

Because in Romania the research of feline calivirus infection is limited, and also due the importance of the disease, the aim of this scientific paper is to describe the clinical manifestations of feline calivirus infection and correlate between different predisposing factors and the frequency of the disease in indoor cats from Iassy.

It is important to understand the association of the external and internal risk factors with the previous respiratory tract infections, so to diagnose in time and to improve the procedures for the decrease of the disease incidence.

Material and methods

For the investigation, the study group consisted in cats presents in Internal and Infectious Diseases Clinics from the Faculty of Veterinary Medicine of Iassy, during April 2010 - April 2012.

For each case, from which biological samples were collected for laboratory tests, a clinical chart was maintained.

Based on the epidemiological and clinical investigations, a presumptive diagnosis has been established.

Results and discussions

From the total of 160 examined cats, 28 indoor cats presented clinical signs specific to feline calicivirus infection (Table 1).

The clinical manifestations specific to feline calicivirus infection were represented by: fever; anorexia; cortical inhibition; rhinitis with serous and sero-mucous secretions; stertorous breathing; dyspnea; pneumonia; superficial ulcerations with regular circumscriptions, on the dorsal side of the tongue and on the nose; moderate salivation; stomatitis; gingivitis; pharyngitis; less often conjunctivitis with serous and sero-mucous secretions (Figures 1- 4).

Table 1. Cats with symptoms indicating feline calicivirus infection, presented at the Internal and Infectious Disease Clinics of the Faculty of Veterinary Medicine Iassy, between April 2010 and April 2012

Indoor Cat	Sex	Age	Breed
1	Female	8 months	Siamese
2	Female	12 years	European
3	Male	8 years	European
4	Female	two months	Russian Blue
5	Female	1 – 2 years	Siamese
6	Female	2 – 3 years	European
7	Male	4 – 5 years	European
8	Female	11 months	Birman
9	Female	10 months	European
10	Female	3 years	Birman
11	Female	2 years	European
12	Male	11 – 12 months	European
13	Female	10 months	Birman
14	Male	5 years	European
15	Female	5 years	Birman
16	Female	9 months	Siamese
17	Female	2 years	Siamese
18	Male	2 – 3 years	European
19	Female	11 – 12 months	Birman
20	Female	10 – 11 months	European
21	Female	4 months	European
22	Male	9 years	Siamese
23	Male	two months and 15 days	Persian
24	Male	2 years and 6 months	European
25	Male	4 months	Birman
26	Female	two – three months	European
27	Female	3 – 4 months	European
28	Female	10 years	European

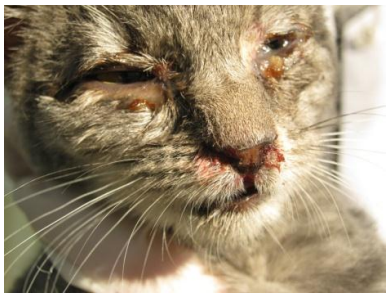


Fig. 1. Mucous suppurative conjunctivitis
Mucous suppurative rhinitis and nasal hemorrhage
Female, European, 4 months old

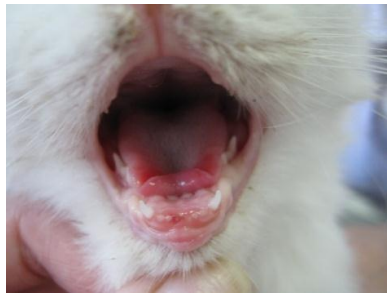


Fig. 2. Superficial ulcerations
on the dorsal side of the tongue
Female, European, 3 - 4 months old

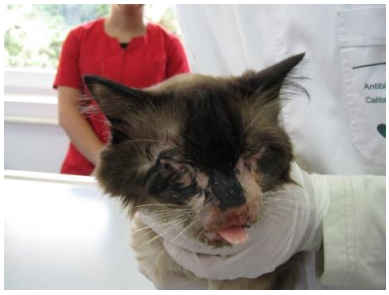


Fig. 3. Serous and mucous conjunctivitis
and rhinitis Lingual ulcerations
Male, Siamese, 9 years old

Fig. 4. Sero-mucous rhinitis
Female, European, 2 - 3 years old

Regarding the correlations of the internal factors (age, sex and breed) and the prevalence of feline calicivirus infection in indoor cats (Figures 5 - 7), were observed that the high sensibility is present in cats aged from one month to three years old, and the females are more susceptible than males. Even if the majority of the cases belongs to European breed, a direct correlation between breed factor and the prevalence of feline calicivirus infection in indoor cats could not be established.

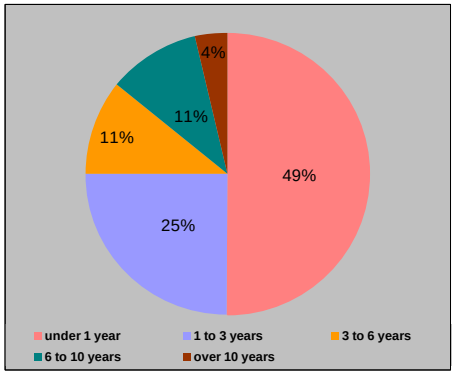


Fig. 5. Case prevalence in cats with symptoms indicating feline calicivirus infection by age

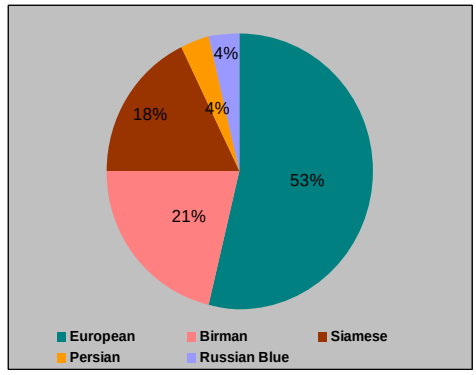


Fig. 6. Case prevalence in cats with symptoms indicating feline calicivirus infection by breed

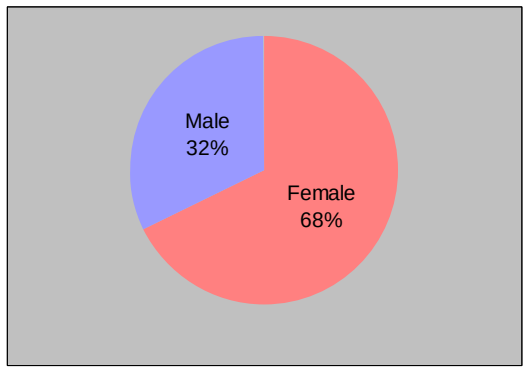


Fig. 7. Case prevalence in cats with symptoms indicating feline calicivirus infection by sex

The clinical manifestations were moderate in indoor cats submitted to the study, cats who lived in small number, in hygienic conditions and with a strict diet, evidence that the external factors (lifestyle and the growth of cats, the presence of other diseases) plays an important role in the severity of disease.

Conclusions

1. The clinical manifestations specific to feline calicivirus infection are moderate in indoor cats.
2. For the indoor cats, who live in small number and in hygienic conditions, the prevalence of feline calicivirus infection is low.
3. In the species, receptivity depend first on age and sex factors and after that on breed factor.

References

1. Binns Sarah şi Dawson Susan, 1995 - *Feline infectious upper respiratory disease,* Companion Animal Practice, pag. 458-461, available on-line at http://inpractice.bvapublications.com/.
2. Helps C.R. şi colab., 2005 - *Factors associated with upper respiratory tract disease caused by feline herpesvirus, feline calicivirus, Chlamydophila felis and Bordetella bronchiseptica in cats: experience from 218 European catteries,* Veterinary Record 156, pag. 669-673, available on-line at http://veterinaryrecord.bvapublications.com/.
3. Vasiu C., 2003 – *Viroze şi boli prionice la animale,* Editura Nereamia Napocae, Cluj-Napoca.
4. Perianu T., Ştefan N., Carp-Cărare M. şi Velescu Elena, 2005 – *Boli infecţioase ale animalelor, Viroze,* Vol. II, Editura Universitas XXI, Iaşi.
5. Radford A.D. şi colab., 2007 – *Feline calicivirus,* Veterinary Research 38, pag. 319-335, available on-line at http://publications.edpsciences.org/.

PROPER USE OF THE SECOND LINE ANTIMICROBIALS IN ORDER TO AVOID RESISTANCE

Alina DRAGHICI, Anca BITOIU, Simona STURZU
Institute for the Control of the Biological Products and Medicines for the Veterinary Use, Bucharest 6, 39 Dudului Street, 060603, Romania
draghici.alina@icbmv.ro, anca.bitoiu@icbmv.ro, sturzu.simona@icbmv.ro

Abstract
The aim of this paper is to communicate about the importance of prudent use of antibiotics in order to turn the tide on antibiotic resistance. Antibiotic resistance is a moving target. Developing and marketing of new antibiotics with novel mechanisms of action represents a further essential strategy against antibiotic resistance as resistance inevitably builds over time. Prudent use of antibiotics is not the only strategy for fighting antibiotic resistance, but is one of the most important one, especially when it comes about the second line antimicrobials. This category of antimicrobials should be use with great care, as an alternative to the first line antimicrobials. Extent use of them will lead to an undesirable effect: resistance of bacteria's to this category of antimicrobials.
Keywords: resistance, second, line, antimicrobials.

In the last years there were a lot of meetings which had as the discussing subject resistance to antimicrobials. This is a very actual and important subject.

Denmark is one of the countries which understood the importance of this phenomena and organized a conference on this subject at the beginning of this year. Her Majesty the queen of Denmark sent her message to the participants. She underlined the importance of this subject and she ensured the participants that minimization of the resistance to the antimicrobials is one of the priority actions on the president's agenda.

The concern on this subject is very actual and many countries took new measures in order to reduce as much as possible the spread of this phenomena. The international organizations as EMA, WHO, FVE, European Commission are deeply involved in this.

Antibiotic resistance is a moving target but also an easy "target" to achieve. If penicillin has been discovered in 1928 and streptomycin in 1944, the first signs of AMR (antimicrobial resistance) appears in 1960.

In Europe, due to the tourism in the last 30 years, 40 new diseases appeared.

Much more: there are bacteria which developed great resistance to the antimicrobials: for example Klebsiela pneumoniae is resistant to the fluoroquinolone, cephalosporines and carbapenen (the latest generation of antimicrobials). E coli resistant to cephalosporines and fluoroquinolone appeared in 2010.

Every year 25000 people die because of the resistance to the antimicrobials.

Antimicrobials should be used with more wisdom. As much as you use them as soon you will lose them – "use it and lose it".

If the actual trend of using of the antimicrobials will be the same in the couple of years the results will be the end of the antimicrobials efficacy.

Of course, the international organizations are working on solutions. Production of new antimicrobials would be one of them. The problem is that taking into account the costs of production (money, time) and the short life of the new product on the market due to the unwise use of it, the producers are not really interested in this.

So, we have to deal with what we have and to have great care to extend the life of the current antimicrobials.

A major importance should be granted to the using of the "second line antimicrobials" or "critical antimicrobials".

But what are the "second line antimicrobials"? Any antimicrobial agent that is not the drug of choice or the 1st normally used to treat a particular infection; the 2nd-line antimicrobials are used when standard 'first-line' antimicrobials, fail. Generally these 2nd-line antimicrobials are the most recently discovered. We are talking about cephalosporines (the third and the forth line), quinolones, some macrolides and some beta-lactamic antimicrobials (carbapemeni).

The use of these antimicrobials should not be extended, but keep them as a second option, always based on the results of the sensitivity test of the bacteria isolated from sick animals.

Some countries understood that, for now, these antimicrobials are, sometimes, our last hope. That's why the use of them is very restricted and controlled by the national authorities. They can be used only when the results of the treatment with others antimicrobials where not a success and only in the following situations: for treatment, for prevention of mastitis, for prevention of septicaemia after aseptic intervention, for metaphylaxis. Use of the antimicrobials (from de first or second line) as prophylaxis in different infectious disease is out of discussion. This is why is important, in this war against resistance, to take also measures to improve de hygiene and the health of animals, in order to minimize the use of antimicrobials.

In some countries, for example in Denmark, it is forbidden to use the second line antimicrobials in farms. In the same context of minimization of the risk of resistance, the vets from some European countries are not allowed to sell antimicrobials, but only to prescribe them.

"We must act rapidly and firm if we don't want to lose the antimicrobial medicines as a basis treatment against bacterial infections in humans but also in animals." This is the message of the president of the European Commission for all doctors (vets, humans), for producers. We all should enjoy this work and try to ensure that we will still be in the position to treat with success infected animals.

It is in our hands!

Bibliography

1. Conference "Prevention of antimicrobian resistance- time for combined action"-, Copenhaga, 14-15 martie 2012
2. The Swedish Presidency of the European Union [website on the Internet]. Stockholm: Conference Innovative Incentives for Effective Antibacterials. Available from: www.se2009.eu/en/meetings_news/2009/9/17/conference_innovative_incentives_for_effective_antibacterials [accessed 12 November 2009]
3. Centers for Disease Control and Prevention [website on the Internet]. Atlanta: Get Smart: Know When Antibiotics Work. Available from:www.cdc.gov/getsmart [accessed 12 November 2009].
4. The White House President Barak Obama [website on the Internet]. Washington(DC): The White House, Office of the Press Secretary. U.S.-EU Joint Declaration and Annexes. 2009 U.S.-EU Summit Declaration. November 3, 2009 Available from: http://www.whitehouse.gov/the-press-office/us-eu-joint-declaration-and-annexes [accessed 12 November 2009].

5. Hensgens MP, Goorhuis A, Notermans DW, van Benthem BH, Kuijper EJ. Decrease of hypervirulent Clostridium difficile PCR ribotype 027 in the Netherlands. Euro Surveill. 2009;14(45): pii=19402. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19402
6. Arvand M, Hauri AM, Zaiss NH, Witte W, Bettge-Weller G. Clostridium difficile ribotypes 001, 017, and 027 are associated with lethal C. difficile infection in Hesse, Germany. Euro Surveill. 2009;14(45): pii: 19403. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19403
7. Bauer MP, Notermans DW, van Benthem BHB, Wilcox MH, Monnet DL, van Dissel JT, et al. First results of the European Clostridium difficile infection survey (ECDIS). 19th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Helsinki, 16-19 May 2009.
8. Souli M, Galani I, Giamarellou H. Emergence of extensively drug-resistant and pandrug-resistant Gram-negative bacilli in Europe. Euro Surveill. 2008;13(47):pii=19045. Available from: www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19045
9. Lepape A, Monnet DL, on behalf of participating members of the European Society of Intensive Care Medicine (ESICM). Experience of European intensive care physicians with infections due to antibiotic-resistant bacteria, 2009. Euro Surveill. 2009;14(45): pii:19393. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19393
10. Joint opinion on antimicrobial resistance (AMR) focused on zoonotic infections. Scientific Opinion of the European Centre for Disease Prevention and Control; Scientific Opinion of the Panel on Biological Hazards; Opinion of the Committee for Medicinal Products for Veterinary Use; Scientific Opinion of the Scientific Committee on Emerging and Newly Identified Health Risks. Stockholm, European Centre for Disease Prevention and Control ; Parma, European Food Safety Agency; London, European Medicines Agency & Brussels, Scientific Committee on Emerging

METAPHYLAXIS- A WAY OF MINIMIZATION/ELIMINATION OF RESISTANCE TO ANTIMICROBIALS

Alina DRAGHICI, Anca BITOIU, Simona STURZU
Institute for the Control of the Biological Products and Medicines for the Veterinary Use,
Bucharest 6, 39 Dudului Street, 060603, Romania
draghici.alina@icbmv.ro, anca.bitoiu@icbmv.ro, sturzu.simona@icbmv.ro

Abstract
Antibiotics are one of the most important therapeutic discoveries in medical history. They have revolutionized the way we treat patients with bacterial infections and have contributed to reducing the mortality and morbidity from bacterial diseases. Unfortunately, antibiotics have been liable to misuse. They are often unnecessarily prescribed for viral infections, against which they have no effect. Similarly when diagnoses are not accurately made, broad-spectrum antibiotics, i.e. antibiotics that kill a large proportion of various bacteria and not only the bacteria responsible for the disease, are prescribed because the causative micro-organism is not known. Misuse of antibiotics leads to the emergence and selection of resistant bacteria. Doctors in Europe and worldwide are now facing situations where infected patients cannot be treated adequately because the responsible bacterium is totally resistant to available antibiotics. The correct use of antimicrobials is one of the most important tools which could limit the spread of this phenomenon - resistance to antimicrobials.
Keywords: antimicrobials, resistance, metaphylaxis

Antibiotics are one of the most important therapeutic discoveries in medical history. They have revolutionized the way we treat patients with bacterial infections and have contributed to reducing the mortality and morbidity from bacterial diseases.

Unfortunately, antibiotics have been liable to misuse. They are often unnecessarily prescribed for viral infections, against which they have no effect. Similarly when diagnoses are not accurately made, broad-spectrum antibiotics, i.e. antibiotics that kill a large proportion of various bacteria and not only the bacteria responsible for the disease, are prescribed because the causative micro-organism is not known.

Misuse of antibiotics leads to the occurrence of resistant bacteria. Doctors in Europe and worldwide are now facing situations where infected patients cannot be treated adequately because the responsible bacterium is totally resistant to available antibiotics.

This is why many European organizations (WHO, European Commission, European Medicines Agency, FVE, HMA) are deeply involved in the campaign concerning minimization of spread of the resistance to the antimicrobials.

In a recent meeting of the European Commission, John Dalli, the European commissary for health and consumers, said: “We must act rapidly and firm if we don’t want to lose the antimicrobial medicines as a basis treatment against bacterial infections in humans but also in animals.” During this meeting 12 measures were proposed to be taken in order to minimize the risk of antimicrobial resistance. One of them is the correct use of antimicrobials.

The message diffused by WHOM

As we all know the antimicrobials should be used in the following situations:

- treatment
- prevention
- metaphylaxis

Treatment of the sick animals, infected with bacterial should be based on the results of the sensitivity test, made on the bacteria isolated from the sick animal/s which will be treated.

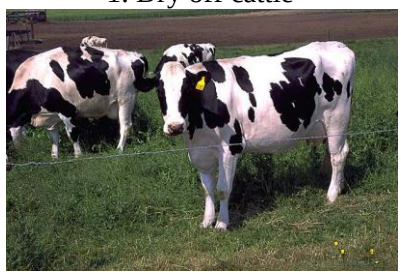
In some cases, from different causes, it is not possible to perform this test (due to the urgent situation generally). Only in this justified cases it is permitted to use antimicrobials, taking into account the epidemiological data from the region or farm. It is important that such cases are isolated and do not become a habit.

1. Sick animal

2. SENSITIVITY TEST
3. TREATMENT
4. Cured animal

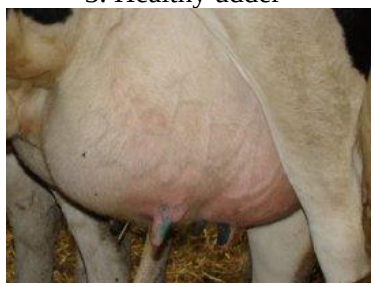
Prevention is a very controversial term, because it has different understanding in different countries. Nowadays, prevention is accepted only in cattle in the dry period, in order to "prevent" mastitis from the calving period. It is also recommended prevention only in septic operations.

1. Dry off cattle



2. PREVENTION

3. Healthy udder



"Metaphilaxis" for Romanian veterinarians is a new term. This world came from Greek: "meta"- nearly, "phylaxix"- prevention. In many countries, in veterinary medicine, this term has a very clear meaning: is prevention in the herds (groups) of animals were some animals were diagnosed with an infectious disease.

What does it mean? It means that even so, it is necessary to identify the cause of the disease (to isolate and to perform the sensitivity test of the strain isolated from the group) and than to treat sick animals, but also the contacts which are asymptomatic carriers.

1. Sick animals and carriers

2. METAPHYLAXIS
3. Healthy animals

These are the only three situations when the use of antimicrobials is permitted.

Of course, it depends on the authorities from each country in how will apply the antimicrobial politics. But what we have to keep in our mind is that the life of the antimicrobials is limited. Extend and unwise use of them will finish with unsatisfactory results of the treatment or even failure due to the resistance to antimicrobials.

Bibliography

1. ECDC Antimicrobial Resistance and Healthcare-Associated Infections Programme. Antibiotic resistance in Europe: the challenges ahead. Euro Surveill. 2009;14(45):pii=19405.
2. Earnshaw S, Monnet DL, Duncan B, O'Toole J, Ekdahl K, Goossens H, et al. European Antibiotic Awareness Day, 2008 – the first Europe-wide public information campaign on prudent antibiotic use: methods and survey of activities in participating countries . Euro Surveill.2009;14(30):pii=19280.Available,from: www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19280.
3. Magiorakos AP, Suetens C, Boyd L, Costa C, Cunney R, Drouvot V, et al. National Hand Hygiene Campaigns in Europe, 2000-2009. Euro Surveill. 2009;14(17):pii=19190. Available from: www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19190.
4. Council of the European Union. Council Recommendation of 9 June 2009 on patient safety, including the prevention and control of healthcare associated infections (2009/C 151/01). Available,from: http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:C:2009:151:0001:0006:EN:PDF
5. European Centre for Disease Control and Prevention/ European Medicines Agency. The bacterial challenge: time to react. Stockholm, European Centre for Disease Prevention and Control2009.Available,from:http://ecdc.europa.eu/en/publications/Publications/0909_TER_The_Bacterial_Challenge_Time_to_React.pdf.

MORPHOLOGICAL CHANGES OF THE ACROPODIA SOFT TISSUES AND PHYSICAL PROPERTIES OF PHALANGEAL BONES IN NECROBACILLARY PODODERMATITIS OF SHEEP

Gr. DUMITRAŞ, N. NAFORNIŢA
State Agrarian University of Moldova, Chisinau, grigore.dumitras@gmail.com

Absract
Necrobacillary pododermatitis of sheep is one of the most widespread diseases of this species in the Republic of Moldova. Histopathological changes revealed consist of congestion, hemorrhagic, then purulent, infiltration of epidermal crests. In the chronic form of the disease a severe disturbance of the bone structure takes place manifested by the osteoporosis, thus reducing the resistance of the distal phalangeal bones.
Key words: necrobacillary pododermatitis, soft tissues, bones

Introduction

Necrobacillary pododermatitis of sheep is one of the most widespread diseases of this species in the Republic of Moldova. The disease is characterized by a high contagion and massive affecting of animals. Acropodia lesions lead to the limitation and even impossibility of sheep to move and reduces animals ability to eat [1]. As a result, sheep lose their weight until cahexia, which compromises them in terms of production and predisposes to various diseases. In the initial phase the disease begins with the involvement of the hooves and soft tissues of the fingers, then the process includes bones of distal phalanges [2, 3].

In the present investigation we aimed to study pathological processes in soft tissues of acropodia and physical properties of phalangeal bones necrobacillary pododermatitis of sheep.

Material and methods

To study morphopathological changes of the acropodia soft tissues of sheep damaged by necrobacillary pododermatitis we used histopathological method. For this purpose 157 pieces have been made. Mechanical properties of phalangeal bones collected from 48 healthy and diseased animals with necrobacillary pododermatitis were tested using mechanical presses (compression test) in the Laboratory of Material resistance of State Agrarian University of Moldova.

Results and discussion

Our data demonstrated that inflammatory processes, skin necrosis and changes of physical and chemical properties of phalangeal bones were highlighted in the soft tissues of sheep acropodia affected by necrobacillary pododermatitis. The disease begins with penetration of the pathological agent - *Fusobacterium necrophorum* - into interdigital space, which initiates an inflammatory process in soft tissues of acropodia [1, 4]. The surface of the interdigital fissure skin is moist, covered by whitish-grey mucus of unpleasant odor. Further process spreads in horn, forming pockets in the calcanean regions, which detach it from the base of the acropodial skin. As the horn is detached and ulcers on the base of the skin appear, the pathological process develops a chronic course with necrotizing of skin, subcutaneous tissue, tendons and joints.

Histological changes found out by us on microscope are installed progressively. In the first phase, they consist of congestion and hemorrhagic infiltration, then of purulent infiltration of epidermal crests. Then this infiltration also includes horny plates. The cells are separated from secretion, this determines their balloon degeneration, especially at the level of epidermis. By rupture of cellular membranes small vesicles are produced, further they are invaded by leukocytes, this, in turn, determines dissociation of the horn layer of living tissues. At the level of sections through the walls of the organs affected by chronic pododermatitis we found a total disorganization of connections between epidermal crests and horny plates of the hooves. At the level of sole, in the horn tissue of epidermal crests and in the horn tubular tissue appear changes that determine the aspect of a decayed horn. At the same time, disorganization and edema of epidermal crests appear.

Pathological changes in necrobacillary pododermatitis are manifested not only at the level of soft tissues but also in phalangeal bones. By comparing visually the bones of phalanges of healthy and diseased sheep we observed essential differences. The bones of diseased animals are larger in size, the medial and caudal parts being strongly dilated. The surface of affected bones represents numerous rough thickenings. Coronary and sesamoid bones placed at greater distance from the pathological process are less changed, presenting some deformities and insignificant outgrowth.

Mechanical properties of the bones collected from healthy animals also differ from those collected from diseased sheep. The compression test showed that bones from healthy animals could resist a force of 290-320 kg, while those from diseased animals were destroyed at a force of 120 kg. The reducing of resistance of affected bones can be explained by the fact that in the chronic form of the disease, i.e. more than 6 months, a severe disturbance of the bone structure takes place manifested by osteoporosis and the increase of bone cavities [4].

This fact was confirmed by the X-ray method that found osteoporosis at the level of the third phalanx and demineralization. Knowing that osteoporosis can be revealed by X-ray only after resorbtion of at least 30% of bone mineral complex, as well as the fact that in case of advanced disease the hard layer of the third phalanx is attacked thinning a lot and having a spongious structure, we can get a true picture of the severity of injuries installed.

Conclusions

1. Necrobacillary pododermatitis of sheep is characterized by the involvement in the pathological process of soft tissues, hooves and bones of distal phalanges.
2. Study of the physical properties set considerable differences between macroscopic appearance and mechanical properties of distal phalangeal bones obtained from healthy animals and from those affected by necrobacillary pododermatitis

Bibliography

1. Edwards J.F., Davis D.S., Roffe T.J., et al. (2001) - Fusobacteriosis in captive wild-caught Pronghorns. Vet. Pathol. 38: 549-552.
2. Gyles C.L., Prescott J.F., Songer J.G., et al. (2010) – Pathogenesis of bacterial infections in animals. Blackwell Publishing, 643 pp.
3. Jacob W., Schroder H.D., Rudolph M., et al. (2000) - Necrobacilosis in free-living European bison in Poland. J. Wild Dis. 36: 248-256.
4. Nagaraja T.G., Narayanan S.K., Stewart G.G., et al. (2005) – Fusobacterium necrophorum infections in animals: pathogenesis and pathogenetic mechanisms. Anaerobe 11: 239-246.

FEMORAL HEAD NECROSIS CONSEQUENCE OF SEPTICEMIA WITH APEC STRAINS OF BROILERS

Ionica FODOR*, Ioana GROZA*, Oana PETREC*, Iancu S., Nicolae CĂTANA***
*Faculty of Veterinary Medicine Timisoara, **SC Ando Tours SRL Timisoara
Ifodor2001@yahoo.com

Abstract
Necrosis of the femoral head was diagnosed for the first time in 1972, to chickens, at that time the etiology of this disease is controversial. Subsequently, several research teams have studied the etiology and pathogenesis of this disease, proving that occur: E. coli (APEC strains), staphylococci, enterococci and avian reovirus. Research was made in a number of broilers, increased ground, with a herd of 6500 heads. Prevalence of disease was followed by pathological examinations conducted twice weekly starting on the first day of life until the 7 week - when a flock of chickens was slaughtered, there were 153 necropsied cadavers. To identify potential pathogens were performed bacteriological and serological examinations. The pathological examination determined the prevalence of this disease was, throughout the series, this indicator with a value of 63.40% relative, reported the number of bodies autopsied and 13.16% reported in mortalitaea cumulative. Bacteriologic analysis of samples performed revealed the presence of one bacterial species, namely E. coli, all isolates were classified in the APEC pathotype. The results of serological examination confirmed the presence of avian reovirus infection in flocks of chickens under observation.

Keywords: femoral head necrosis, broiler, APEC

Introduction

Necrosis of the femoral head was diagnosed in 1972 for the first time, to chickens, at that time the etiology of this disease was controversial. Subsequently, several research teams have studied the etiology and pathogenesis of this disease, proving that occur: E. coli (APEC strains), staphylococcus, enterococcus and avian reovirus. These biotic pathogens act according to the preference of many predisposing factors associated with the intervention represented by: hybrid increased age, feeding chickens, growing technology and hygiene conditions (1, 3, 5, 6).

This disease has been reported in recent years in broiler farms in west of the country, with varying frequency.

The research was conducted in a broiler farm in the Timis county, where the necrosis of the femoral head evolved in different frequency in chickens.

Materials and methods

This study was made, on a herd of 6500 heads increased to ground.

Pathological examinations were conducted twice weekly, starting on the first day of life until the 7 week of July, when a flock of chickens was slaughtered. In total there were 153 chickens necropsy and was tracked the frequency of necrosis of the femoral head and the presence of other infectious diseases.

From cadavers with necrosis of the femoral head and others specific lesions were sampled, long bone respectively, for bacteriological examination, primary sowings were made in broth and on agar, from long bone.

After 18-20 hours of incubation at 37 ° C in aerobically condition, were appreciated morphologic and cultural characters, isolates strains were biochemically identified.

Cultures identified as E. coli, was subsequently passed on API 20E galleries and interpreted according to the methodology.

Fixing the dye Congo Red agar was made on TSA (Tryptic Soy Agar) with added 0.15% bile salts and 0.03% dye.

Serological examination was carried out in order to confirm infection with avian reovirus. Blood samples were taken from chickens in a randomized mode, as follows:

-R1: 17 days old (20 samples)

-R2: 31 days old (20 samples)

Specific antibodies were detected by ELISA (Enzyme Linked Immunosorbent Assay), using the kit FlockChek Avian Reovirus Antibody Test Kit, furnished by IDEXX Laboratories, Inc.

Results and discussion

Research has provided important results on the etiology of necrosis of the femoral head and its prevalence depending of broilers age.

Laboratory examination results revealed that the main etiological agents are avian reovirus and E. coli (APEC strains).

Pathological examination who was performed twice weekly, starting on the first day of life of chickens revealed the presence of necrosis of the femoral head in varying degrees, depending on the age-old broilers. The prevalence of this disease is given in Table 1.

Necrosis of the femoral head was unilateral or bilateral. In some chickens necrosis was more pronounced, and in others was less pronounced, affecting only the articular cartilage.

By this test was established prevalence of this disease throughout the effective, this indicator with the relative value of 63.40% reported the number of chickens necropsied and 13.16% reported on cumulative mortality. Lowest prevalence was reported in the weeks I, II and IV, and in the weeks III, V, VI, VII it has had the highest values. Unilateral necrosis was present in 30.07% of necropsied chickens and bilateral necrosis was present in 33.33% of necropsied chickens.

Necrosis of the femural head as a consequence of colisepticemia, was associated with mycoplasmosis and pseudomonosis, at 81 cadavers necropsy, representing 52.94%.

At broilers chicken taken in this study, was determined and cumulative mortality also who had the, and the relative value was 6.7%.

Bacteriologic analysis of samples performed revealed the presence of one bacterial species, namely E. coli, all strains isolates were classified in the APEC pathotype. From a total of 97 bacteriological samples examined, 41 respectively 42.27% were positive, were only strains of E.coli isolated. Isolations frequency of E. coli strains is given weekly Table 2.

The results of serological examination this examination, performed by ELISA, are shown in Table 3.

At the first harvest at 17 days old, six groups of titers were identified (0-5), with a minimum titer of 12 D.O. and the maximum titre of 1022 D.O.

At the secound harvest at 31 days old, have been identified 8 groups of titers (0-7), the minimum titre being of 63 D.O., and maximum titre being of 1453 D.O..

Analyzing these results, we note that at 17 days old (R1) were 10 positive serum samples, respectively 50%, and at age of 31 days (R2), 19 samples were positive for serum, respectively 95%.

The results show that in a period of 14 days the proportions of positive sera increased 1.9 times, suggesting that the avian reovirus infection have horizontal extension in the flock under observation.

The results of serological examination confirmed the presence of infection with avian reovirus of chicken flocks. Postinfection antibody titers obtained after second harvesting demonstrate the presence of the seroconversion phenomenon consecutive evolution reovirosis avian.

Necrosis of the femoral head develops in recent years, frequently in broiler chickens being produced in association with some strains of avian reovirus with some bacteria, frequently with E. coli (APEC strains), with staphylococcus and enterococcus (1, 2, 5, 8).

This value is similar to the prevalence reported of other authors. Thereby (2000), through research conducted established that the disease McNamee Perpetua prevalence was 17.3%. Similar values of prevalence were reported from other authors (5).

Dinev's (2009) studies show that *E. coli* was isolated more than 90% of the bacteriological test samples, with femoral head necrosis (4).

Results of bacteriological examination revealed the presence of E. coli strains based on phenotypic testing that character, the setting of red Congo, allow discrimination APEC strains responsibles for extraintestinal infections, having a place of penetration of the body, respiratory system. These APEC strains consistently produce at meat chickens, septicaemic infections, who in this researchs, was confirmed by the presence of fibrinous polyserositis lesions and isolation of a number of 41 strains of E. coli. These strains who have set Congo red which allows integration into APEC pathotype. Several researchers have demonstrated the association of necrosis of the femoral head with colisepticemia, demonstrating in this way the pathogenic role of these strains (5, 6, 8).

Because don't have been isolated strains of staphylococcus and enterococcus, as communicated Oktay (2009) in one study, isolated E. coli with a larger number of strains of staphylococcus and enterococcus, we can not confirm the role of these bacteria in disease etiopathogenesis (7).

Table 1. The frequency of necrosis of the femoral head and cumulative mortality

Weeks	**Number of cadavers**		**Number of necropsy cadavers**		**Cumulative mortality**		**Necrosis of the femoral head**	
	No	%	No	%	No	%	No	%
I	87	11,80	16	10,46	87	0,80	12	7,84
II	98	13,30	12	7,84	98	0,90	9	5,88
III	103	13,98	24	15,69	103	0,94	16	10,46
IV	85	11,67	15	9,80	86	0,78	10	6,54
V	125	16,96	27	17,65	125	1,14	16	10,46
VI	116	15,74	30	19,61	116	1,05	18	11,76
VII	122	16,55	29	18,95	122	1,11	16	10,46
Total	**737**	**100**	**153**	**100,00**	**737**	**6,7**	**97**	**63,40**

Table 2. The result of bacteriological examination

Weeks	Number of examinated samples	Positive samples		Bacterial species	Negative samples	
		Number	%		Number	%
I	12	7	58,33	*E.coli*	5	41,67
II	9	4	44,44	*E.coli*	5	55,56
III	16	8	50,00	*E.coli*	8	50,00
IV	10	7	7,00	*E.coli*	3	3,00
V	16	8	50,00	*E.coli*	8	50,00
VI	18	4	22,22	*E.coli*	14	77,78
VII	16	3	18,75	*E.coli*	13	81,25
Total	**97**	**41**	**42,27**	***E.coli***	**56**	**57,73**

Table 3. The result of serological examination

No.	**R 1/ 17 days/ 20 samples**		**R2/ 31 days/ 20 samples**	
	Titer group	**No. of samples**	**Titer group**	**No. of samples**
1.	0	10	0	1
2.	1	3	1	1
3.	2	4	2	2
4.	3	3	3	2
5.	4	-	4	3
6.	5	-	5	5
7.	-		6	3
8.	-		7	3
9.	Maximum titer	1022 D.O	Maximum titer	1453 D.O.
10.	Minimum titer	12 D.O.	Minimum titer	63 D.O.
11.	Geometrical mean titers	89	Geometrical mean titers	245

Conclusions

1. Necrosis of the femoral head having a prevalence of 13.16% raported on cumulative mortality and one of 63.40% raported of number of necropsied cadaver.
2. The disease was reported starting from the first week of life the chickens, the weekly prevalence ranging between 5.88% -11.76%.
3. By bacteriological examinations were performed isolated strains of E. coli, classified based on phenotypic characters, in APEC pathotype, proportion of these strains isolation was of 42.27%.
4. By serological tests carried out by immunoassay test, were put in evidence antibodies of avian antireovirus , confirming the infection with this virus, and the phenomenon of seroconversion confirmed active development of an infectious process.

5. By bacteriological examination were not identified staphylococcus and enterococcus that was considered by some researchers as etiologic and bacterial agents that occur in the etiology of this disease.

Acknowledgements

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References

1. Barnes,H.J. (2008) - Other Bacterial Diseases, in Disease of Poultry, 12 th Edition, Editor-in-chief, SAIF.Y.M., Blackwell Publishing, 891-970.
2. Cătană, N., Popa, Virgilia, Herman, V., Fodor, Ionica (2008) - Cercetări anatomoclinice şi serologice într-un focar de reoviroză la puii de carne, Lucr. Şt. Med. Vet. Iaşi, 51 (10).
3. Catană, N. (2001) - Infecţii produse de germeni din genul Staphylococus,în Boli Infecţioase ale Animalelor, sub redacţia MOGA MÂNZAT,R., Ed.Brumar, Timişoara, 325-345.
4. Dinev, I. (2009) Clinical and morphological investigations on the prevalence of lameness associated with femoral head necrosis in broilers, Br. Poult. Sci, 50, (3), 284-290.
5. Ewers, Christa, Janßen, Traute, Wieler, L. H. (2003) - Aviäre pathogene Escherichia coli (APEC), Berl. Münch. Tierärztl. Wschr., 116, 381-395.
6. McNamee,T. Perpetua (2000) - Bacterial chondronecrosis with osteomyelitis („femoral head necrosis") of broiler chickens: a review, Avian Pathology 29,253-270.
7. Oktay, N., Temelli, S., Çarli, K.T. (2009) – Phenotypic characterisation of Enterococcus spp. from femoral head necrosis lesions of chickens, Turk. J.Vet. Anim. Sci., 33 (6), 509-516.
8. Swaminathan, T. R., Chandran, N. D. J., Dorairajan, N. (2004) - Virulence attributes of Escherichia coli associated with colisepticaemic chickens, Indian Journal of Animal Sciences, 74, (3), 248-852.

RESEARCH ON THE PREVALENCE OF VIRULENCE GENES IN APEC STRAINS WITH ZOONOTIC RISK

Ionica FODOR*, Ioana GROZA*, Virgilia POPA, Nicolae CĂTANA***
*Faculty of Veterinary Medicine Timisoara; **SN Institute Pasteur SA Bucharest
Ifodor2001@yahoo.com

Abstract
In the species E. coli isolates from sick birds, but also in humans, are classified based on characters, phenotypic and genotypic, in several pathotypes. Because of the pathogenic factors may cause extraintestinal infections in humans, thus having recognized zoonotic risk. Have been examined a total of 3146 corpses, and according to existing lesions were sampled for laboratory examination.A total of 587 broiler corpses were subjected to routine bacteriological examination, which confirmed the infection colibacilare a number of 552 cases. These biochemical characteristics were positive at all 46 isolated strains. This way their belonging to the Escherichia coli species was confirmed. On the red Congo agar the colonies were identified by their dark red color and dry, wrinkled appearence. Identification of virulence genes was performed by PCR, which allowed the classification the isolates in APEC pathotype. The results obtained show us that the association of two genes was more frequent, as follows: the association of the ompA + iss was present at 4 stems (8,69 %), the association of the ompA + fimH genes was present at 4 stems as well (8,69%), and the association of the iss + fimH was present at 5 stems (10,86%). Because virulence factors described above, in birds, APEC strains have as penetration sites the areas of gas exchange, such lungs and air sacs, of which they enter in the bloodstream and cause septicemia.
Keywords: virulence factors, APEC, zoonotic risk.

Introduction

In the species E. coli isolates from sick birds, but also in humans, are classified based on characters, phenotypic and genotypic, in several pathotypes.

In last year's, are well defined extraintestinal infections in poultry and humans (Extraintestinal Pathogenic E. coli-ExPEC), produced by E. coli strains classified in pathotypes: APEC (Avian pathogenic E. coli), UPEC (Uropathogenic E. coli), NMEC (Newborn Causing E. coli Meningitis) and E. coli septicemia.

Strains classified in these pathotypes has many pathogenic factors: adhesin (fimbriae P and S), of iron acquisition factors (aerobactine), capsule, somatic O antigens, toxins, complement resistance. They are known as extraintestinal virulence factors.

In birds, *APEC* strains most frequently isolated belong to several serogroups, O_1, O_2 and O_{78}, and produce septicaemic and localized infections, especially in broilers. Because of the pathogenic factors may cause extraintestinal infections in humans, thus having recognized zoonotic risk.

Materials and methods

Investigations have been performed in broiler farms in western Romania. In the farms studied were conducted epidemiological, clinical and pathological.

Have been examined a total of 3146 corpses, and according to existing lesions were sampled for laboratory examination.

A total of 587 broiler corpses were subjected to routine bacteriological examination, which confirmed the infection colibacilare a number of 552 cases.

Bacteriological exam was realized in chickens that developed avian colibacillosis. From pathological examination cases, depending on existing lesions, samples were taken

(long bone), which were made primary isolations by common methodology in broth and agar medium. After 18-20 hours of incubation at 37°C temperature, in aerobical conditions, the cultural characteristics were noted, then being made Gram stained smears from the colonies. The cultures that presented Gram negative bacillary form bacteria were passed on Levine and S - S media, biochemical characteristics were either determined by *API 20E* kit (1).

The fixing of the dye Congo Red WAS made on TSA agar (Trypticase Soy Agar) with additional year 0.15% 0.03% biliary Salts and dye, the inseminations having been made through exhaustion, with the bacteriological dowser, in order to obtain isolated colonies (3, 5).

Antibiosensibilitatea isolates was carried out with sensitivity testing by Kirby Bauer method.

Identification of virulence genes was performed by PCR, which allowed the classification the isolates in APEC pathotype.

Results and discussion

From a total of 587 bodies were isolated 46 strains of E. coli that had behavior typical of APEC strains.

After the insemination, made after the methodology described before, cultures characteristic of the *Escherichia coli* species were obtained. This way, a dense enough turbidity was noticed in bouillon, and on the agar, colonies type S with a 2-6 mm diameter opaque and with no pigmentation.

On the S-S environment, the lactose positive cultures, namely *Escherichia coli*, have formed pink colonies, and on the Levin environment, the colonies had dark colors, with metallic gloss.

We mention that the isolated strains that could not reach these criteria, characteristic of the *Escherichia coli* species were excluded.

Biochemical exams, carried out after the described methodology, have shown characters with phenotypical characteristics for the *Escherichia coli.*

This way, on the TSI environment, on the right side, the fermentation of the glucose took place. It is characterized by the production of gas and yellow dye, and on lean surface, after the fermentation of the lactose, the color turned to yellow.

The mobility was shown on the MIU environment, the production of indol and the hydrolysis of the urea.

These biochemical characteristics were positive at all 46 isolated strains. This way their belonging to the *Escherichia coli* species was confirmed.

Most isolated strains were sensible to florfenicol and ciprofloxacin, moderately sensible to colistin sulphate, spectinomycin and enrofloxacin and resistant to neomycin, tetracycline, doxycycline and erythromycin.

On the red Congo agar the colonies were identified by their dark red color and dry, wrinkled appearence.

The screening carried out in the multiplex (multi primer) variant, shown the presence of the 3 genes that govern the synthesis of some virulent factors characteristic of the APEC stems, at a number of 34 stems (73,91%) of the 46 stems tested.

The results obtained show us that the association of two genes was more frequent, as follows: the association of the *ompA + iss* was present at 4 stems (8,69 %), the association of

the *ompA + fimH* genes was present at 4 stems as well (8,69%), and the association of the iss + *fimH* was present at 5 stems (10,86%).

The results show the presence of the APEC stems, at chickens for meat, genotypically characterized by the presence of at least one gene, out of the 3 that are governing the main pathogen factors of this patotype and, can be transmitted directly or indirectly in incubation centers and shelters.

Researches have shown the presence of the 3 genes, responsible for the codification of the main pathogen factors of the APEC stems, which produce avian colibacillosis. This way, the *ompA* gene which codifies the synthesis of a protein of external membrane, responsible for the bacterial attachment at the level of the respiratory epithelium cells was present at 52,17% of the tested stems, and the *fimH* gene, which is a component of the *fim* operon, responsible for the synthesis of the type 1 fimbria with whom *Escherichia coli* attaches itself to the respiratory epithelium cells was also present at, was present at 52,17%. The *iss* gene responsible for the synthesis of a protein of external membrane that induces resistance to the complement and, implicitly, favors this way the colonization and multiplication of the colbacilli in the blood, was present at 63,04% of the tested stems.

E. coli strains of APEC pathotype have constant genotypic and phenotypic characteristics which gives them a special pathogenicity for birds, but also for humans. Because virulence factors described above, in birds, *APEC* strains have as penetration sites the areas of gas exchange, such lungs and air sacs, of which they enter in the bloodstream and cause septicemia. The same genetically coded virulence factors, especially protectine and intimine, give special pathogenicity to *APEC* strains also in human subjects by producing urinary infections (Uropathogenic *E. coli-UPEC*) and meningitis (*NMEC* - Newborn Meningitis *E. coli*). Urinary tract infections in humans are a consequence of the *pap* operon presence in these strains, with *papG* alleles that govern the synthesis of pathogenic factors for production of pyelonephritis. Several authors (1,2, 3, 4, 5) studied a large number of *APEC* strains isolated from birds, especially in chickens, and noted the presence of several genes and several operons which govern the virulence factors mentioned above, responsible for developing of extraintestinal infections in birds and humans. This data shows the risk of zoonotic infections with *APEC* strains, and also the epidemiological importance of birds as a natural reservoir of these infections.

Conclusions

1. The PCR technique used during these researches is a molecular technique used to show the *ompA, iss* and *fimH* genes using three multiplex amplification reactions.
2. The multiplex PCR screening carried out, established a high frequency of the APEC stems, isolated from bursts of avian colibacillosis.
3. The results have shown the fact that these 3 genes, which codify pathogen factors are, usually, associated by two or three.
4. The presence of these genes is correlated to the capacity of tying the red Congo, a phenotypical character of discriminating the APEC stems.

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References

1. Barnes,H.J., Nolan L.C., Vaillancourt J.P. (2008)- Colibacilosis, in Disease of Poultry, 12 th Edition, Editor-in-chief, SAIF.Y.M., Blackwell Publishing, 691-739.
2. Ewers, Christa, Esther-Maria Antao, Ines Diehl, Philipp, H.C., Wieler, L. H (2009) Intestine and environment of the chicken as reservoirs for extraintestinal pathogenic Escherichia coli strains with zoonotic potential, Applied and Environmental Microbiology, 75, 1, 184–192
3. Ewers, Christa, Janßen, Traute, Wieler, L. H. (2003) - Aviäre pathogene Escherichia coli (APEC), Berl. Münch. Tierärztl. Wschr., 116, 381-395.
4. Giovanardi, D., Campagnari, E., Ruffoni, L. S., Pesente, P., Ortali, G., Furlattini, V. (2005) - Avian pathogenic Escherichia coli transmission from broiler breeders to their progeny in an integrated poultry production chain, Avian Pathology, 34, (4), 313-318.
5. Rodriguez-Siek, Kylie E., Giddings, Catherine W., Doetkott, C., Johnson, T. J., Nolan, Lisa K. (2005) - Characterizing the APEC pathotype, Vet. Res. 36, 241-256.

PHOSPHOLIPASE PRODUCTION OF SOME *CANDIDA SPP.* STRAINS ISOLATED FROM HUMANS AND ANIMALS

George Cosmin NADĂŞ, Marian TAULESCU, Nicodim FIŢ, Flore CHIRILĂ, Cosmina BOUARI, Sorin RĂPUNTEAN, Pompei BOLFĂ, Vasile RUS
University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca
3-5 Mănăştur Street, Faculty of Veterinary Medicine, gnadas@usamvcluj.ro

Abstract
Phospholipase production of yeasts is a rapid method for determining the pathogenicity of such strains. Twenty-two Candida spp. strains isolated from humans and animals were tested regarding phospholipase production on culture medium supplemented with egg yolk. The isolates were represented by mastitis cow milk, external ear channel secretions from dogs, urine samples from humans, faeces samples from pigeons and parrots, pharyngeal exudates from humans and dogs, rumen liquid from cows and yeast strains that contaminated different culture mediums. The phospholipase activity zone (Pz) is represented by the ratio between the diameter of the colony and the diameter of the opaque white area around it. A total of 12 strains (54,54%). Presented phospholipase activity with values from 0,81 to 0,23.

Key words: phospholipase, *Candida spp*., animals, pathogenicity

Introduction

Fungal diseases of mammals, mycoses, range from the common mild cutaneous or subcutaneous skin infections, to the potentially lethal acute or chronic infection of deep tissues that are typically caused by *Candida* species(2). Of the *Candida* species affecting animals, *Candida albicans* is by far the most common. *Candida albicans* belongs to the class *Ascomycetes* and the family *Saccharomycetaceae*. This yeast can live as harmless commensal in many different body locations, and is carried in almost half of the population (2). However, in response to a change in the host environment, *Candida* can convert from a benign commensal into a disease-causing pathogen, causing infections in the oral, gastrointestinal, skin and genital tracts(1).

C*andida albicans* can be found in the intestinal tracts and in the oral cavities of healthy individuals, and is also the predominant causative agent of human candidosis. In addition, all domestic animals like cattle, horses, pigs, cats, and dogs as well as birds are susceptible to *Candida* infections. This suggests that animals could be vectors of transmission or reservoirs of strains causing human disease and may present a risk for immunocompromised patients(2). Although, many case reports of candidiasis in animals are published, very little is known about the identity and origins of these infecting strains and the genetic relationship among *C. albicans* isolates from human and animal sources(3).

Phospholipases are important pathogenicity determinants in *Candida spp*. They play a significant role in damaging cell membranes and invading host cells. High phospholipase production is correlated with an increased ability of adherence and a higher mortality rate in animal models(3).

The aim of the study was rapid detection of phospholipase activity in case of candida isolates from humans and animals using plate method on culture medium supplemented with egg yolk.

Materials and methods

The researches took place between October-December 2011 within the Microbiology Laboratory of the Faculty of Veterinary Medicine Cluj-Napoca. Twenty-two *Candida spp.* strains isolated from mastitis cow milk, external ear channel secretions from dogs, urine samples from humans, faeces samples from pigeons and parrots, pharyngeal exudates from humans and dogs, rumen liquid from cows and yeast strains that contaminated different culture mediums were isolated and identified by morphological and cultural characters.

The strains were then subcultured on Sabouraud agar and 24 h colonies from this culture medium was tested following the spot method on nutrient agar containing 10% egg yolk. Usually two *Candida* strains were tested on the same plate.

This plate method allows rapid detection and measurement of the extracellular activity in a number of clinical isolates. The ratio of colony diameter to diameter of the dense white zone of precipitation around phospholipase positive colonies, (Pz value), correlates with hydrolysis of phosphatidylcholine by concentrated culture filtrates of selected test isolates. A large variation in phospholipase activity is found between different isolates, however the Pz value is constant for any one isolate regardless of the site from which it is recovered in the patient(4).

The egg yolk plates containing the tested samples were then incubated for 7 days at 32˚C and examined daily.

Results and discussions

Regarding the results, the Pz value 1 was recorded for 10 (45,45%) strains, the lack of phospholipase activity being correlated with the absence of pathogenicity for these strains. For the rest of 12 (54,54%) strains the Pz value ranged from 0,81 to 0,23.

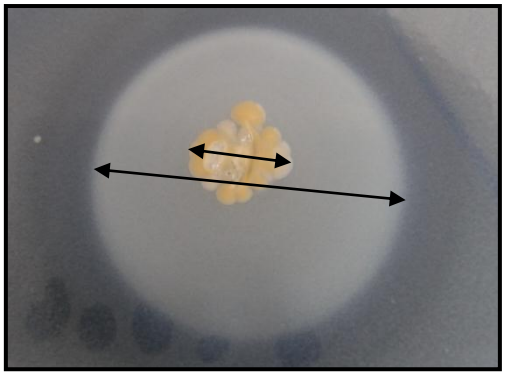

Fig. 1. *Candida spp.* strain isolated from mastitis cow milk with Pz value of 0,33 (0,5 cm /1,5 cm) – detail image

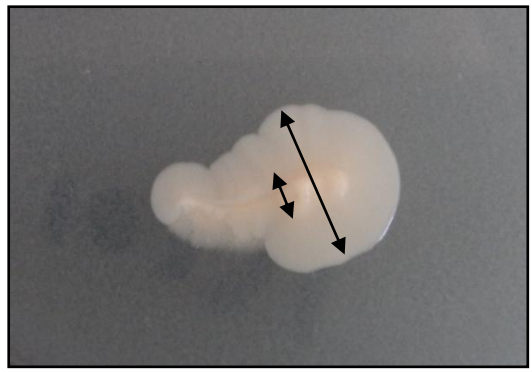

Fig. 2. *Candida spp*. strain isolated from pharyngeal exudate with Pz value of 0,30 (0,2 cm /0,65 cm) – detail image

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The highest percentage of phospholipase production strains was observed for the isolates of human origin (both pharyngeal exudates and urine samples) followed by milk samples and isolates from dogs.

The percentage of strains with phospholipase production of 54,54 is similar to the results of Birinci A. et all, 2005, who obtained 61,3 percentage of phospholipase positive strains. In this study is also mentioned that none of the other *Candida spp*. except albicans is exhibiting phospholipase activity.

Conclusions

1. The percentage of positive strains of 54,54 can be easily explained mainly because only *Candida albicans* is producing phospholipase and secondly because most probably some of the strains, even if *C. albicans* are only commensal.
2. These results suggest that clinical isolates of *Candida spp.* exhibit varying degrees of phospholipase activity being also correlated with yeast sensitivity to antimycotics, subject also approached in a similar study.
3. Evaluation of phospholipase activity for *Candida spp*. is a quick and easy to use method for determining pathogenicity of yeasts.

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Bibliography

1. Birinci A., Cihan CC, Bilgin K, Acuner C, Durupinar B., 2005, Phospholipase activity of Candida species isolated from different clinical samples, Mikrobiyol Bul., 39(2):205-9.
2. Fotedar R, Al-Hedaithy SS., 2005, Comparison of phospholipase and proteinase activity in Candida albicans and C. dubliniensis, Mycoses, 48 (62-67).
3. Kothavade R . J. and M. H. Panthaki, 1998, Evaluation of phospholipase activity of Candida albicans and its correlation with pathogenicity in mice, J. Med. Microbiol, 47 (99 102).
4. Price MF, Wilkinson ID, Gentry LO, 1982,Plate method for detection of phospholipase activity in Candida albicans, Sabouraudia. 1982 Mar; 20(1):7-14.

EVOLUTION OF CERTAIN PHYSICOCHEMICAL FACTORS OF THE DANUBE RIVER WATER DURING YEAR 2009

Lucica GERU[1], Angela TROFIMOV[2], Flavia RUXANDA[3], Vasile RUS[3], Ionel RADU[4], Viorel MICLĂUŞ[3]
[1]Sanitary-Veterinary Directorate and for Food Safety, Brăila
[2]Institute of Research and Development for Aquatic Ecology, Fishery and Aquaculture, Galaţi
[3]Faculty Of Veterinary Medicine Cluj-Napoca, 3-5 Mănăştur Street
[4]Sanitary-Veterinary Directorate and for Food Safety, Galaţi
vasilerus2002@yahoo.com

Abstract
During year 2009 water samples were harvested from the Danube River monthly, at the sample station Brăila and the main physicochemical parameters were determined. The concentration of the physicochemical parameters unstandardized by the law (carbonates, bicarbonates, alkalinity, fixed residue, suspensions), did not exceed the the maximum values recommended by the speciality literature. Most of the physicochemical parameters, whose concentration is standardized by Ord. MMGA no. 161/2006, range in close values to the admissible ones (pH, organic substance, nitrite, chloride, sulphate and ammonia concentration). On the other hand, the Ca^{2+}/Mg^{2+} and NO_3^-/ PO_4^{3-} proportions were totally inadequate, which makes the Danube water not able to assure the optimal reproduction and alimentation conditions for fish (especially spawn).
Keywords: Danube, fish, physicochemical, proportion, reproduction.

Introduction

Generally, the Danube River water is characterized by a relative stability of the chemical composition. This is especially due to the large flow rate, capable of assuring a significant dilution of the tributary streams, the urban and industrial contribution, and the high capacity of the natural biological self-cleaning system (Bud *et al.*, 2010). The chemical elements in the water are at a certain rate, and the necessity for one element or the other is dictated by the biocoenosis itself, through the so called law of the limiting factors (Antonescu, 1963). Oxygen, carbon dioxide, calcium and magnesium salts, nitrogen and phosphorus, oligoelements can act as limiting factors.

Lately, frequently changes of the water chemism are registered, which are determined by a series of anthropic factors, and a deviation of some substances or chemical elements' concentrations can be noticed (Păltenea and Popa, 1995), with direct consequences on fish reproduction and breeding (Bud and Bud, 1995).

Material and methods

For a more correct physicochemical assessment of the Danube River water, samples were harvested at equal intervals (one month), at the same sample station and the same parameter groups were analyzed, using the same analysis method. Some of the parameters were determined by direct measurement (pH), others volumetrically (organic substance, calcium, magnesium, chloride, carbonates, bicarbonates, alkalinity, total hardness), gravimetrically (sulphate, suspensions, fixed residue), spectrophotometrically (nitrites, nitrates, phosphates, ammonia, ammonium).

Physicochemical parameters of water were analyzed according to the limit values established by Order 161/2006 for water quality classes II and IV or admissible limits in the speciality literature.

Results and discussion

The monthly evolution of the physicochemical parameters of the Danube River is presented in Table 1 and 2.

Table 1. The dynamics in the physicochemical parameters of the water harvested from the Danube River in the 1st semester of year 2009 – Brăila station

Analyzed parameters	U.M.	Period						Admissible limits
		Jan.	Feb.	Mar.	Apr.	May	Jun.	
pH	upH	7,9	7,52	7,5	7,63	7,58	7,7	6,5-8,5
Organic substance	mg $KMnO_4$ /l	31,47	25,97	23,54	30,0	24,5	30,8	5-60
CCO-Mn	mg O_2/l	7,87	6,49	5,88	7,5	6,15	7,7	10-20
Calcium (Ca^{2+})	mg/l	82,3	58,32	20,83	54,0	54,16	51,77	100-200
Magnesium (Mg^{2+})	mg/l	25,26	35,36	47,9	38,8	50,5	47,22	50-100
Ca^{2+}/Mg^{2+}		3,3	1,65	0,43	1,39	1,0	1,09	5
Chlorides (Cl^-)	mg/l	63,7	78,6	68,16	60,2	58,8	44,2	50-250
Sulphates (SO_4^{2-})	mg/l	75	94,3	95,5	45,3	70,6	84,2	120-250
Carbonates (CO_3^{2-})	mg/l	missing	missing	missing	missing	missing	missing	0-80
Bicarbonates (HCO_3^-)	mg/l	328,7	353,09	268,4	288,6	240,0	275,9	20-600
Nitrites (NO_2^-)	mg/l	lipsă	0,132	0,198	0,132	0,06	0,132	0,03-0,06
Nitrates (NO_3^-)	mg/l	2,55	1,50	4,3	3,81	4,10	3,85	3-5,6
Phosphates (PO_4^{3-})	mg/l	0,67	0,35	0,48	0,54	0,82	0,54	0,4-0,75
Ammonia (NH_3)	mg/l	0,0013	0,0044	0,048	0,01	0,067	0,087	0,005-0,3
Ammonium (NH_4^+)	mg/l	0,038	0,195	0,95	0,3	0,293	0,210	0,8-1,2
Total alkalinity	ml HCl/l	5,4	5,8	4,4	4,7	4,8	4,5	0,2-6
Total hardness	^oD	16,83	15,71	13,4	18,5	19,1	12	8-20
Fixed residue	mg/l	250,2	289,5	362,5	470,5	510,5	312,5	750-1000
Suspensions	mg/l	20,5	22,5	38,5	32,6	35,8	28,5	15-80

The concentration of some of the analyzed physicochemical parameters is standardized by the law, whereas others' is not, but for the latter, the speciality literature recommends maximum admitted values.

The concentration of the physicochemical parameters unstandardized by the law (carbonates, bicarbonates, alkalinity, fixed residue, suspensions), did not exceed the the

maximum values recommended by the speciality literature, which shows that from this point of view, breeding and reproduction of the fish is not endangered.

Table 2. The dynamics in the physicochemical parameters of the water harvested from the Danube River in the 2nd semester of year 2009 – Brăila station

Analyzed parameters	U.M.	Period						Admissible limits
		Jul.	Aug.	Sept.	Oct.	Nov.	Dec.	
pH	upH	7,8	7,76	8,1	7,94	7,72	7,8	6,5-8,5
Organic substance	mg $KMnO_4$ /l	35,09	42,53	32,43	30,12	25,28	20,9	5-60
CCO-Mn	mg O_2/l	8,77	10,63	8,11	7,53	6,32	5,22	10-20
Calcium (Ca^{2+})	mg/l	62,49	30,08	41,6	50,69	29,16	58,32	100-200
Magnesium (Mg^{2+})	mg/l	37,15	20,55	29,05	22,14	26,52	35,36	50-100
Ca^{2+}/Mg^{2+}		1,67	1,46	1,43	2,28	1,09	1,65	5
Chlorides (Cl^-)	mg/l	74,3	69,18	42,51	50,45	64,2	65,80	50-250
Sulphates (SO_4^{2-})	mg/l	63,8	34,2	44,7	51,1	49,8	40,0	120-250
Carbonates (CO_3^{2-})	mg/l	missing	missing	17,64	3,0	remnants	missing	0-80
Bicarbonates (HCO_3^-)	mg/l	268	280,04	131,51	198,23	221,18	185,56	20-600
Nitrites (NO_2^-)	mg/l	0,09	0,06	0,0132	0,006	0,06	0,0132	0,03-0,06
Nitrates (NO_3^-)	mg/l	3,74	4,48	3,80	3,06	2,56	2,47	3-5,6
Phosphates (PO_4^{3-})	mg/l	0,50	0,36	0,78	0,51	0,36	0,43	0,4-0,75
Ammonia (NH_3)	mg/l	0,024	0,006	0,016	0,007	0,0045	0,067	0,005-0,3
Ammonium (NH_4^+)	mg/l	0,876	0,194	0,384	0,313	0,1455	0,293	0,8-1,2
Total alkalinity	ml HCl/l	4,4	4,6	2,75	4,0	4,5	4,7	0,2-6
Total hardness	^oD	15,4	8,96	12,06	10,65	10,9	11,57	8-20
Fixed residue	mg/l	435,2	293,7	275,5	354,2	226,3	312,5	750-1000
Suspensions	mg/l	32,4	26,5	24,2	30,0	22,9	28,7	15-80

The assessment of the physicochemical parameters whose concentration is standardized, show that according to the standards imposed by Ord. MMGA no. 161/2006 regarding the surface water quality, the majority range in close values to the admissible ones, even though they are not at an optimum level. The pH, organic substance, nitrite, chloride, sulphate and ammonia values are in this situation. We can state that concerning these parameters, Danube water is appropriate for breeding and reproduction of fish. Unfortunately, some of the most important parameters are at concentrations not only far from the optimum values, but from the admissible ones also. Thus, Ca^{2+} and Mg^{2+} ions must be in a certain proportion, with the optimal one recommended by the speciality literature being 5:1. It is known that the calcium along with magnesium ions are essential for a normal development

and growing up of the aquatic organisms, especially fish. The closer to 5:1 their proportion, the better their assimilation in the organism of the fish. Even though the concentration of the two ions does not exceed the maximum admitted values for neither of them, the Ca^{2+}/Mg^{2+} proportion is very deviated from the optimal one. The smallest values were registered in March (0.43). Remarkable, in none of the year's 2009 months, the 5:1 proportion was reach, not even in the months with highest values. We calculated the semestrial average values because of the high oscillation from one month to another, in order to have a more accurate situation of this proportion, especially for the first trimester, when the most delicate periods of a fish life take place (embryonic and larval stages). Analyzing the semestrial values, we observed that Ca^{2+}/Mg^{2+} proportion was 1.31 in the first semester and 1.59 in the second semester. It is clearly that beside the values lower than the optimal ones, there are large differences between the trimesters, the values of the first semester being lower. Ca^{2+}/Mg^{2+} proportion registered during 2009 does not offer favourable growing up and development conditions of the ichthyofauna from the Danube River. The negative effect of this imbalance will be obvious especially in the young specimens. First developing stages (embryonic and larval) appear and evolve in the first semester and can be significantly influenced by this major imbalance. The growth rhythm in larval, alevin and spawn is very fast, hence the mineral imbalances can have negative consequences on body development and survival rate. The two aspects are crucial for the reproduction and perpetuation of the fish species. Gradual decrease of the survival rate and appearance of underdeveloped and deficient specimens will have negative consequences in time, especially on fish reproduction. This actually leads to a decline of the fish species.

Other ions whose concentration is very important are nitrates (NO_3^-) and phosphates (PO_4^{3-}). Similar to calcium and magnesium, every ions' level matters, especially the proportion between them. Most plausible explanation for this situation is the fact that the water resulted from melting snow "washes" the areas near the waters, collecting nitrates from large areas and takes them to the Danube. The highest phosphate level was also identified in the first semester, but not at the snow melting time, but a little bit later (March-May period). This period coincides with the application of most mineral chemical fertilizers for soil fertilization, utilized for agricol crop and their carriage in the hydrographic system of Danube River by the rain water which washes these surfaces. Nitrates and phosphates are very important for the adequate development of the phytoplankton and zooplankton, which represent essential components in spawn's alimentation. For these assimilation processes to take place at a high level, nitrates and phosphates must be in a certain proportion. According to the existing data in the speciality literature, the optimum NO_3^-/ PO_4^{3-} proportion is 4:1. Unfortunatelly, the values registered in year 2009, in the Danube River water were far from this optimum proportion. Throughout the year, it was observed that the proportion was exceeded, sometimes with large differences from one period to another, in some cases, the value of this proportion being more than double in comparison to the optimum one.

The imbalance of NO_3^-/PO_4^{3-} proportion reverberates negatively on the development of the zooplankton and phytoplankton and compromises the specific food from the first period of life, both quantitatively and qualitatively. The consequences may lie in the abnormal development (underdevelopment) of the young and significant impact on the survival rate. Indirectly, these aspects influence the fish reproduction negatively, whose decline in time is inevitable if the conditions do not change radically.

Although all the physicochemical parameters unstandardized by the law and the majority of the standardized ones were situated in admissible limits, totally inappropriate Ca^{2+}/Mg^{2+} and NO_3^-/PO_4^{3-} proportions determine the Danube water not to assure optimum conditions for fish reproduction and alimentation (especially spawn). These aspects have certainly contributed, along with other factors, in the decline of the fish species in the Danube River, signalized more and more insistently in the past decades.

Conclusions

1. The pH, organic substance, nitrite, chloride, sulphate and ammonia concentration values, range in close values to the admissible ones for an active aquatic ecosystem, according to Ord. MMGA no. 161/2006 regarding the surface water quality.
2. The carbonates and bicarbonates ions, alkalinity, fixed residue and suspensions concentration are not standardized by the law, but the speciality literature recommends maximum admitted values for these parameters, which were not exceeded in the analyzed water.
3. The optimum Ca^{2+}/Mg^{2+} proportion is 5:1, but in the analyzed water its values were much lower, which shows a major imbalance that can negatively influence the fish reproduction, development and growing up processes.
4. The NO_3^-/PO_4^{3-} proportion was high above the optimum value of 4:1 throughout the study period, which can have negative consequences on the normal development of the aquatic organisms which constitute food for fish.
5. Although a large number of parameters are in relatively normal limits, the imbalance of Ca^{2+}/Mg^{2+} and NO_3^-/PO_4^{3-} proportions, can negatively influence the survival and spawn developing rate, leading to the decline of fish species in time.

References

1. Antonescu C.S. (1969), Biologia apelor, Editura Didactică şi Pedagogică, Bucureşti
2. Bud I.,; V. Vlădău; M. Nădăşanu **(**2010**),** Tratat pentru creşterea peştilor, Ed. Texte, Cluj-Napoca
3. Bud I.; A. Bud (1995), Danube Delta faunistic importance genetic reserve for future, Simp. Naţ. Vol. XXI, p. 312-316, Cluj-Napoca
4. Păltenea Elpida; Carmen Popa (1995), Investigaţii biochimice la ştiucă, Aquarom' 95, p. 250-255.
5. Păsărin B.; Tr. Stan (2004), Reproducerea peştilor, Ed. Karro, Iaşi

THE SUBCLINICAL MASTITIS EFFECT ON MILK QUALITY FROM PRIMIPAROUS HOLSTEIN FRIESIAN ROMANIAN CATTLE POPULATIONS

A.C. GRĂDINARU[1], O. POPESCU[2], Şt. CREANGĂ[1]
[1] University of Agricultural Sciences and Veterinary Medicine of Iasi, Romania
[2]Purdue University, Veterinary School, USA
andre_gradinaru@yahoo.com

Abstract
The present work aimed to evaluate correlations between somatic cell counts and some physico – chemical milk quality indexes. A positive correlation between the progressively increasing subclinical mastitis incidence and the somatic cell count values ranging from 400 – 600 (x 1000 cells/ml) was observed. The subclinical mastitis that developed into a clinical form and the highest class of somatic cell count values (> 600,000 cells/ml) were found to be in a high dependency in autumn and winter. A decrease of the fat content in the mastitis milk was also observed. No statistically significant correlation between the subliclinical mastitis incidence and the total protein content variantion was observed. The progressively increasing subclinical mastitis incidence was positively correlated with more than 3% caseins in all seasons. The subclinical mastitis that develops to the clinical form and the lowest class of caseins %(< 2.5) were found to be in a high dependency in autumn and winter seasons. A statistical depedency between the subclinical mastitis incidence and different lactose content classes in autumn was also observed.

Key words: cow's milk quality indexes- somatic cell counts correlations, subclinical masitis

Introduction

Mastitis are one of the most common and damaging dairy cow diseases all over the world, despite of all genetical, feeding systems, housing and milking conditions advances. (Batavani *et al.,* 2005; Carlén *et al.,* 2004). Economic losses are significant and, in most of the cases, related to the diminished milk yield, treatment costs, increased labor, discarded or withheld milk due to mastitis treatments or milk quality changes / increased premature culling (reviewed by Sharif and Muhammad, 2008). Among different types of mammary gland inflamations, the subclinical mastites require a particular attention as their frequency is up to 15 – 40 times higher than the clinical forms; they have extended expression periods and precede clinical forms, and are difficult to detect without a specific test in the dairy farm; they are a permanent microorganisms reservoir on the farm (Bruckmaier *et al.*, 2004; Tuteja and Dixit, 2003).

The negative effects on several reproductive indicators, such as the first estrus delay, increased artificial insemination indicators, conception block and embryo atrophy, were also reported (Cullor, 1990).

The severity of the mammary tissue inflamation are highly correlated with the milk quality parameters. Some studies demonstrated that the fat and protein content are decreasing in the mastitis milk (reviewed by Sharif and Muhammad, 2008). Şonea *et al.,* 2009, found higher values of the total protein content, due to the whey protein increase rather than the casein content increase. The poor feeding or sugar blood low levels have singifiant negative effects on lactose quantity (Lehninger *et al.*, 2000).

The present study aimed to add to the knowledge about the incidence of naturally occurring subclinical mastitis in the primiparous Holstein Friesian Romanian cattle

population and to establish some correlations with the somatic cell count and physico – chemical milk quality indices.

Material and methods

Investigations were conducted on 117 primiparous Holstein Friesian Romanian dairy cows. An subclinical mastitis farm test (Draminski™), which correlates the milk electrical resistance and health of the udder was used.

15 ml milk from each mammary quarter were collected for the subclinical mastitis diagnosis. The results were displayed on the instrument's screen where the tested subjects fell into three major groups, according to the manufacter's specifications:

(−) no subclinical mastitis or the risk for subclinical mastitis is very low;

(±) a progressively increasing of subclinical mastitis incidence;

(+) a subclinical mastitis which progress to clinical form.

Monthly, samples of 60 mL milk samples were collected from each cow in dry clean polyethylene containers and the Bronopol™ (2 – bromo – 2 – nitropropan – 1,3 diol; 0,040g/100 ml) was added as a milk preservative. The milk samples were stored in a freezer at -18 C^0 until they were analysed using the Milko Scan FT 6000™ module, with a FTIR interferometer, a flow system and a PC (Combi Foss 6000, Foss Electric Denmark). The milk somatic cell counts and the milk concentrations of fat, protein, casein and lactose were evaluated. Differences of milk traits depending on subclinical mastitis status were tested using chi-square test (χ^2). Thus, the observed frequencies were compared with the expected ones, the test statistics being reported to the appropriate degrees of freedom. The zero value of the chi - square indicator showed no trend of dependency; the higher the value of chi - square was, the higher the trend of dependency resulted (Badea and Georgescu, 2003; Jaba, 2006).

$$\cdot\chi^2 = \sum_{i=1}^{l} \sum_{j=1}^{c} \frac{\left(f_{ij}^0 - f_{ij}^a\right)^2}{f_{ij}^a}$$

f_{ij}^0 = observed frequencies for line i and column j;

f_{ij}^a = expected frequencies for line i and column j;

l = number of rows from the table (or the number of classes of the effect factor);

c = number of columns from the table (or the number of classes of the active factor).

Results and discussions

The variation of the observed and expected frequencies of cows with different results of the subclinical mastitis test function of different classes of somatic cell count, fat, protein, casein and lactose milk contents are shown in Tabs. 1 – 5. The lack of addictive tendency in summer for all types of correlations was given by the null chi – square calculated test values.

The calculated values of chi – square test for spring, autumn and winter seasons are greater than the theoretical values for the corresponding degrees of freedom and $\alpha = 0.001$, which indicates a statistical significant dependency between different classes of subclinical mastitis test results and the somatic cell count variations. Our investigation showed a positive correlation between the progressively increase of the subclinical mastitis incidence and the somatic cell count values ranging from 400 – 600 (x 1000 cells/ml). The subclinical mastitis that develops to the clinical form and the highest class of somatic cell count values (> 600,000 cells/ml) were found to be in a high dependency in autumn and winter seasons. In

spring and summer, the number of positive results for the subclinical mastitis cases that developed into the clinical form was not enough for a conclusive statistical interpretation.

Table 1. Interpretation of the match test between the subclinical mastitis incidence and different somatic cell count classes in the Holstein Friesian cattle

Season	Subclinical mastitis		Cows #	Somatic cell count (x 1000 cells/ml)			Total	Calculated χ^2
				< 400	400 - 600	> 600		
Spring	***Test diagnostic***	**(-)**	86	6.29	-3.88	-2.41	0.00	**33.00**
		(±)	30	-5.41	3.95	1.46	0.00	
		(+)	1	-0.88	-0.07	0.95	0.00	
	Total		**117**	**0.00**	**0.00**	**0.00**	**0.00**	
Summer	***Test diagnostic***	**(-)**	87	3.99	-3.09	-0.91	0.00	**0.00**
		(±)	30	-3.99	3.09	0.91	0.00	
		(+)	0	0.00	0.00	0.00	0.00	
	Total		**117**	**0.00**	**0.00**	**0.00**	**0.00**	
Autumn	***Test diagnostic***	**(-)**	79	14.96	-10.15	-4.80	0.00	**82.49**
		(±)	32	-10.70	11.08	-0.38	0.00	
		(+)	6	-4.26	-0.92	5.18	0.00	
	Total		**117**	**0.00**	**0.00**	**0.00**	**0.00**	
Winter	***Test diagnostic***	**(-)**	82	10.72	-5.01	-5.71	0.00	**62.87**
		(±)	29	-6.79	5.52	1.27	0.00	
		(+)	6	-3.92	-0.51	4.44	0.00	
	Total		**117**	**0.00**	**0.00**	**0.00**	**0.00**	

A straight association between subclinical mastitis test results and different classes of fat % *($\alpha = 0.05$)*, in relationship with the higher calculated value than the theoretical one for chi – square test in spring and autumn is shown in Tab.2.

A decrease of fat % in milk collected from cows with a progressively increase of the subclinical mastitis incidence or a subclinical mastitis developed into the clinical form as compared with those collected from the healty cows was also observed.

No statistically significant dependency between subliclinical mastitis incidence and the total protein % variantion was observed (Tab. 3).

Less than 3% of total protein in winter and an content ranging from 3 to 4% in spring and autumn in milk samples collected from cows with a progressively increasing subclinical mastitis incidence or a subclinical mastitis developed into the clinical form was identified. For the cows with a progressively increasing subclinical mastitis incidence, an association with total protein content ranging from 3 to 4% was observed.

A statistically significant dependency *($\alpha = 0.05$)* of casein % and the incidence of the subclinical mastitis cases during spring, autumn and winter is shown in Tab. 4. A positive correlation between the progressively increasing subclinical mastitis incidence and the caseins values more than 3% was described in all seasons. The subclinical mastitis that developed to the clinical form and the lowest class of caseins %(< 2.5) were found to be in a high dependency in autumn and winter seasons.

Table 2. Interpretation of the match test between the subclinical mastitis incidence and different fat percentage classes in the Holstein Friesian cattle

Season	Subclinical mastitis		Cows #	Fat %			Total	Calculated χ^2
				< 4	4.0 – 5.0	> 5		
Spring	***Test diagnostic***	**(-)**	86	-6.20	6.96	-0.76	0.00	**11.33**
		(±)	30	6.51	-7.41	0.90	0.00	
		(+)	1	-0.32	0.45	-0.14	0.00	
	Total		**117**	**0.00**	**0.00**	**0.00**	**0.00**	
Summer	***Test diagnostic***	**(-)**	87	-3.78	4.14	-0.36	0.00	**0.00**
		(±)	30	3.78	-4.14	0.36	0.00	
		(+)	0	0.00	0.00	0.00	0.00	
	Total		**117**	**0.00**	**0.00**	**0.00**	**0.00**	
Autumn	***Test diagnostic***	**(-)**	79	0.67	-1.41	0.74	0.00	**15.37**
		(±)	32	-4.67	3.87	0.79	0.00	
		(+)	6	4.00	-2.46	-1.54	0.00	
	Total		**117**	**0.00**	**0.00**	**0.00**	**0.00**	
Winter	***Test diagnostic***	**(-)**	82	-3.32	-0.93	4.25	0.00	**7.69**
		(±)	29	1.29	1.83	-3.12	0.00	
		(+)	6	2.03	-0.90	-1.13	0.00	
	Total		**117**	**0.00**	**0.00**	**0.00**	**0.00**	

Table 3. Interpretation of the match test between the subclinical mastitis incidence and different total protein percentage classes in the Holstein Friesian cattle

Season	Subclinical mastitis		Cows #	Total protein %			Total	Calculated χ^2
				< 3	3.0 – 4.0	> 4		
Spring	***Test diagnostic***	**(-)**	86	1.06	-1.91	0.85	0.00	**2.17**
		(±)	30	-1.03	1.82	-0.79	0.00	
		(+)	1	-0.03	0.09	-0.06	0.00	
	Total		**117**	**0.00**	**0.00**	**0.00**	**0.00**	
Summer	***Test diagnostic***	**(-)**	87	0.00	-0.83	0.83	0.00	**0.00**
		(±)	30	0.00	0.83	-0.83	0.00	
		(+)	0	0.00	0.00	0.00	0.00	
	Total		**117**	**0.00**	**0.00**	**0.00**	**0.00**	
Autumn	***Test diagnostic***	**(-)**	79	1.60	-3.17	1.57	0.00	**3.28**
		(±)	32	-1.19	2.20	-1.01	0.00	
		(+)	6	-0.41	0.97	-0.56	0.00	
	Total		**117**	**0.00**	**0.00**	**0.00**	**0.00**	
Winter	***Test diagnostic***	**(-)**	82	-0.50	0.32	0.19	0.00	**3.08**
		(±)	29	-0.24	-0.29	0.53	0.00	
		(+)	6	0.74	-0.03	-0.72	0.00	
	Total		**117**	**0.00**	**0.00**	**0.00**	**0.00**	

Table 4. Interpretation of the match test between the subclinical mastitis incidences and different casein percentage classes in the Holstein Friesian cattle

Season	Subclinical mastitis		Cows #	Caseins %			Total	Calculated χ^2
				< 2.5	2.5 – 3.0	> 3		
Spring	***Test diagnostic***	**(-)**	86	0.53	3.15	-3.68	0.00	**15.71**
		(±)	30	-0.51	-3.21	3.72	0.00	
		(+)	1	-0.02	0.06	-0.04	0.00	
	Total		**117**	**0.00**	**0.00**	**0.00**	**0.00**	
Summer	***Test diagnostic***	**(-)**	87	-1.71	4.34	-2.63	0.00	**0.00**
		(±)	30	1.71	-4.34	2.63	0.00	
		(+)	0	0.00	0.00	0.00	0.00	
	Total		**117**	**0.00**	**0.00**	**0.00**	**0.00**	
Autumn	***Test diagnostic***	**(-)**	79	0.17	5.68	-5.85	0.00	**22.79**
		(±)	32	-3.20	-2.79	5.98	0.00	
		(+)	6	3.03	-2.90	-0.13	0.00	
	Total		**117**	**0.00**	**0.00**	**0.00**	**0.00**	
Winter	***Test diagnostic***	**(-)**	82	-5.72	7.13	-1.41	0.00	**23.47**
		(±)	29	1.79	-3.82	2.03	0.00	
		(+)	6	3.92	-3.31	-0.62	0.00	
	Total		**117**	**0.00**	**0.00**	**0.00**	**0.00**	

A statistical depedency between the subclinical mastitis incidence and different lactose % classes in autumn season was observed (Tab.5).

Table 5. Interpretation of the match test between the subclinical mastitis incidence and different lactose percentage classes in the Holstein Friesian cattle

Season	Subclinical mastitis		Cows #	Lactose %			Total	Calculated chi-square
				< 4.5	4.5 – 4.7	> 4.7		
Spring	***Test diagnostic***	**(-)**	86	-2.82	-0.61	3.43	0.00	**6.20**
		(±)	30	2.92	-0.03	-2.90	0.00	
		(+)	1	-0.10	0.63	-0.53	0.00	
	Total		**117**	**0.00**	**0.00**	**0.00**	**0.00**	
Summer	***Test diagnostic***	**(-)**	87	-0.80	0.20	0.61	0.00	**0.00**
		(±)	30	0.80	-0.20	-0.61	0.00	
		(+)	0	0.00	0.00	0.00	0.00	
	Total		**117**	**0.00**	**0.00**	**0.00**	**0.00**	
Autumn	***Test diagnostic***	**(-)**	79	-7.21	-4.28	11.49	0.00	**22.86**
		(±)	32	6.44	2.97	-9.41	0.00	
		(+)	6	0.77	1.31	-2.08	0.00	
	Total		**117**	**0.00**	**0.00**	**0.00**	**0.00**	
Winter	***Test diagnostic***	**(-)**	82	-1.82	0.86	0.96	0.00	**1.81**
		(±)	29	1.05	0.34	-1.39	0.00	
		(+)	6	0.77	-1.21	0.44	0.00	
	Total		**117**	**0.00**	**0.00**	**0.00**	**0.00**	

No addictive tendency during other seasons among the investigated traits was observed; the calculated chi – square values were less than the theoretical value in spring and winter, and zero in summer. Positive correlations between the progressively increasing subclinical mastitis incidence and less than 4.5% lactose in spring and summer, and with less than 4.5% and between 4.5 – 4.7% lactose milk content in autumn and winter were observed.

The healthy udder maintenance is an important objective for farmers, as the subclinical mastitis lowers the quality of milk. Even if the intake of milk with higher levels of somatic cell count did not represent a direct risk on the health (Hogan, 2005), a higher SCC level associated with milk antibiotic residues was reported to determine a higher risk (Ruegg, 2005; Grădinaru, 2010). The presence of antibiotic residues in milk can delay or stop the fermentative processes in milk. Thus, the monitoring of somatic cell count could become an useful indicator of the udder's health and milk quality, with significant implications on the milk product markets.

Conclusions

1. A positive correlation between the progressively increasing subclinical mastitis incidence and the somatic cell count values ranging from 400 – 600 (x 1000 cells/ml) was observed; the subclinical mastitis that developed into the clinical form and the highest class of the somatic cell count values (> 600,000 cells/ml) was found to be in a high dependency in autumn and winter.
2. A decrease of fat % in milk samples from cows with a progressively increasing subclinical mastitis incidence or a subclinical mastitis that developed to the clinical form ascompared to those from healty cows was also observed.
3. No statistically significant dependency between the subliclinical mastitis incidence and the total protein % variantion was observed.
4. The progressively increasing subclinical mastitis incidence and the casein values over 3% was in a high correlation during the entire period of investigations. The subclinical mastitis that develops to the clinical form and the lowest class of caseins (< 2.5) were found to be in a high dependency in autumn and winter seasons.
5. Positive correlations between the progressively increasing subclinical mastitis incidence and less than 4.5% lactose in spring and summer, and with less than 4.5% and between 4.5 – 4.7% milk lactose content in autumn and winter were identified.

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References

1. Atasever S., Erdem H., 2009 – *Estimation of milk yield and financial losses related to somatic cell count in Holstein cows raised in Turkey*, J. Anim. Vet. Adv., 8 (8): 1491 – 1494,
2. Badea P., Georgescu D., 2003 - *Introduction to Biostatistics,* (in Romanian), University Medical Publishing House, Craiova, Romania.

3. Batavani R.A., Asri S., Nalbzadeh H., 2007 – *The effect of subclinical mastitis on milk composition in dairy cows*, Iran.J.Vet.Res., 8 (3): 205 – 211;
4. Bruckmaier R.M., Ontsouka C.E., Blum J.W., 2004 – *Fractionized milk composition in dairy cows with subclinical mastitis,* Vet.Med. – Czech, 49 (8): 283 – 290;
5. Carlén E., Strandberg E., Roth A.., 2004 – *Genetic parameters for clinical mastitis, somatic cell score, and production in the first three lactations of Swedish Holstein cows,* J.Dairy Sci., 87: 3062 – 3070;
6. Cullor J.S., 1990 – *Mastitis and its influence upon reproductive performance in dairy cattle, Int. Symp. Bovine Mastitis*, Indianapolis IN, 176 – 180;
7. Grădinaru A.C., 2010 – *Assessement of antibiotic and elementary xenobiotic residues in milk and milk products and their risk for consumers*, (in Romanian), PhD thesis, Faculty of Veterinary Medicine, Iaşi;
8. Hogan J., 2005 – *Human health risks associated with high SCC milk*, Proceedings of the british Mastitis Conference Stoneleigh, 21 – 24;
9. Jaba E., 2006 - *Fundamentals of Statistics*, "Al.I. Cuza", University Iasi, Romania;
10. Ruegg P.L., 2005 – *Relationship between bulk tank milk somatic cell count and antibiotic residues*, NMC Annual Meeting Proceedings, 28 – 35;
11. Sharif A., Muhammad G., 2008 – *Somatic cell count as an indicator of udder health status under modern dairy production: a review*, Pakistan Vet.J., 28 (4): 194 – 200;
12. Şonea C., Colceri D., Băcilă V., 2009 – *Research on subclinical mastitis effect on milk quality,* Scientific Papers Anim. Husbandry and Biotech. Timişoara 42 (2): 337 – 340;
13. Tuteja F.C., Dixit S.K., 2003 – *Control of mastitis in dairy animals*, Veterinary Practitioner, 4(2): 130 – 133.

CORRELATIONS BETWEEN KAPPA – CASEIN AND BETA – LACTOGLOBULIN GENOTYPES AND MASTITIS INCIDENCE IN HOLSTEIN FRIESIAN AND MONTBÉLIARDE ROMANIAN CATTLE POPULATIONS

A.C. GRĂDINARU[1], O. POPESCU[2], Şt. CREANGĂ[1]
[1] University of Agricultural Sciences and Veterinary Medicine of Iasi, Romania
[2]Purdue University, Veterinary School, USA
andre_gradinaru@yahoo.com

Abstract
The present work aimed to establish some correlations between different genotypes for κ – CN and β – LG and the incidence of subclinical mastitis in the primiparous Holstein Friesian and Montbéliarde Romanian cattle populations. Investigations were conducted on 109 primiparous Holstein Friesian and 62 primiparous Montbéliarde dairy cows. Phenotyping of the skimmed milk samples was carried out according to the Isoelectrical Focusing electrophoresis(IEF) method on a 0.3 mm thin polyacrylamide gel. The Draminski™ farm test was used for the subclinical mastitis diagnostic. Differences of subclinical mastitis case groups function of genotypes for κ – CN and β – LG were calculated using the chi-square test (χ^2). A high correlation between the subclinical mastitis incidence and BB and AB genotypes for κ – CN in Holstein Friesian population was found during the summer and winter seasons, respectively. The association among the AE, BE and EE genotype carring cows and the higher prevalence of subclinical mastitis was registered in the spring and autumn. The correlation of the κ –CN BB genotype and the number of positive subclinical mastitis cases that developed into clinical forms in Montbéliarde cows during the spring, summer and winter was observed. In the autumn, the AB genotype of κ –CN and the increased number of sublinical mastitis test results were highly positively correlated. During the spring, autumn and winter, a depedency between AB genotype of β – LG and the subclinical mastitis cases that developed into clinical forms seemed to occur in the Holstein Friesian population. No correlation tendency between the positive mastitis results and the β – LG genotypes in the Holstein Friesian cows was observed in summer. In the Montbéliarde cattle, the positive sublinical mastitis test reactions during the spring and summer, and the autumn and winter seasons seemed to be associated with the BB and AB genotypes, respectively.

Key words: IEF, cattle milk, mastitis-genotype correlation

Introduction

The milk proteins have a genetic polymorphism, e.g., one or two aminoacid substitutions and, occasionally, a segment deletion within the polypeptid chain being involved (Caroli *et al.*, 2010; Grosclaude, 1976; Ng – Kwai – Hang *et al.*, 1990). The four caseins (– CN) are encoded by four clustered autosomal genes, which are located on chromosome 6 at position 6q31 – 33, on a range of 185 – 250 kb (Formaggioni *et al.*, 1999; Caroli *et al.*, 2009) in the following order: α_{S1} –, β –, α_{S2} – , κ – CN. The first three allelic variants are of 17.5 kb, 8.5 kb and 18.5 kb total lengths, respectevely (Caroli *et al.*, 2009) being more closely linked than the κ – CN gene, which is of 13 kb total length and it is at least 70 kb away from them (Ferreti *et al.*, 1990). The α – lactalbumin (- LA) gene is of 2 kb total length and it is located on chromosome 5; the β – lactoglobulin (- LG) gene is of 4 kb total length and it is located on chromosome 11 (Caroli *et al.*, 2009). All of these genes have different alleles coding for several genetic variants of the corresponding protein.

During the last 20 years, the bovine milk protein polymorphism has become an important factor for the dairy industry in comercial terms, as many investigations on the certain milk protein genetic variants showed that the genetic heritage determined the

quantities and/or qualities of yield traits, composition and the technological milk properties (Aleandri *et al.*, 1990; Çardak, 2005; Caroli *et al.*, 2004;. Ng – Kwai – Hang *et al.*, 1990). A correlation between the mastitis incidence and the high level of milk production has been also previously reported (Oltenacu and Ekesbo, 1994).

Among proteins, κ – casein (– CN) and β – lactoglobulin (– LG) possess a great influence on milk production and milk constituents (Erhardt, 1993). Many investigations demonstrated that the BB genotype of κ – CN was associated with higher casein concentrations (Ikonen *et al.*, 1997; Graml and Pirchner, 2003); protein, fat and milk yield (Aleandri *et al.*, 1990; Caroli *et al.*, 2004); higher protein percentage (Aleandri *et al.*, 1990). The BB genotype of β – LG was associated with higher fat percentage, whereas the AA genotype was associated with the protein yield (Aleandri *et al.*, 1990; Caroli *et al.*, 2009).

The present study aimed to establish some correlations between different genotypes for κ – CN and β – LG and the incidence of subclinical mastitis in primiparous Holstein Friesian and Montbéliarde Romanian cattle populations.

Materials and methods

Investigations were conducted on 109 primiparous Holstein Friesian and 62 Montbéliarde dairy cows. Phenotyping of the 171 total skimmed milk samples was carried out by IEF in 0.3 mm thin polyacrylamide gel (acrylamide / NN' methylene – bisacrylamide / urea), using carrier ampholytes (pH 3.0 – 7.0) and catalysts (TEMED™ and ammonium persulphate), according to the method reported by Erhardt, 1993.

The Draminski™ farm test, which correlates the milk electrical resistance and health of the udder was used for the subclinical mastitis diagnostic. Three major groups, according to the manufacter's specifications, were established:

(−) no subclinical mastitis or the risk for subclinical mastitis is very low;

(±) a progressively increasing of subclinical mastitis incidence;

(+) a subclinical mastitis which develops into the clinical form.

Differences of the diagnosed subclinical mastitis groups depending on genotypes for κ –CN and β – LG were tested using the chi-square test, χ^2 (Badea and Georgescu, 2003; Jaba, 2006):

$$\chi^2 = \sum_{i=1}^{l} \sum_{j=1}^{c} \frac{\left(f_{ij}^0 - f_{ij}^a\right)^2}{f_{ij}^a}$$

f_{ij}^0 = observed frequencies for line i and column j;

f_{ij}^a = expected frequencies for line i and column j;

l = number of rows from the table (or the number of classes of the effect factor);

c = number of columns from the table (or the number of classes of the active factor).

Results and discussions

Separations of κ – CN and β – LG phenotypes in skimmed Holstein Friesian milk samples by IEF is shown in Fig.1.

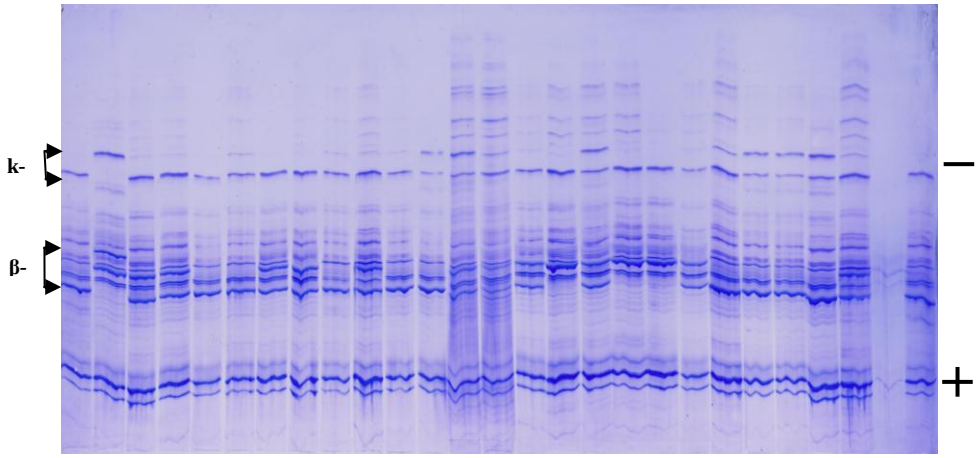

Fig. 1: Patterns of Holstein Friesian milk samples by IEF technique

In order to establish a correlation between the results of subclinical mastitis test and some of the κ – CN and β – LG genotypes, the chi - square test was applied. The differences between the observed and expected frequencies of cows belonging to the three subclinical mastitis diagnostic groups, function of the κ – CN and β – LG genotypes, are shown in Tab. 1 – 4. The most of the calculated values of chi – square test (excepting the winter correlations for κ – CN genotypes in Holstein Friesian cows) are smaller than the theoretical value for the corresponding degrees of freedom and $\alpha = 0.05$; this indicates a statistical non-significant dependency between each of the subclinical mastitis diagnostic groups and the κ – CN and β – LG genotypes. However, the lack of addictive tendency is given by the null chi – square value test; the greater the chi – square value is, the higher the addictive tendency is.

The different types of additive tendency between the sublinical mastitis test results and the genotypes for κ – CN in Holstein Friesian and Montbéliarde populations are shown in Tab.1 and Tab.2.

A high correlation between the subclinical mastitis incidence and each of the BB and AB genotypes for κ – CN in Holstein Friesian population was observed during summer and winter, respectively. The association between each of the AE, BE and EE genotype carring cows and the higher prevalence of the subclinical mastitis was observed in spring and autumn.

The correlation of the BB genotype and the number of positive subclinical mastitis cases that progressed to the clinical form in the Montbéliarde cows during spring, summer and winter was observed. In autumn, the correlation of the AB genotype and the increased number of sublinical mastitis cases was highly positive in the Montbéliarde cattle. Çardak, 2005, showed that milk yield and protein content were significantly influenced by κ – casein (AB > AA > BB and AA > AB = BB, respectively) in the Simmentaler cows (the Montbéliarde breed belongs to the Simmental family); there was no reported influence on the

protein content at the CSN3 locus, but the milk yield was significantly influenced by the AA genotype (AA > AB) in the Holstein Friesian cattle.

Table 1. Seasonal variation of the subclinical mastitis incidence and its match with each of the κ – CN genotypes in the Holstein Friesian cattle

Season	Subclinical mastitis		Cows #	κ – CN *genotypes*				Total	Calculated χ^2
				AA	AB	AE, BE, EE	BB		
Spring	*Test diagnostic*	(-)	**92**	1.67	-0.88	-1.20	0.40	0.00	**8.33**
		(±)	**12**	0.30	-0.85	-0.63	1.18	0.00	
		(+)	**5**	-1.98	1.73	1.83	-1.59	0.00	
	Total		**109**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	
Summer	*Test diagnostic*	(-)	**96**	-0.30	0.85	0.63	-1.18	0.00	**0.00**
		(±)	**13**	0.30	-0.85	-0.63	1.18	0.00	
		(+)	**0**	0.00	0.00	0.00	0.00	0.00	
	Total		**109**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	
Autumn	*Test diagnostic*	(-)	**93**	0.93	-0.20	-0.49	-0.24	0.00	**4.42**
		(±)	**12**	0.30	-0.85	-0.63	1.18	0.00	
		(+)	**4**	-1.23	1.05	1.12	-0.94	0.00	
	Total		**109**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	
Winter	*Test diagnostic*	(-)	**91**	-3.54	-2.10	1.76	3.88	0.00	**20.97**
		(±)	**12**	4.02	-0.27	-1.17	-2.59	0.00	
		(+)	**6**	-0.49	2.37	-0.59	-1.29	0.00	
	Total		**109**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	

Table 2. Seasonal variation of the subclinical mastitis incidence and its match with each of the κ – CN genotypes in the Montbéliarde cattle

Season	Subclinical mastitis		Cows #	κ – CN *genotypes*			Total	Calculated χ^2
				AA	AB	BB		
Spring	*Test diagnostic*	(-)	**53**	-0.84	2.52	-1.68	0.00	**8.91**
		(±)	**5**	1.35	-1.06	-0.29	0.00	
		(+)	**4**	-0.52	-1.45	1.97	0.00	
	Total		**62**	**0.00**	**0.00**	**0.00**	**0.00**	
Summer	*Test diagnostic*	(-)	**53**	-1.84	1.52	0.32	0.00	**6.93**
		(±)	**5**	1.35	-0.06	-1.29	0.00	
		(+)	**4**	0.48	-1.45	0.97	0.00	
	Total		**62**	**0.00**	**0.00**	**0.00**	**0.00**	
Autumn	*Test diagnostic*	(-)	**46**	1.06	-0.19	-0.87	0.00	**2.62**
		(±)	**10**	-1.29	-0.13	1.42	0.00	
		(+)	**6**	0.23	0.32	-0.55	0.00	
	Total		**62**	**0.00**	**0.00**	**0.00**	**0.00**	
Winter	*Test diagnostic*	(-)	**48**	-0.19	1.58	-1.39	0.00	**1.88**
		(±)	**3**	-0.39	0.16	0.23	0.00	
		(+)	**11**	0.58	-1.74	1.16	0.00	
	Total		**62**	**0.00**	**0.00**	**0.00**	**0.00**	

Our investigation showed a positive correlation between the AA genotype and the number of subclinical mastitis test results but it was not very high. Most of the reports agreed that κ – casein BB genotype was associated with a higher protein rate (or only a higher casein percentage) and a higher protein yield (Aleandri *et al.*, 1990; Caroli *et al.*, 2009; 2010, Grosclaude *et al*., 1976). A positive correlation between the milk yield and the mastitis incidence in cows has been long known (Oltenacu and Ekesbo, 1994). The subsequent question this work tried to find an answer for was whether or not the higher cow's milk protein content itself was associated with a higher incidence of various types of mastitis.The E allele of κ – CN was observed only in the Holstein Friesian population. Most of reports showed that the E allele and its associated genotypes were correlated with negative effects on the milk production parameters and protein quality, and weaker milk coagulating properties (reviewed by Caroli *et al.*, 2009).

During the spring, autumn and winter, a depedency between the AB genotype of β – LG and the subclinical mastitis that developed into the clinical form seemed to occur in the Holstein Friesian population (Tab. 3).

No addictive tendency between positive mastitis results and β – LG genotypes in the Holstein Friesian cows was observed in summer. In the Montbéliarde cattle, the sublinical mastitis positive test results seemed to be associated with the BB genotype during spring and summer and those associated with the AB genotype during autumn and winter, respectively (Tab. 4). This observation seemed to be consistent with the findings of Çardak (2005) regarding the milk production yield. This trait was found to be significantly influenced by the β – lactoglobulin genetic variants (AB > BB > AA) in the Holstein Friesian cows, whereas in the Simmentaler cows the different genotypes of β – lactoglobulin (BB > AA > AB) had influence on the fat and protein content.

Table 3. Seasonal variation of the subclinical mastitis incidence and its match with each of the β – LG genotypes in the Holstein Friesian cattle

Season	Subclinical mastitis		Cows #	β – LG *genotypes*			Total	Calculated χ^2
				AA	AB	BB		
Spring	***Test diagnostic***	**(-)**	**92**	-0.10	-2.42	2.52	0.00	**2.73**
		(±)	**12**	0.25	1.94	-2.19	0.00	
		(+)	**5**	-0.15	0.48	-0.33	0.00	
	Total		**109**	**0.00**	**0.00**	**0.00**	**0.00**	
Summer	***Test diagnostic***	**(-)**	**96**	0.98	-1.44	0.46	0.00	**0.00**
		(±)	**13**	-0.98	1.44	-0.46	0.00	
		(+)	**0**	0.00	0.00	0.00	0.00	
	Total		**109**	**0.00**	**0.00**	**0.00**	**0.00**	
Autumn	***Test diagnostic***	**(-)**	**93**	-0.33	-1.93	2.26	0.00	**3.91**
		(±)	**12**	1.25	0.94	-2.19	0.00	
		(+)	**4**	-0.92	0.98	-0.06	0.00	
	Total		**109**	0.00	0.00	0.00	0.00	
Winter	***Test diagnostic***	**(-)**	**91**	1.13	-0.92	-0.21	0.00	**1.13**
		(±)	**12**	-0.75	-0.06	0.81	0.00	
		(+)	**6**	-0.38	0.97	-0.60	0.00	
	Total		**109**	**0.00**	**0.00**	**0.00**	**0.00**	

Table 4. Seasonal variation of the subclinical mastitis incidence and its match with each of the β – LG genotypes in the Montbéliarde cattle

Season	Subclinical mastitis		Cows #	β – LG *genotypes*			Total	Calculated χ^2
				AA	AB	BB		
Spring	***Test diagnostic***	**(-)**	53	0.58	0.35	-0.94	0.00	**1.06**
		(±)	5	-0.32	-0.42	0.74	0.00	
		(+)	4	-0.26	0.06	0.19	0.00	
	Total		**62**	**0.00**	**0.00**	**0.00**	**0.00**	
Summer	***Test diagnostic***	**(-)**	53	0.58	-0.65	0.06	0.00	**0.89**
		(±)	5	-0.32	0.58	-0.26	0.00	
		(+)	4	-0.26	0.06	0.19	0.00	
	Total		**62**	**0.00**	**0.00**	**0.00**	**0.00**	
Autumn	***Test diagnostic***	**(-)**	46	0.03	-2.26	2.23	0.00	**3.71**
		(±)	10	0.35	0.16	-0.52	0.00	
		(+)	6	-0.39	2.10	-1.71	0.00	
	Total		**62**	**0.00**	**0.00**	**0.00**	**0.00**	
Winter	***Test diagnostic***	**(-)**	48	0.90	-1.23	0.32	0.00	**2.40**
		(±)	3	-0.19	-0.45	0.65	0.00	
		(+)	11	-0.71	1.68	-0.97	0.00	
	Total		**62**	**0.00**	**0.00**	**0.00**	**0.00**	

The use of IEF technique, as an efficient tool for screening of big populations at the milk protein phenotypic level, is recommended for both the low costs involved and the possibility to have six gene polymorphism in one picture for 27 or 54 individuals. Using the milk protein genes as a marker in the process of animal selection for milk yield and quality, and the integration of certain alleles in a National Selection Program for Bulls, would have an economically positive impact on the dairy product market.

More studies on the correlations between κ – CN and β – LG genotypes and the subclinical mastitis incidence need to be made in order to establish different connections between some genotypes and economical advantages due to higher milk yields, on the one hand, and financial losses, due to the higher subclinical mastitis incidence, on the other.

Conclusions

1. Phenotyping of 179 total skimmed milk samples from Holstein Friesian and Montbéliard Romanian cattle populations by the IEF technique on a 0.3 mm thin polyacrylamide gel was performed; the Draminski™ farm test for subclinical mastitis diagnostic was used.
2. A high correlation between the subclinical mastitis incidence and both BB and AB genotypes for κ – CN in Holstein Friesian population was obtained during the summer and winter seasons; the association among the AE, BE and EE genotypes carring cows and the high incidence of the subclinical mastitis was observed in spring and autumn.
3. A positive correlation between the BB genotype of κ – CN and the number of subclinical mastitis results identified in the Montbéliarde cows during the spring, summer and winter was observed; in the autumn, the AB genotype and the increased

number of sublinical mastitis test results were highly positive correlated in the Montbéliarde cattle.

4. During the spring, autumn and winter, a correlation between the AB genotype of β – LG and the subclinical mastitis form occurs in the Holstein Friesian population; no addictive tendency between positive mastitis results and β – LG genotypes in the Holstein Friesian cows was observed in summer.
5. In the Montbéliarde cattle, the BB and AB genotypes of β – LG were found to be associated with a high incidence of the sublinical mastitis during the spring and summer, and the autumn and winter, respectively.

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References

1. Aleandri R., Buttazzoni L.G., Schneider J.C., Caroli A., Davoli R., 1990 – The effects of milk protein polymorphisms on milk components and cheese – producing ability, J.Dairy Sci. 73: 241 – 255;
2. Badea P., Georgescu D., 2003 - *Introduction to Biostatistics,* (in Romanian), University Medical Publishing House, Craiova, Romania;
3. Çardak A.D., 2005 – Effects of genetic variants in milk protein on yield and composition of milk from Holstein Friesian and Simmentaler cows, S. Afr. J. Anim. Sci. 35 (1): 41 – 47;
4. Caroli A.M., Chessa S., Bolla P., Budelli E., Gandini G.C., 2004 – Genetic structure of milk protein polymorphism and effects on milk production traits in a local dairy cattle, J. Anim. Breed. Genet., 121: 119 – 127;
5. Caroli A.M., Chessa S., Erhardt G.J., 2009 – Invited review: Milk protein polymorphisms in cattle: Effect on animal breeding and human nutrition, J.Dairy Sci. 92: 5335 – 5352;
6. Caroli A.M., Chessa S., Erhardt G.J., 2010 – *Milk protein polymorphism in cattle: effect on animal breeding an human nutrition*, 9th World Congress on Genetics Applied to Livestock Production, 01.06 – 06.08.2010, Leipzig;
7. Erhardt G., 1993 – Allele frequencies of milk proteins in German cattle breeds and demonstration of α_{S2} casein variant by isoelectric focusing, Arch.Tierz. 36 (2): 145 – 152;
8. Formaggioni P., Summer A., Malacarne M., Mariani P., 1999 – Milk protein polymorphism: detection and diffusion of the genetic variants in *Bos* genus, Annali Facoltà Medicina Veterinaria, Università Parma, 19: 127 – 165;
9. Ferreti L., Leone P., Sgaramella V., 1990 – Long range restriction analysis of the bovine casein genes, Nucleic Acids Res., 18: 6829 – 6833;

10. Graml R., Pirchner F., 2003 – Effect of milk protein loci on content of their proteins, Arch. Tierz., Dummerstorf 46 (4): 331 – 340;
11. Grosclaude F., Mahé M.F., Mercier J.C., Bonnemaire J., Teissier J.H., 1976 – Polymorphisme des lactoprotéines de bovinés népalais, Ann.Génét.Sél.Anim. 8(4): 461 – 479;
12. Ikonen T., Ojala M., Syväroja E.L., 1997 – Effects of composite casein and β – lactoglobulin genotypes on renneting properties and composition of bovine milk by assuming an animal model, Agr. Food Sci. Finland 6: 283 – 294;
13. Jaba E., 2006 - *Fundamentals of Statistics*, "Al.I. Cuza", University Iasi, Romania;
14. Ng – Kwai – Hang K.F., Monardes H.G., Hayes J.F., 1990 – Association between genetic polymorphism of milk proteins and production traits during three lactations, J. Dairy Sci. 73: 3414 – 3420;
15. Oltenacu P.A., Ekesbo I., 1994 – Epidemiological study of subclinical mastitis in dairy cattle, Vet. Res. 25: 208 – 212.

ADAPTATION OF FISHING COMMUNITIES IN COASTAL RECLAMATION AREAS IN MANADO CITY

Jardie Androkles ANDAKI, Gybert E. MAMUAYA[1]
Hendronoto Arnoldus W. LENGKEY[2]
[1)] Faculty of Fishery and Marine Science, Sam Ratulangi University, Manado Indonesia
[2)] Faculty of Animal Husbandry, Padjadjaran University, Bandung, Indonesia
j_a_andaki@yahoo.com

Abstract
The phenomenon of coastal development through reclamation needs to be studiedrelated to the existence of coastal communities, particularly fishing communities. Fishing communities have a high dependence on coastal ecological resources for social and economic activities. Study of post-reclamation fishermen adaptation is the knowledge needed to determine the positive and negative impacts on coastal communities in relation to coastal development.This study uses a case study approach, which is studying the adaptation aspectspost-reclamation fishing communities in the city of Manado.Adaptation of fishing communities after reclamation is done through the struggle to get right a boat moorings. Jetty into the critical issues related to the sustainability of fishingopportunities. Similarly, fishermen who moor boats licensed by the developer, get thedouble benefit of the fishing and easy access to jobs outside the fisheries.
Keywords: adaptation, fishermen, reclamation, boat moorings

Introduction

Adaptability *(adaptive capacity)* is the ability to react to challenges through learning, risk management and its impact, in the knowledge that the new development with a more effective approach (Marshall *et al.,* 2009).

On social systems, adaptability is a characteristic of conscious, able to develop institutions, cooperation in harnessing knowledge and experience, and create flexibility in problem solving, have the ability to cope and adapt to change (Armitage 2005; Holling and Meffe 1996; Nelson *et al.,* 2007; Scheffer *et al.,* 2001). Adaptability is largely influenced by the sensitivity of the community will change and environmental damage and climate (Adger, 2006; Adger *et al.,* 2005; Rapport *et al.,* 1998).

Changes in coastal environments due to increased human activity in coastal areas need different adaptation strategies of coastal communities, including fishermen community. Geographically fishing communities are communities that live, grow, and thrive in the coastal area, which is a transition area between land and sea areas (Kusnadi, 2009). As a system, a fishing community consists of social categories that make up social unity.

Concentration of human activities in coastal areas led to the phenomenon of coastal settlement. Arrangement referred to, namely to maximize the function of the beach to the pressure of population and activities. One form of the arrangement of the beach is through reclamation. Reclamation conducted by stockpiled coastal and marine areas to serve new land, both for housing and infrastructure supporting the socio-economic activities.

Technically able to change the configuration of the beach reclamation and closing some areas to the sea. Although as a solution to the fulfillment of the coastal space, environmental and ecological problems often occur in the process of reclamation, in the form of beach erosion and sedimentation, changes in coastal wetlands, coastal habitat loss, and

changes in the marine environment (Barnes, 1991; Noske, 1995; Ni *et al*. 2002; Terawaki *et al*. 2003).

The phenomenon of coastal development through coastal reclamation needs to be studied related to the existence of coastal communities, particularly fishing communities. That which needs to be underlined that fishing communities have a high dependence on coastal ecological resources for social and economic activities. This dependence is a consequence of vulnerability in the event of significant changes in the region. If the nature of the vulnerability of the region are not addressed, it will display the conflict between the interests of coastal resource use for subsistence and economic development in the short term generation needs to come to the coastal resources.

Study the adaptation of post reclamation fishing communities in the Manado of North Sulawesi Province, Indonesia, is the knowledge needed to determine the positive and negative impacts on coastal communities in relation to coastal development.

Materials and methods

This study uses a case study approach, which is studying the adaptation aspects post-reclamation fishing communities in Manado. The case study is a research strategy that is used in various situations that contribute to the understanding of phenomena associated with individuals, groups, organizations, social and political (Yin, 2003).

The objective of this study, namely the family and fishermen family members who are directly affected of coastal reclamation activities in Manado. Object of study is taken from the family and family members living at the site reclamation, which is in the Titiwungen, South Wenang and Sindulang Village.

Data analysis was performed with qualitative techniques, ie since the beginning of the researchers took the data, for data retrieval, to the end of the accumulation of all data according to issues and do more intensive research after returning from the field. All data are available are reviewed and reduced and abstracted to form an information. Unit information is interpreted and processed in the form of research results to a conclusion at this stage.

Results and discussion

Coastal Communities and Coastal Reclamation in Manado

Manado is the capital of the North Sulawesi province located between: latitude $1^{0}25'88"-1^{0}39'5"$ S dan longitude $124^{0}47'00"-124^{0}56'00"$ E. Manado as a city located in the coastal region has five districts coast.

The main tribes located in Manado, ie Minahasa, Sangir, Bajo, Bolaang Mongondouw, Gorontalo, Bantik, and ethnic mix (Padang, Arabs, Bugis, and Javanese). More mixed tribes inhabiting the center of Manado, general merchant by profession. Tribal mix also includes six major tribes have been mixed in the bond of marriage.

Although Manado said to be a beach town, but the development until the year 2008, Manado city tend to be classified as trade and services. This is because the contribution of industrial sector and the services of employment and to the formation of the Gross Regional Domestic Product (GDP) is still relatively dominant compared to that in which the agricultural sector including fisheries sub-sector.

Table 1. The number of coastal districts in Manado

No	Sub-district	Number of Village	Total Population (people)	Length of coastline (m)
1.	Bunaken (Siladen, Bunaken and Manado Tua	8	36,555	40,050
2.	Tuminting	6	21,171	5,225
3.	Wenang	2	4,519	2,325
4.	Sario	4	15,588	2,610
5.	Malalayang	5	27,868	8,502
	Total	25	105,701	58,712

Source: CRMP (2002)

Table 2. Composition of the Working Population by Group Business Sector, 1998, 2003 and 2008

Field Work	1998	2003	2008
	---------- % ----------		
Agriculture	5.96	5.10	4.98
Industry	15.24	18.23	16.83
Services	78.79	76.67	78.19

Sources: National Social Economic Survey 1998, 2003, Sakernas 2008

The services sector is still dominated the past 10 years, while the proportion of people working in the agricultural sector continued to decline from 5.96 percent to 4.98 percent during the period 1998 to 2008. Spatial structure in Manado in 2000, showing most of Manado included in the services and trade, office and marine tourism.

Increasing the proportion of people who work in Manado related services with the launching of a government policy of Manado in 1990, the reclamation. The project was rejected by many local residents who received support from a number of environmental activists and non-governmental organizations, given the social impact would threaten the lives and future of fishing in the area or location of the reclamation project.

Along with the loss of some parts of the coastal city of Manado coastal communities also experience a variety of livelihood-related loss of coastal fisheries and the relocation of people going into the new settlements.

Livelihood Adaptation to First: Fishermen to the City

Although Manado has developed rapidly into a city of trade and services, but the family fishing can still be found, either as fishermen, fishing workers, and multiple livelihood of fishermen. Reclamation is very pronounced influence on the family group *in-shore fishermen*, ie fishermen who do fishing effort around the beach and the sea city of Manado. This group of fishermen who normally anchor the boat as a means of catching fish in the seashore city of Manado. Fishing operations carried out at any time depending on the weather, so the need for a safe boat mooring space when the weather is absolutely necessary unfriendly.

The presence of *in-shore* fishing communities post-reclamation can still be found, but have been scattered. This condition occurs as a result of reclamation carried out by developers and for developers. The struggle to get the boat mooring location, must be done with great effort.

The group is promoting the rights of fishing jetty from the developer. This struggle to fruition in the form of granting approval jetty location. Locations in the reclamation area is often used to sell the catch of fishermen, which is on the edge of the road mall locations.

Consumers want it because it catches fish freshness is guaranteed. The observations of researchers in one of the fishermen in the boat mooring location Titiwungen Village, fishermen sell the fish is still alive. Consumers usually come from people who exercise in the morning around the site reclamation and also mothers who live around the Titiwungen Village.

Source: photographs of the study, 2011.

Fig. 1. Location of boat moorings facility (reclamation areas) in the Titiwungen Village, Manado, and transaction fresh fish catch fisherman

A big advantage for fishermen, because the conditions are still fresh fish to sell high-value, in addition to marketing is not difficult because consumers are familiar with the locations of traditional fishing boat moorings.

The catch of fishermen in the city not only enjoyed by urban households. Employers restaurant, which provides a culinary specialty of grilled fish in desperate need of high quality

fish. Grilled fish restaurants in the area of reclamation has always accepted the catch of local fishermen around the coastal city of Manado for guaranteed freshness.

Livelihood Adaptation to Double: Cities for Fishermen

Season causes the dependence of fishermen on fishing operations can not be done throughout the year. There was a time "peceklik" where fishermen can not go to sea because the sea wave conditions. In such conditions the fishermen hope to earn money for family needs can be disrupted, so that fishing effort needs to be done outside the family fishing business.

Manado with a variety of social and economic activities provide employment opportunities to all walks of life including fishing. A bad season will be the day the guerrillas said one informant, in order to find a job through acquaintances who have contacts an acquaintance. Sometimes the work is not only located in Manado, but acquaintances who have contacts outside Manado is often also offered a job.

Fishermen who have the skills to choose to drive a motor vehicle towing a motorcycle taxi or choose a job working in shops, factories, restaurants, or the choice of a construction worker. Sell energy services is the only option that can be done when a bad season fishermen.

The life cycle of fishing there is always a bad season and the ease of getting a replacement job is a strong motivation to stay afloat fishermen on the coast of Manado. Various efforts, including forming groups of fishermen and in cooperation with nongovernmental organizations in Manado is a form of self defense to defend the right to marine resources, in addition to the awareness of fishermen weak position if the work is far from a substitute source of employment other than fishing.

Adaptation of Production Facilities: Boat Modifications

Modifications made boat fishing communities as a form of livelihood adaptation of fishermen into the water transportation service providers with the boat. Most types of boats available in the Village Sindulang I site of reclamation for the construction of the coast road is a type of boat "Londe". Boat "Londe" is a kind of small boat fishermen who often used the traditional "hand line" for fishing operations around the coast of Manado.

This type of boat can accommodate two people, paddle fixtures or machinery furnished "katinting" as a driver of the boat. Difficulties that arise as a result of environmental changes that have implications for the reluctance of coastal fishermen go to sea pushed the boat fishermen utilizing the services of craftsmen to modify the boat "Londe" a boat that can be used as a means of water transportation. Modifications carried out with the aim of improving the function of the boat "Londe" from one function, that means fishing a dual function, which can be used as fishing boats and means of transport.

Than as a means of water transportation, boat modification is often used as a boat for fishing through rent to the owner of the boat. Modification is a bit much to give double benefit to the families of fishermen, even though the capacity of the estuary of the river becomes a limiting factor on the number of modifications to be able to operate a boat.

Source: photographs of the study, 2011.

Fig. 2. Boat modification and transportation activities which cross the Tondano river, Manado

Conclusion

1. Social groups were formed to increase the "*bargaining position*" of traditional fishermen in order to gain concessions of natural resources for the continuation of his life. Indication is shown by the success of a boat mooring space in site coastal reclamation.
2. Substantiality of traditional fishermen occupy the coastal city of Manado is attributed demanding equal rights to resources including coastal areas of fisheries resources, substantiality is also based on the awareness of fishermen weak position if the work is far from a substitute source of employment other than fishing. In other words, fishermen protest against reclamation is not absolute because of disruption of fishing activity but rather due to the fear loss of access to employment outside the fishing activity.
3. Employment opportunities offered by social economic activities in Manado is a guaranteed source of income and livelihood sustainability, while the fishing job is a job that will be retained since it is the business for generations. Kinship as draftsman (fishermen) or the local lived close to the access to sources of income outside of fishing effort, foster social interaction in solving the problem of scarcity of fish catches in order to find other solutions to the social networks for a variety of possible bid livelihood.

References

1. Adger, W.N., 2006. Vulnerability. Global Environmental Change 16, 268-281.
2. Adger, W.N., N.W. Arnell, E.L. Tompkins, 2005. Successful adaptation to climate change across scales. Global Environmental Change.
3. Armitage, D., 2005. Adaptive Capacity, Social Capital and Co-Management. In 'Adaptive Co-Management Symposium'. Waterloo, Ontario p. online at: http://www.omrn.ca/documents/Armitage.pdf.
4. Barnes, R.S.K., 1991. Dilemmas in the theory and practice of biological conservation as exemplified by British coastal lagoons, *Biological Conservation* 55(3).
5. CRMP, 2002. Altas Sumberdaya Wilayah Pesisir Minahasa-Manado-Bitung. Kerjasama Pemenrintah Provinsi Sulawesi Utara, Kabupaten Minahasa, Kota Manado dan Kota Bitung. Proyek Pesisir (USAID Indonesia, Coastal Resources Management Project), Manado, Indonesia.
6. Holling, C.S, and G.K. Meffe, 1996. Command and Control and the Pathology of Natural Resource Management. Conservation Biology 10.
7. Kusnadi, 2009. *Keberdayaan Nelayan dan Dinamika Ekonomi Pesisir*. Diterbitkan atas Kerjasama Pusat Penelitian Wilayah Pesisir dan Pulau-Pulau Kecil Lembaga Penelitian, Universitas Jember dengann Penerbit Ar-RuzzMedia. Yogyakarta.
8. Marshall, N.A., P.A. Mashall, J. Tamender, D. Obura, D. Malleret-King, dan J.E. Cinner, 2009. *A Framework for Social Adaptation to Climate Change*. Sustaining Tropical Coastal Communities & Industri. IUCN (International Union for Conservation of Nature) Publications Services. Switzerland.
9. Nelson, D.R., W.N. Adger, and K. Brown, 200. Adaptation to Environmental Change: Contributions of a Resilience Framework. Annual Review of Environment and Resources 32.
10. Ni, J.R., A.G.L. Borthwick and H.P. Qin, 2002. Integrated approach to determining postreclamation coastlines, *Journal of Environmental Engineering-Asce*, 128 (6).
11. Noske, R.A., 1995. The ecology of mangrove forest birds in peninsular malaysia, *IBIS* 137 (2).
12. Rapport, D.J and C. Gaudet, 1998. Evaluating Landscape Health: Integrating Societal Goals and Biophysical Process. Journal of Environmental Managemen.
13. Scheffer, M., S. Carpenter, J.A. Foley, C. Folke and B. Walker, 2001. Catastrophic Shifts in Ecosystems. Nature 413.
14. Terawaki, T., K. Yoshikawa, G. Yoshida, M. Uchimura and K. Iseki, 2003. Ecology and restoration techniques for Sargassum beds in the Seto Inland Sea, Japan, *Marine Pollution Bulletin* 47 (1-6)
15. Yin, R.K., 2003. Case Study Research. Design and Methods. Third Edition. Sage Publication.

NATIVE CHICKEN EGG CHOLESTEROL CONTENT WHICH HAS BEEN FED OF SKIPJACK TUNA WASTE MEAL *(Katsuwonus pelamis L)*

Jein Rinny LEKE[1], Osfar SJOFJAN[2], Marie NAJOAN[1]
[1]Animal Husbandry, Sam Ratulangi University, Manado, Indonesia
[2]Animal Husbandry , Brawijaya University, Malang. Indonesia

Abstract
Waste in the form of Skipjack tuna waste meal {fish heads, entrails, the rest of the assortment (Arachon), the rest of filleting (bone)} can be used in formulating chicken feed to produce low cholesterol-egg laying chicken. The research was conducted at the Faculty of Animal Science University of Sam Ratulangi, Manado, Indonesia; between August 2011 to February 2012, using 180 native chickens' 32-weeks old. The chickens was kept in battery cages each of four birds. Feed used is containing skipjack waste meal {the head and entrails, the rest of filleting (bone) and the rest of the assortment (arachon)} with a level of 0%, 5%, 10%, 15%, and 20%. Feed and drinking water provided ad libitum. Completely Randomized Design (CRD) nested pattern was used with three treatments and three replications of five levels. Each test used four chickens. The data obtained were tested by analysis of variance and differences between treatments were tested with Duncan test. Observed variable is the content of cholesterol as well as consumption, conversion, and egg mass chicken egg. The sampling performed by taking the eggs randomly on each test for all treatments (cholesterol content) was analyzed in eight weeks study. Results indicated that skipjack tuna waste meal (bone and arachon) were not significantly differ ($P<0.05$) of cholesterol, feed intake and egg mass. The analysis of diversity at the level of 0%, 5%, 10%, 15%, and 20%; skipjack tuna waste has non-significant effect ($P>0.05$) on cholesterol content and feed consumption. Conversion of skipjack tuna waste meal (bone and arachon) were highly significant ($P<0.01$). The results of diversity analysis showed that the level of 0%, 5%, 15%, and 20% of skipjack tuna waste meal, has highly significant effect ($P>0.01$) on the conversion and egg mass. The conclusions of this study, that the skipjack tuna waste meal (heads and entrails) till the level of 10%, results a low cholesterol egg content compared with skipjack tuna fish waste meal (bones and arachon). Native chickens that consumed 15% skipjack tuna waste meal, gives best conversion and egg mass.

Keywords : Cholesterol content, Skipjack tuna waste meal, Native chicken egg.

Introduction

Chicken egg production is still very low in Indonesia, as well as weight gain and growth rate. Therefore, efforts to improve the productivity of native chickens is still very necessary, by an improvement of food quantitatively and qualitatively in order to achieve optimal growth rates by utilizing locally available foods. This shift has an impact on the feeding systems; but the source must be available continuously.

Fish waste of the tuna fishing industry, which will cause odors and pollution has a low value because it is easily damaged, but by mechanical processing in a sewage plant the fish waste will improve the value of protein, calcium, phosphorus, omega-3 that only comes of fish; can be used as animal feed, especially for poultry.

In general, the problem in chicken eggs is a fairly high fat content, making it less attractive to middle and upper part of the population. Efforts to overcome these problems need to be reviewed chicken maintenance pattern in which one aspect is the aspect of feed. There should also note that the presence of cholesterol in chicken eggs is essential to the needs of the cell. Fat and cholesterol is needed, since both compounds are very big function, but there was a wide - range of diseases caused by these compounds. Efforts to overcome these problems in this study do not eliminate fat and cholesterol eggs in chicken eggs, but

make a low-order chicken egg cholesterol content. According to Lengkey et al (2011), using Skipjack tuna gill meal in crumbled ration, can replace the function of fish meal and also has average 2.08% on abdominal fat of broiler. Sretenovic et al (2009), said that the role of omega-3 fatty acids in animal nutrition has influence improvement of their production, reproduction and health performance. Using waste of skipjack tuna fish silage until 4% level in ration had optimal response on the final body weight, carcass percentage and the meat protein conversion of broiler (Widjastuti et al, 2011). Using feed contain skipjack waste meal, will increase the production of chicken eggs that low in cholesterol; utilizing waste materials tuna heads and entrails, bones, and arachon.

Materials and methods

180 native chickens age 32 weeks was used, the initial body weight 861 g with a coefficient of variability 24.57%. Chickens kept in battery cages each of four birds. Feed used is containing skipjack waste meal {the head and entrails, the rest of filleting (bone) and the rest of the assortment (arachon)} with a level of 0%, 5%, 10%, 15%, and 20%. Feeding and drinking water provided ad libitum, done twice a day i.e. morning and afternoon. Completely Randomized Design (CRD) nested pattern was used with three treatments and three replications of five levels. Each test used four chickens. Differences in responses were tested with Duncan's multiple range analysis according to various Steel & Torrie (1980). Feed composition of the research contained in Appendix 1. The chicken eggs was determined the cholesterol content and the observed variables are consumption, conversion, and egg mass. The study lasted in 8 weeks and the egg quality was sampled weekly.

Results and discussion

1.The Effect of Skipjack Tuna Waste Meal on Consumption, Conversion, Egg Mass and Egg Cholesterol.

The consumption, conversion, egg mass and egg cholesterol, are shown in Table 1.

Table 1. The Effect of Skipjack Tuna Waste Meal on Consumption, Conversion, Egg Mass and Egg Cholesterol, for 8 weeks

Skipjack TunaWaste Meal	Consumption (gram/bird)	Conversion	Egg Mass (gram/bird)	Egg Cholesterol (mg/g)
The head and entrails	2.178,912 ± 322,761[a]	9,764 ± 1,054[a]	224,949 ± 31,312[a]	592,666 ± 50,739[a]
Bone	2.248,337 ± 290,911[a]	10,051 ± 1,098[ab]	231,662 ± 33,378[a]	571,457 ± 40,720[a]
Arachon	2.312,835 ± 282,908[a]	10,696 ± 1,164[b]	220,830 ± 18,862[a]	571,539 ± 66,609[a]

Notes : Different notations in the same column indicate significant differences (P<0.05)

The highest feed consumption contained in the Arachon meal (2312.835 ± 282 908) g/bird, and then bone meal (2248.337 ± 290.911) g/bird, and the lowest are the head and entrails meal (2178.912 ± 322.761) g/bird. But by the diversity analysis, results indicated that the type of skipjack tuna waste meal, has no significantly different ($P<0.05$) of consumption. Amount of feed consumed during the period of egg-laying chicken fed with skipjack tuna waste meal (tuna head and entrails and of arachon, are 2178.912 to 2312.885 g/bird with the average per bird during the 8-week study of 70 g/bird/day. Suprijatna (2002) suggested that the ration consumption was not significantly different ($P>0.05$). The average consumption of medium type chicken, production age 20-44 weeks was 3179.46 g (12% protein), 3191 g (15% protein), and 3160 grams (18% protein); distinguishes the consumption is still below the medium type, because of the declining period in feed intake, and the average consumed only as much as 28.5 grams/bird/day, or only a third of the consumption during the spawning period (Mufti, 2005). The feed consumption during the starting time to lay eggs for hatching period, will decrease. Feed consumption continues to decline until it reaches the lowest, on the 16th day of hatching. Low feed intake during hatching occurs because the chickens do not produce eggs, so the body needs little bid nutrients. In addition, the activity of hatching chickens is very low because most of the time, the chickens are in cages in a sitting position so that the opportunity to consume less feed.

The highest feed conversion is the skipjack waste meal (arachon) 10.696 ± 1.164, and bone meal (10.051 ± 1.098), and the lowest is the head and entrails (9.764 ± 1.054). The analysis showed that the diversity of tuna waste meal gives a highly significant different effect ($P>0.01$) on the conversion. Further testing showed that the head and entrails skipjack tuna meal, has not significant effect ($P>0.05$) compared to bone meal; and the bone meal also has no significantly different ($P>0.05$) compared to arachon. The head and entrails compared to arachon, has highly significant effect ($P<0.01$) because the feed conversion is determined by the rate of growth. High growth rate will result in increased efficiency of feed use, but the high growth rate is offset by the consumption of too high then the efficiency of feed use is low, resulting in improved feed conversion. This is especially true due to the rate of growth that has begun decreased as it enters the final growth period.

The highest egg mass, found in bone meal (231.662 ± 33.378), then head and entrails meal (224.949 ± 31.312), and lowest is the arachon meal (220.830 ± 18.862). But by the diversity analysis results, showed that the skipjack tuna waste meal has no significantly different ($P<0.05$) against egg mass, because of the decisive factor is the number of egg mass and egg weight. The increase of the egg numbers or egg weight can improve egg mass. Thus, the difference egg mass in this study was a result of differences in the number of eggs in the chicken produced in the early period and began to decline when it begins to enter the hatching time, and also of the difference in egg weight during production time.

The highest egg cholesterol present in head and entrails meal (592.666 ± 50.39) and then arachon meal (571.539 ± 66.609), and the lowest in bone meal (571.457 ± 40.720). However, the analysis showed that the diversity of skipjack tuna waste meal has no significantly different ($P<0.05$) on egg cholesterol. According to Sudibya (1998), low cholesterol eggs was produced from chickens that has fed with 30% of shrimp head meal (802.22 mg/dl), while the highest are the control treatment (957.31 mg/dl). The ration fat content, causes the yolk fatty acid content changes. Chao et al., (2001) suggested that peroxisomes are important organelles involved in various aspects of cell metabolism, including fatty acids and other lipids.

2. Effect of Skipjack Waste Meal Level on Consumption, Conversion, Egg Mass and Egg Cholesterol

The results of the average level of consumption, conversion, egg mass and egg cholesterol, are shown in Table 2.

Table 2. The average effect of a variety of levels of consumption, conversion, egg mass and egg cholesterol for 8 weeks

Level	Consumption (gram)	Conversion	Egg Mass (gram/bird)	Egg Cholesterol (mg/g)
0%	2,369.962 ± 312.060[a]	10.741 ± 0.929[b]	215.968 ± 16.323[b]	529.549 ± 32.883[a]
5%	2,203.157 ± 307.874[a]	10.921 ± 1.069[b]	207.489 ± 14.361[b]	590.684 ± 41.502[b]
10%	2,268.683 ± 311.195[a]	9.806 ± 1.327[b]	241.583 ± 30.030[a]	565.260 ± 60.655[ab]
15%	2,259.072 ± 314.650[a]	9.626 ± 1.014[a]	241.854 ± 34.010[a]	578.206 ± 44.451[ab]
20%	2,132.598 ± 254.343[a]	9.760 ± 0.899[b]	222.173 ± 28.249[ab]	629.070 ± 36.548[b]

Notes : Different notations in the same column indicate significant differences (P<0.05).

The highest feed consumption are at level 0% (2369.962 ± 312.060), then 10% (2268.683 ± 311.195), 15% (2259.072 ± 314.650), 5% (2203.157 ± 307.874), and lowest at 20% level (2132.598 ± 254.343). The analysis showed that the diversity of skipjack tuna waste meal levels are not significantly different (P<0.05) of consumption.

The highest feed conversion are at level 5% (10.921 ± 1.069), then the 0% (10.741 ± 0.929), level 10% (9.806 ± 1.327), level 20% (9.760 ± 0.899) and the lowest at 15% level (9.626 ± 1.014). The analysis showed that the level of diversity of skipjack tuna waste meal gives a highly significant effect (P>0.01) on the conversion. Further testing showed that the level of 0% gives no significant (P>0.05) compared with the level of 5%, 10% and 20% level successively, while the level of 15% has highly significant (P<0.01) compared to 0%, 5%, 10%, and 20% skipjack tuna waste meal levels.

Egg mass highest level found in 15% (241.854 ± 34.010), then the level of 10% (241.583 ± 30.030), 20% (222.173 ± 28.249), 0% (215.968 ± 16.323) and the lowest is 5% level (207.489 ± 14.361). The analysis showed that the level of diversity of skipjack waste meal, has highly significant effect (P>0.01) against egg mass. Further testing showed that the 15% skipjack tuna waste meal levels, are highly significant (P<0.01) compared to the level of 20%, 5% and 0%. But the level of 15% has not significant effect (P>0.05) compared to the level of 10%, and 20%. The next level of 5% skipjack tuna waste meal, has a highly significant effect (P<0.01) compared to the level of 10%, and 20%. However, the level of 5% has no significant effect (P>0.05) compared with 0% and 20% level.

The highest level of cholesterol present in 20% (629.070 ± 36.548), then the level of 5% (590.684 ± 41.502), the level of 15% (578.206 ± 44.451), 10% (565.260 ± 60.655) and the lowest is 0% (529.549 ± 32.883). The analysis showed that the level of skipjack tuna

waste meal has highly significant effect (P<0.05) on cholesterol. Furthermore, the level of 20% has highly significant effect (P<0.01) than 0%. While the level of 20% has no different significant (P>0.05) compared to the level of 5%, 10% and 15%.

Conclusion

10% skipjack tuna waste meal (heads and entrails) has lowest cholesterol egg content compared with the bone skipjack tuna waste meal; and arachon. And also the 15% (head and entrails) has the best conversion and egg mass.

References

1. Chao, P.M.., C.Y. Chao, F.J. Lin and C.J. Huang. 2001. Oxidized frying oil up-regulates hepatic acyl-Co Oxidize and Cytochrome P450 4 A1 Genes in rats and activates PPAR . J. Nut, 131, 3166-3174.
2. Lengkey, Hendronoto A.W., Tuti Widjastuti and Maya Ludong. 2011. Various Levels Effect of Skipjack tuna gill meal (*Katsuwonus pelamis* L) in ration on broiler carcass and abdominal fat. Lucrari Stiintifice Seria Zootehnie Vol. 56 (16). Editura Ion Ionescu De La Brad, Iasi. Romania. p. 121 – 123.
3. Mufti. M. 2005. Tingkah Laku Mengeram, dan Pengendaliannya Guna Meningkatkan Produksi Telur Ayam Kampung. Pascasarjana Institut Pertanian Bogor.
4. Sretenovic, Lj, V Pantelic and Z Novakovic, 2009. Importance of Utilization of Omega-3 Fatty Acid in Human and Animal Nutrition. Biotechnology in Animal Husbandry 25 (5-6). Institute for Animal Husbandry, Belgrade-Zemun. p. 439 – 449.
5. Suprijatna. E. 2002. Manifestation of Grower Dietary Protein Level on the Growth of Reproduction Organ and the Effects on Production Performance of Medium Type Layer. Program Pascasarjana . Universitas Padjadjaran. Bandung.
6. Sudibya. 1998. Manipulasi Kadar Kolesterol Dan Asam lemak Omega 3 Telur Ayam Melalui Penggunaan Kepala Udang Dan Minyak Ikan Lemuru. Program Pascasarjana. Institut Pertanian Bogor.
7. Steel RGD, Torrie, Dickey DA. 1997. Principles and Procedures of Statistics: A Biometrical Approach. 3rd Ed. New York: Mc Graw- Hill Co. Inc.
8. Tuti Widjastuti, Hendronoto Arnoldus W. Lengkey, R. Wiradimadja and D. Herianti. 2011. Utilizing Waste Product of Tuna (*Thunnus atlanticus*) Fish Silages and Its Implementation on the Meat Protein Conversion of Broiler. Lucrari Stiintifice Seria Zootehnie Vol. 55 (15) Editura Ion Ionescu De La Brad, Iasi. Romania. p. 83-88.

SCREENING OF ANTIBIOTICS IN POULTRY LIVER USING THE MICROBIOLOGICAL METHOD AND TETRASENSOR TEST

Oana-Mărgărita GHIMPEŢEANU[1], Guy DEGAND[2], Narimene MANSOURI[3], Laurenţiu TUDOR[1], Manuella MILITARU[1], Marie Louise SCIPPO[2]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, [2]Laboratory of food analysis, Faculty of Veterinary Medicine, University of Liège, Belgium; [3] Institute of Veterinary Science, el tarf University Centre, Algeria; ghimpe_marga@yahoo.com

Abstract

Microbiological methods and rapid tests, such as TetraSensor, are the most commonly used techniques to screen residues of antibiotics applied in animal production, including poultry industry. The election organ for antibiotics, in poultry, is liver, so a careful monitoring of the residues should be made in order to provide safe foodstuff for consumers. The microbiological screening method uses two media at different pH seeded with a single bacteria strain (Bacillus subtilis). The method was used to detect the antibiotics from four groups: tetracycline, quinolones, macrolides and sulfonamides. The rapid test, TetraSensor, was used for confirmation of presence of tetracycline at lower concentrations than the microbiological screening. Poultry livers sampled on Romanian and Belgian markets were analyzed using both the microbiological and the TetraSensor method. For all samples, the results were negative either using the microbiological method or the rapid test.

Keywords: antibiotics, poultry liver, TetraSensor Test, microbiological method

Introduction

The presence of residues of antibiotics in animals foodstuff can have three causes: failure of respecting the withdrawal period specific for each antibiotic (Paige, 1994; Nollet, 2004), errors of treatment or of registration made by the veterinarian or grower, or error concerning treated animal identification (Sundlof, 1989).

Many studies showed that the presence of antibiotics in foodstuff, of residues in animal products or of their degradation products in the environment can have potentially dangerous effects for human and animal health (Le, 2004).

The first effect of antibiotic residues is the modification of the intestinal flora of animals. Secondly, consumer's health can be affected after consumption of contaminated products, because of both toxic effects of antibiotic residues and the selection of microorganisms resistant to antibiotics which can pass from animals to humans through the food chain.

The monitoring of the presence of antimicrobial resistant bacteria in the food chain is mandatory in the European Union for certain bacteria (Commission Regulation (EC) N^{o} 1831/2003).

In 2011, the World Health Organization established an action plan against the resistance to antimicrobials accompanied by the slogan «Antimicrobial resistance: no action today, no cure tomorrow» (WHO, 2011).

The overusing of antibiotics in animal production, including poultry, is a worldwide problem, so monitoring of their residues should be a constant concern.

The most used groups of antibiotics in poultry industry in Romania are: tetracycline, quinolones, macrolides and sulfonamides.

The aim of this study was to monitor the concentration of antibiotics in commercial poultry liver samples.

Materials and methods

This study was conducted on four collective commercial poultry liver. Two samples came from Romanian slaughterhouses (one from Muntenia region and one from Transilvania region) and two from Belgium slaughterhouses (one from Flanders and one from Wallonia).

Two methods of screening of antibiotics were used: the NTPT (New Two Plate Test) using culture media with two different pH (Dang et al., 2010) and the rapid TetraSensor Test (KIT306_Tetrasensor_Tissue_insert_ V1.0).

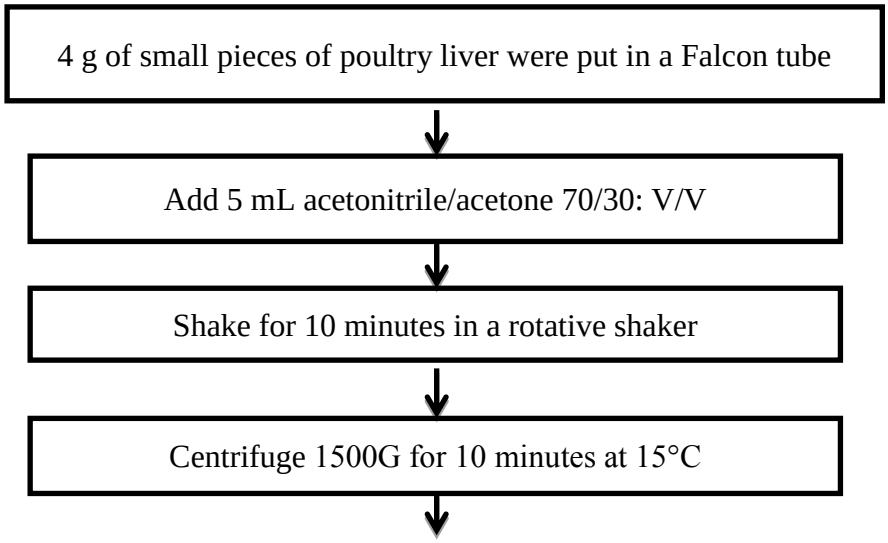
In addition to unknown liver samples, spiked liver samples were analyzed as positive control (by adding 20µL standard solution for each antibiotic).

Method culture media with different pH

Two types of culture media were used: one with pH 6 (prepared from Test Agar pH 6, Merck 1.10663) and the second with pH 7, 5 (prepared from Standard II Nutrient Agar pH 7.5, Merck 1.07883). To each 250 mL of culture media batch, were added 3,30 mL glucose stock solution (45%) and 250µL of bacterial suspension (*Bacillus subtilis* strain BGA spore suspension containing 10^{-7} spores mL^{-1}, Merck 1.10649).

The culture media with pH 6 was used for screening of tetracycline and quinolones, while that with pH 7, 5 for sulfonamides and macrolides.

For the first method, samples were prepared using the following protocol, adapted from the method developed for chicken meat by Dang and collaborators (2011) (Fig. 1).



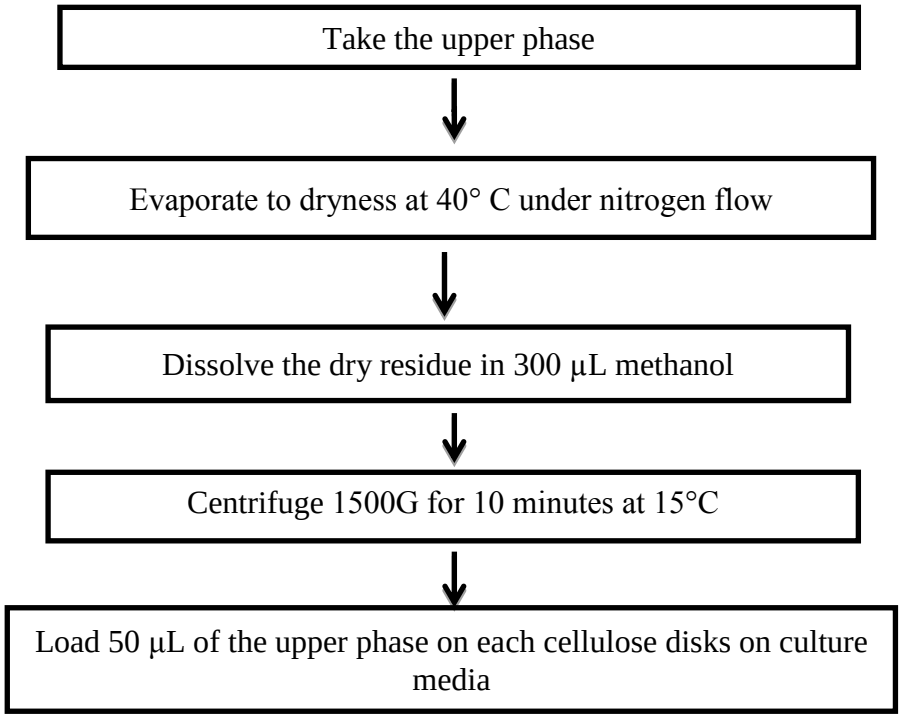


Fig. 1. Preparation of samples for antibiotics screening using the NTPT (adapted from Dang et al, 2011)

The NTPT assay (fig. 2) is based on the inhibition of the growth of *Bacillus subtilis*, which is sensitive to a large number of antibiotic molecules. It is however possible to discriminate between antibiotics by using two different pH and by using PABA (para-aminobenzoic acid), as explained below. Tetracyclines and quinolones will give a higher response on the plate with pH 6 while plate with pH 7,5 will be used to identify sulfonamides and macrolides..

The minimum inhibitory quantity (MIQ) is the minimum quantity of antibiotic able to produce an inhibitory zone of at least 1,5 mm of width (sample considered positive) on a disc of 12,7 mm of diameter. The MIQ is determined by using standard antibiotic solutions.

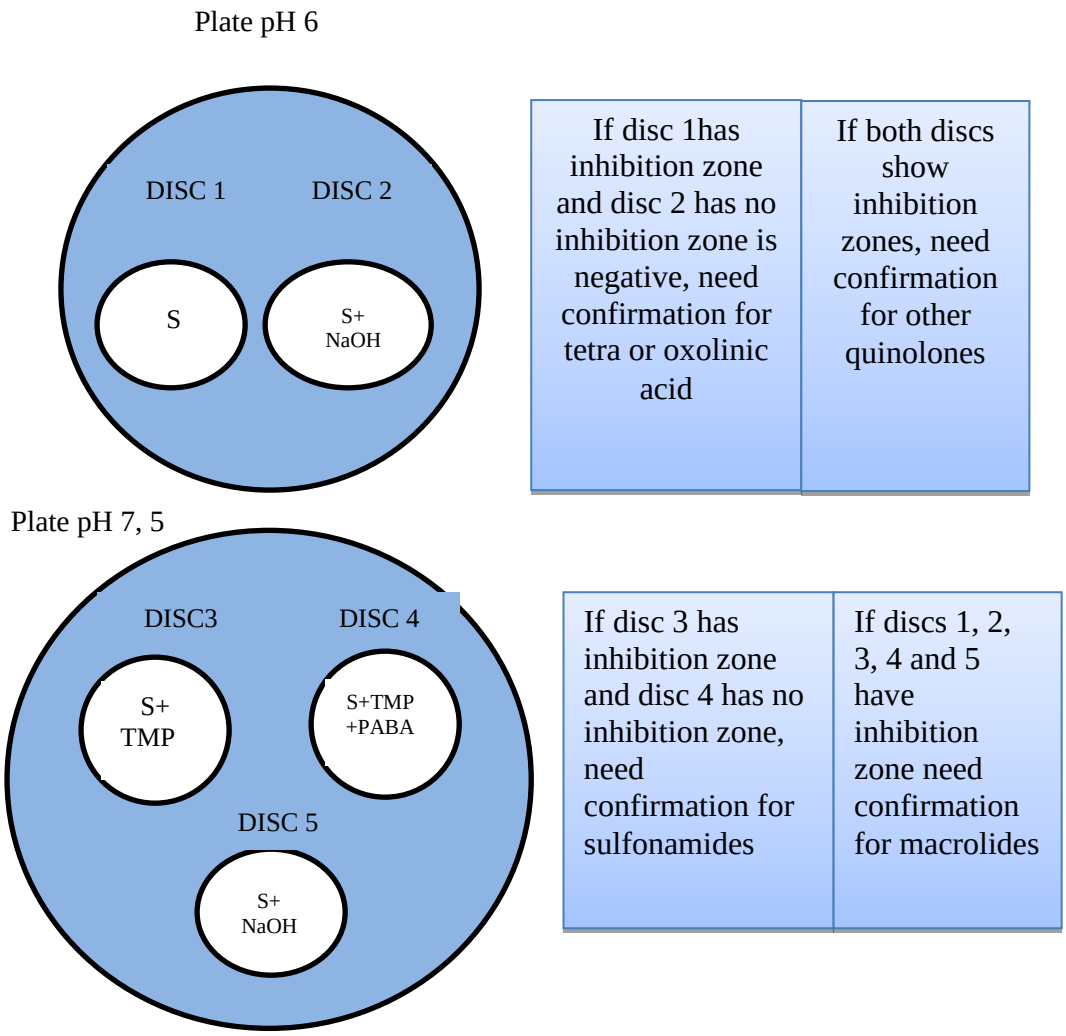


Fig. 2. General scheme for the NTPT (from Dang et al, 2010)

S= 50 µL of sample extract, TMP = 10 µL of trimethoprim solution (10µg mL^{-1}), NaOH = 20 µL of 1% NaOH solution, PABA = 10 µL of para-aminobenzoic acid solution (10µg mL^{-1})

For the second method, the TetraSensor Test, samples were prepared using the protocol described above (Fig.3)

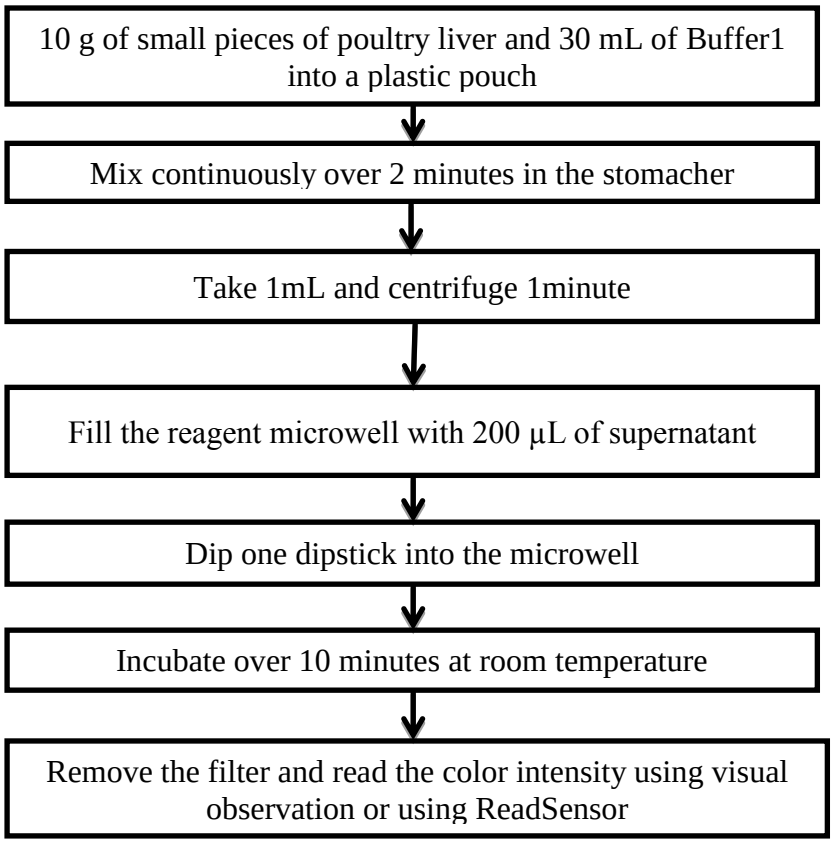


Fig. 3. Preparation of samples for antibiotics screening using TetraSensor Test

Results and discussions

For all antibiotics types, we worked with a quantity of sample that allowed us to detect the antibiotic residues under the maximum residue limit (MRL). The limit of detection was around 100 ppb for all types of antibiotics (Dang et al, 2011), while the MRL, in poultry liver, for tetracycline is 300 ppb, enrofloxacin –200 ppb, sulfadiazine – 100 ppb and tylosin – 100 ppb, respectively. (Commission Regulation (EC) N^{o}37/2010).

The medium pH affects the activity of certain antibacterials, the tetracyclines are more active in acid pH (amines groups are then charged), while most of the quinolones are more active in alkaline pH (carboxylics groups are then charged).

Tetracyclines potency is rapidly destroyed by alkali hydroxide solutions. For this reason, the addition of 20 µl NaOH 1% has been used for the identification of tetracyclines and to improve the sensibility towards the quinolones and macrolides antibiotic groups. All macrolides tested on plate pH 7,5 showed larger inhibition zones when NaOH is added

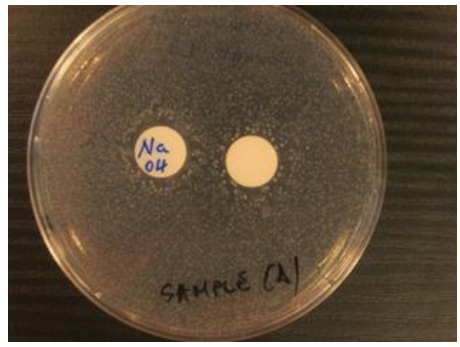
The bacteria synthetize the folic acid, an essential precursor for the DNA synthesis. The sulfonamides act by inhibiting the synthesis of folic acid (by taking the place of the normal substrate) and consequently, the replication of the bacteria. In the presence of an

excess of exogenous of PABA, the sulfonamides are not able anymore to inhibit the bacterial synthesis of folic acid, because bacteria uses the external source of PABA. So, if sulfonamide are present in the sample extract, the positive response seen on Disc n°3 ("S+TMP") will disappear on Disc n°4 ("S+TMP+PABA").

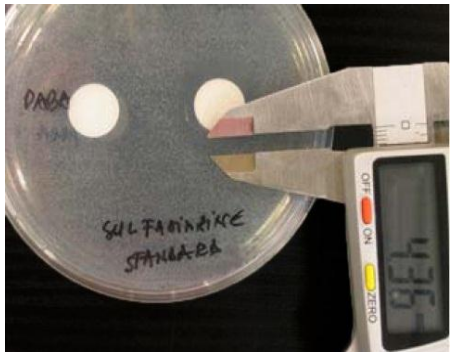
According to the publications of Dang et al. (2010, 2011), samples are considered positive if the width of the inhibition zone around the paper disc is equal or higher than 1.5 mm.

None of the liver samples show any positive response on the five discs of both plates (pH 6 or pH 7,5) and for comparation spiked samples (positive control) were presented (fig.4).

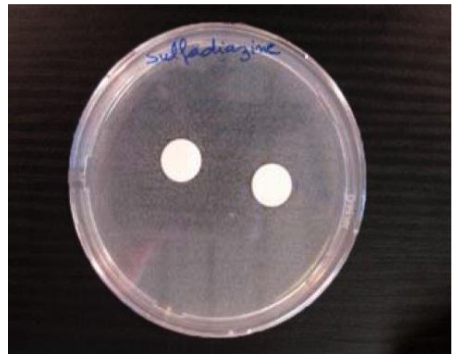
Spiked sample for tetracycline and quinolones with 20µl standard solution conc 20µg/ml(positive control)



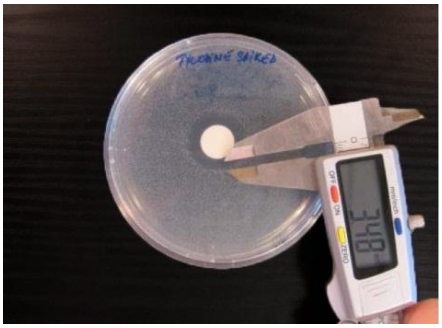
Example of one liver sample giving a negative result on plate at pH 6 (more sensitive to tetracyclines and quinolones)



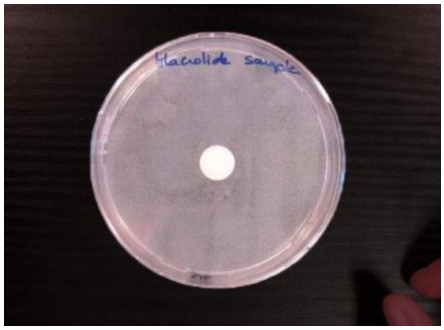
Spiked sample for sulfonamides with 20µl standard solution , conc 20µg/ml(positive control)



Example of one liver sample giving a negative result on plate at pH 7,5, on both discs n° 3 and 4 (without and with PABA, respectively)



Spiked sample for macrolides with 20µl standard solution , conc 1µg/ml (positive control)



Example of one liver sample giving a negative result on plate at pH 7,5, Disc n°5 (more sensitive to macrolides)

Fig. 4. Results of microbiological screening for spiked and real poultry liver samples

All liver samples were also analyzed using the rapid test TetraSensor, a screening test which allows detecting all tetracycline molecules at levels of least at 10 ppb, i.e. with a better sensitivity than the microbiological screening.

Because the extract from poultry liver had a strong red color (hemoglobin), all samples had a false positive result by using ReadSensor (fig.5). The pigmentation was removed by using hydroxylapatite, a form of calcium phosphate. After this, all results were negative (fig. 6)

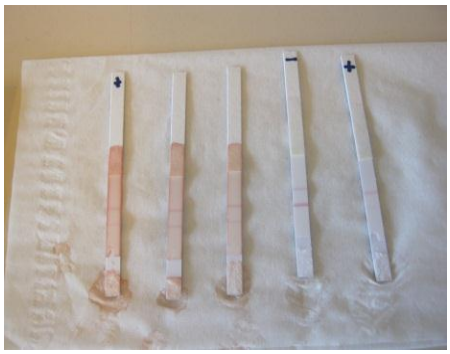


Fig. 5. False positive results using TetraSensor

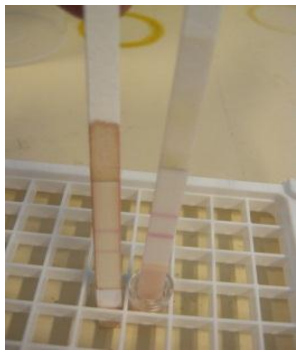


Fig. 6. Results after using hydroxylapatite

Conclusion

1. The results of rapid test can be influenced by some components of the sample, such as the pigments, and can reveal false positive results.

2. The microbiological method is one of the most used screening techniques, because of its low cost and good results.

Acknowledgments

This study is part of the POSDRU project 88/1.5/S/52614 "Doctoral scholarships for high quality training for young researchers in the field of agronomy and veterinary medicine" and it is part of the PhD thesis "Correlations between liver pathology in broiler chickens and food safety"- Oana-Mărgărita Ghimpeţeanu

References

1. Commision Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. OJ L 268, 18.10.2003b, p. 29–43
2. Commision Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin,OJ L 15/1,20.01.2010
3. Dang K.P,Degand G., Danyi S.,Pierret G., Delahaut P,Tona V.D., Guy Maghuin-Rogister G.,Scippo ML
4. Validation of a two-plate microbiological method for screening antibiotic residues in shrimp tissue, Analytica Chimica Acta, 2010, 672, p 30–39
5. Dang.P.K.., Degand G., Douny C., Dinh T.V., Maghuin-Rogister G., Scippo M.L., Optimization of a new two-plate screening method for the detection of antibiotic residues in meat, International Journal of Food Science and Technology,2011,p 1-7
6. Le, T. X., Munekage, Y. Residues of selected antibiotics in water and mud from shrimp ponds inmangrove areas in Viet Nam. Marine Pollution Bulletin, 2004, 49, 922-929.
7. Paige, J. C. Analysis of tissue residues. FDA Vet., 1994, 9, 4-6.
8. Nollet, L.M.L. Handbook of food analysis, vol2. Residues and other food component analysis.NewYork, 2004, 1738 .
9. Sundlof, S. F. Drug and chemical residues in livestock. Veterinary Clinics of North America. Food Animal Practice, 1989, 5, 411- 449.
10. WHO (WORLD HEALTH ORGANIZATION), World Health Day – 7 April 2011,http://www.who.int/world-health-day/2011/en/index.html 30/04/2012.
11. ***KIT036_Tetrasensor_Tissue_insert_V1.0)

BETA-LACTAM RESISTANCE PHENOTYPES OF *ESCHERICHIA COLI* STRAINS ISOLATED FROM BROILERS

Elena-Iuliana MĂCIUCĂ, Mihai OBADĂ, Cătălin CARP-CĂRARE, Cristina RIMBU
Eleonora GUGUIANU, Mihai CARP-CĂRARE
University of Agricultural Sciences and Veterinary Medicine
''Ion Ionescu de la Brad'' Iași Faculty of Veterinary Medicine
Aleea Mihail Sadoveanu nr. 8, 700489, Iaşi, România
e_iulia70@yahoo.com

Abstract
The aim of this study was to determine antimicrobial susceptibility to beta-lactam antibiotisc and resistance phenotypes of Escherichia coli strains, isolated from broiler chickens. During the study we isolated 40 bacterial strains belonging to the species Escherichia coli. 22,5% of Escherichia coli strains were wild phenotype, 15% low penicilinase producers, 7,5% high penicillinase producers, 15% were extended spectrum beta-lactamase(ESBL) producing strains, 10%proved to be cephalosporinase-producing strains, and 30% were producers of inhibitor-resistant beta-lactamases. None of the isolated strains had produced carbapenemases.
Keywords-*Escherichia coli* β-lactamase, antibiotic resistance

Introduction

Emergence of resistance to β-lactam antibiotics began even before the first β-lactam, penicillin, was developed. The first β-lactamase was identified in *E. coli* prior to the release of penicillin for use in medical practice(1).

Many genera of gram-negative bacteria possess a naturally occurring chrosomally mediated β-lactamase. These enzymes are thought to have evolved from penicillin-binding proteins(PBP), with which they show some sequence homology.

This development was likely due to the selective pressure exerted by β-lactam-producing soil organisms found in the environment (1).

Today resistance to antibiotics through β-lactamases is a phenomenon that creates serious therapeutic problems worldwide(5).

Materials and methods

Transmission of multidrug-resistant germs from animals to humans via the food chain can be achieved, so the presence of such germs at this level, has created concern that these strains may represent a significant risk to public health(3).

During November 2011 to February 2012 the sampling was done through monthly visits in the slaughterhouse where samples were collected.

In the laboratory caecal samples were enriched overnight in buffered peptone water.

To identify strains were used API Rapid ID 32 tests.

Susceptibility testing of isolates to β-lactam antibiotics was performed by disk-diffusion method.

Working protocol has been followed step by step:

-turbidity of the suspension was controlled and adjusted to 0.5 McFarland (1.5 $x10^8$CFU / ml) using sterile saline(2,4).

- a sterile non-toxic swab was inserted into the adjusted suspension, and then the swab was rotated several times, pressing firmly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab(4);

- the dried surface of an agar plate was inoculated by streaking the swab over the entire sterile agar surface; this procedure was repeated two more times, to ensure an even distribution of inoculum(4);

- after 15 min. of waiting, time for any excess surface moisture to be absorbed, the antibiotic disks(Bioanalyse) were applied using an automated dispencer

- the plates were placed in an incubator at 37°C, after 16-18 hrs. of incubation, each plate was examined and the diameters of the zones of complete inhibition measured, including the diameter of the disk(4);

- interpretation of the zone sizes was made by referring to BSAC Methods for Antimicrobial Susceptibility Testing Version 10.2 May 2011 and the strains were reported to be either susceptible,intermediate or resistant(2).

The isolates were tested to the antibiotics shown in table 1.

Table 1. Antibiotics tested

Nr.crt	**Class of antibiotics**	**Active substance**	**Abbreviation**
1.	Aminopenicillins	Ampicillin	AM
2.	Aminopenicillins/ beta-lactamase inhibitor	Amoxicillin/ clavulanic ac.	AMC
3.	Carboxypenicilins	Ticarcillin	TIC
4.	Ureidopenicillins	Piperacillin	PRL
5.	Cephalosporins . Ist gen.	Cephalotin	KF
6	Cephalosporin IInd gen.	Cefaclor	CEC
7.	Cephalosporins IIIrd gen.	Cefotaxime	CTX
		Ceftazidime	CAZ
8.	Cephalosporins IIIrd gen. / beta-lactamase inhibitor	Cefotaxime/ Clavulanic ac.	CTC
		Ceftazidime / Clavulanic ac.	CZC
9.	Cephalosporins IVth gen.	Cefpirome	CPO
10.	Cephamicins	Cefoxitin	FOX
11.	Carbapenems	Imipenem	IMP

Results and discussion

Inactivation of beta-lactam antibiotics by beta-lactamases is the currently the most common mechanism of resistance encountered. This enzymes cleaves the beta-lactam ring, causing inactivation of the antibiotic(7).

For detection of ESBL-producing strains was performed double disk test, using IIIrd generation cephalosporins simple and enhanced with clavulanic acid (cefotaxime, cefotaxime /Clavulanic acid and ceftazidime, ceftazidime /Clavulanic acid)(2).

To detect strains producing cephalosporinases cefoxitin was used as an indicator and to detect strains producing carbapenemases we used imipenem .

Susceptibility test results to different groups of beta-lactams are shown in Figure 2.

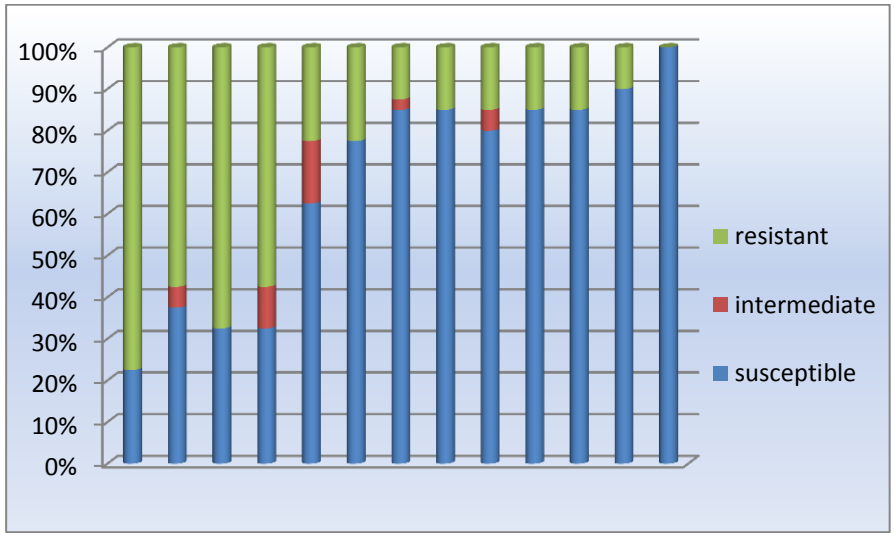


Fig.2. Beta-lactams susceptibility of *E.coli* strains

Antibiotic susceptibility testing of *E. coli* strains revealed a large number of strains with multiple resistance to antibiotics.

Of the 40 strains tested only 9 were sensitive to all antibiotics chosen for testing, representing strains with wild phenotype.

A large number of strains were identified phenotypically as resistant to aminopenicillins, carboxypenicillins and ureidopenicillins.

Depending on the sensitivity /resistance recorded at different β-lactam classes, we divided the 40 strains of *E. coli* isolated in several phenotypes of resistance.

Analyzing the results in Table 2 it can be said that *E. coli* strains studied are producing beta-lactamases in a large number, respectively 77.5% .

Of phenotypes identified, those who make the biggest issues in therapy is ESBL phenotype.

Table 2. Resistance phenotypes to β-lactam antibiotics

Nr crt	Nr.of strains	Wild phenotype	Low penicilinase	High penicillinase	cephalosporinase	Inhibitor resistant beta-lactamaze	ESBL	Carbapenemase
Nr.	40	9	6	3	4	12	6	-
%	100	22,5	15	7,5	10	30	15	-

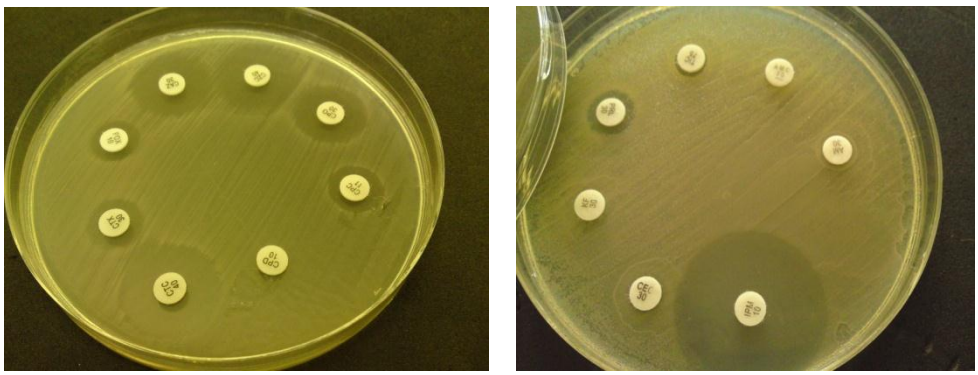


Fig.3. ESBL phenotype
AM-R,AMC-R,TIC-R, PRL-R,KF-R,CEC-R,CTX-R, CTC-S, CPO-R, IMP-S

Strains assigned to this category have multiple resistance to β-lactam antibiotics, except cephamicins and carbapenems(8).

BSAC recommends that organisms inferred to have ESBLs should be reported resistant to all penicillins (except temocillin) and cephalosporins, including the fourth-generation cephalosporins, cefepime and cefpirome (2).

According to a rapport published by EFSA in 2011, recent findings indicate transmission of ESBL genes, plasmid and clones from poultry to humans is most likely to occur thought the food chain (5,3).

The ESBL strains often have associated resistance to other antibiotics, aminoglycosides, quinolones, tetracycline, trimethoprim-sulfamethoxazole and nitrofurantoin(6,7).

Conclusions

1. 40 strains of *Escherichia coli* were studied to determine different phenotypes of resistance to beta-lactam antibiotics.
2. Of the 40 strains of *Escherichia coli* tested, 31 proved to be producing different types of β-lactamases.

3. The main resistance phenotypes identified were: wild phenotype (22.5%), penicillinase low level (15%), high-level penicillinase (7.5%), cefalosparinase (10%), inhibitors resistant β-lactamases (30%) and extended spectrum β-lactamases (15%).
4. ESBL-producing strains isolated from poultry may represent a threat for public health because there are limited therapeutic options left for some of this organisms.
5. Strains with multiple resistance to β-lactam antibiotics often have associated resistance to other classes of antibiotics (quinolones, aminoglycosides).
6. Poultry remains a risk to human health for ESBL-producing strains, however further investigations are needed to see ways in which these strains can be transferred to humans.

The present study was supported by a doctoral studies programme with an ESF (European Social Fund) scholarship **POSDRU–CPP107-DMI1/5/S/77222.**

References

1. Bradford Patricia, 2001, *-Extended-Spectrum β-lactamases in the 21st Century: Characterization, Epidemiology, and Detection of This Important Resistance Threat,* Clinical Microbiology Reviews, Oct. 2001 p. 933-951.
2. *BSAC Methods for Antimicrobial Susceptibility Testing Version 10.2 May 2011*
3. Carattoli, A. 2008. Animal reservoirs for extended spectrum beta-lactamase producers. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases 14 Suppl 1:117-23
4. Carp-Cărare M., Guguianu E., Timofte D., 1997- *Lucrări Practice de Microbiologie Veterinară, Curs litografiat p 100-107* Uz Intern, Iași
5. EFSA Panel on Biological Hazards (BIOHAZ); *Scientific Opinion on the public health risks of bacterial strains producing extended-spectrum β-lactamases and/or AmpC β-lactamases in food and food-producing animals.* EFSA Journal 2011;9(8):2322. [95 pp.] doi:10.2903/j.efsa.2011.2322. Available online*: www.efsa.europa.eu/efsajournal*
6. European Food Safety Authority and European Centre for Disease Prevention and Control; The European Union Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2010. EFSA Journal 2012; 10(3):2598 [233 pp.] doi:10.2903/j.efsa.2012.2598. Available online: *www.efsa.europa.eu/efsajournal*
7. Petraşcu Mirela, Flonta Mirela, Almaş, Ariana Mihaela 2011, Fenotipuri de rezistenţă pentru tulpini de Escherichia coli şi Klebsiella pneumoniae producătoare de beta-lactamaze cu spectru extins (BLSE), izolate din infecţii urinare, Clujul Medical 2011 Vol. 84 - nr. 3 P.371-377
8. Mihăescu Gr., Chifiriuc, M. C, Dițu L. M. 2007- *Antibiotice și substante chimioterapeutice antimicrobiene.* – Editura Academiei Române, București.

WELFARE ASSESSMENT IN DAIRY CATTLE

M. MATEIA, I. ŢIBRU
The Faculty of Veterinary Medicine Timişoara, The Department of Hygiene,
cod 300645, Calea Aradului No. 119, Timisoara, Romania
mnma_maki@yahoo.com

Abstract
This paper compares two methods for assessing the welfare of dairy cows: The Integrative Numeric System ANI 35 and Welfare Quality ® System to the manner for assessing welfare in Romania. The first is based on factors dependent on the shelter which are quantitative expressed and includes 60-70 criteria, which are estimated for seven functional classes, the maximum value given by this numeric system being 200 points and the second is an integrated and standardized welfare assessment system on the technological flow (from farm to slaughterhouse), which assess the welfare of farm animals through direct action, represented by the reaction of the animal. It is based on four principles: housing, feeding, health and the manifestation of a specific behavior, resulting 12 welfare criteria.Evaluation form in Romania provides 9 criteria that make a survey of farm activities, but without quantifying any.

Key words: cow, welfare, assessment

The welfare problem has long been and will be discussed in dairy cattle. To assess and express welfare it must start from its definition. Broom (3) defines an animal welfare as reflected by the degree of adaptation to environment, including efforts that the animal must make to realize it, respectively the extent that get to adapt, and the effects this activity has on his, briefly *the individual state with regard to its attempt to accommodate the environment in which it lives* (5).

World Organisation for Animal Health (OIE) defines animals' welfare as being the way how an animal copes with living conditions.

Decun (2004) considers that the Romanian language would be more appropriate the expression in terms of: full welfare, poor and very poor welfare (4; 5).

In conclusion we can say that animal welfare can be defined by their ability to adapt to the environment and to the group in which they must live, to produce on their genetic potential (economic) and to reproduce normally, without any physical and mental suffering to be present.

The importance of assessing welfare. Was imposed the regular assessment of animal welfare because it first allows to identify deficiencies and, secondly, their removal. This way, it provides the best prerequisites both for expressing the biological production potential, as for improving technology. Designing new technologies for breeding and exploitation of animals, and improving the equipment used to service animals would not be possible without a thorough knowledge of animal welfare. In Switzerland, Sweden, Norway and other countries, assessment of housing conditions and assessment of animal welfare are practiced in the institutes and specialized stations for pre-testing equipment and new breeding and exploitation systems of animals, before obtaining the marketing advice (5).

Systems for assessing dairy cow welfare

1. The Integrative Numeric System ANI 35

In 1985 to the Gumpenstein BAL Institute (Irdning, Austria) it was first developed Tier Gerechtheits Index (TGI) System for farm animal welfare assessment by H. Bartussek, and the name of A.N.I. (Animal Needs Index) appeared in 1991 in England.

In 1994, it was developed by Sundrum, based on Bartussek system, another assessing system that provides some guiding principles in assessing welfare of chickens, cattle and pigs. This concept includes 60-70 criteria, that are estimated for seven functional classes. Maximum value given by this system is 200 points, as this concept allows us to repeat the scores for various maintenance characteristics of multiple categories of animals, which partially explains that high value.

Any assessment of the degree of animal welfare from a farm by ANI system focuses on five criteria (categories) in terms of maintenance, which were chosen because of their importance for animal welfare. These are:

1. Freedom of movement (locomotion);
2. Facilitate social interactions (social behavior);
3. The type and degree of integrity of the floor (floor);
4. The degree of lighting and ventilation (microclimate);
5. Quality of human intervention (care).

The ANI system is a system of points used to assess the extent the housing meets animals' needs in various ways. This system is based on the principle that animals live better if they have more space to move, more rest areas, better air, more light, a greater paddock having access throughout the whole year, clean and dry areas, better relationships to the breeder etc. In addition, animals are considered to have the ability to balance the external factors that help them to offset negative influences with positive ones from the living environment. For example, inconvenience and frustration arising from lack of space in shelters or polluted air can be attenuated by the access to the paddock. The same principle applies to the absence of light in the stable. A good attentive contact to animals can compensate for many shortcomings in the used stabulation system.

Points are awarded for each category through some specific parameters; the sum of all points awarded on the five categories gives the final ANI note. The final grade is higher, the housing conditions are better in terms of welfare. This note, given in the ANI system of assessment, theoretically, may have any value between- 9 and+ 45.5. In any case, note that the minimum requirements are required to be fulfilled.

Table 1. Minimum standards for free uncompartmented

stabulation system (Bartussek 2010) **Category**	**Stables with stalls (m²/animal)**	**Common shelter (m²/animal)**	**Free stabulation without stalls**		**Space requirement of food (m/animal)**
			Rest area (m²/anim)	Feeding, defecation area (m^2)	
heifers to 350 kg	2,2	3,0	1,8	1,8	0,54
up to 500 kg	2,5	5,0	2,2	2,0	0,60
over 500 kg	2,7	5,0	2,5	2,0	0,70
dairy cows		5,0	3,0	2,2	0,75

However, the minimum standards should be defined in addition, if welfare standards imposed by law (also EU directives or national legislation regarding the welfare of farm animals) do not specify such requirements. If those minimum requirements are not met by the

evaluated housing system, the calculated grade is valid only if the deficiencies are removed in a reasonable time. Temporarily, it is granted an ANI provisional-note.

There are 28 criteria evaluated and they are divided into five areas. The maximum number of points is 3 and the minimum is - 0.5. Theoretical the amount of points will be between -9 and 45.5 points (maximum absolute value = 36.5 points) and maximum number of points is 54.5, the average being +18.25.

As a means of assessing welfare by this method (ANI) is completing of the seven records the assessor made during his visit to the farm. Records from 1 to 5 are for the 5 criteria mentioned in the above table, record 6 comprises a summation to calculate the total ANI score and record 7 is a summary description of the farm.

In a farm where all segments of the housing needs an ANI assessment, these have to be made in the worst season (late winter for cattle). In a group of animals or in a maintenance system where conditions vary widely from individual to individual, will be used for evaluation, the conditions affecting worst approximately 25% of the livestock, the detriment of the average group. This will ensure that requirements for welfare of all individuals in that maintenance system are adequate.

2. Welfare Quality® System

The first European protocol to assess the welfare of farm animals: **Welfare Quality ®** was an integrated research project, funded by the European Commission (FP6), Priority axis 5: food quality and safety, involving 44 institutes and universities representing thirteen European countries and four countries from Latin America. The project was conducted over a period of five years, from 2004 to 2009 (12).

One of its main objectives was to develop an integrated and standardized system of assessment of farm animal welfare, respectively for cattle, swine and poultry, all the technological flow (from farm to slaughterhouse). In this European project, researchers have developed some systems for assessing farm animal welfare, based on direct measures, represented by the reaction of the animal. System combines a science-based methodology for assessing farm animal welfare through a standardized way to integrate this information through which to assign farms and slaughterhouses, one of the three categories of wealth.

The developed protocols can be used not only to assess animal welfare, but also to provide feedback and support for producers, thus helping them to benefit from entering markets with higher value. In addition, they will provide traders and consumers clear and reliable information on the status of animal welfare from which food derive (12).

The assessment of welfare must be considered a multidisciplinary process as a variety of different parameters provides a more comprehensive welfare assessment in any given system. Animal welfare is also an important attribute in terms of quality and food safety concept, and consumers expect that animals from which food derive to be grown in conditions resembling their natural environment.

Recent surveys made by the European Commission and studies in Welfare Quality ® project confirm that animal welfare is a matter of considerable importance for European consumers and that the European citizens show a strong commitment to animal welfare (12).

Welfare Quality ® is based on four principles to assess animal welfare: housing, feeding, health and the manifestation of a specific behavior. In these four principles have been identified 12 animal welfare criteria (Table 2). The system was tested in 700 farms from nine European countries (12).

Table 2. Principles and criteria for assessing farm animal welfare developed by *Welfare Quality* ® project (Botreau et al., 2007)

Principles of welfare	Criteria of welfare
Good feeding	Absence of prolonged hunger
	Absence of prolonged thirst
Good housing	Comfort around resting
	Thermal comfort
	Ease of movement
Good health	Absence of injuries
	Absence of diseases
	Absence of pain induced by management procedures
Appropriate behaviour	Expression of social behaviours
	Expression of other behaviours
	Good human-animal relationship
	Positive emotional state

In Romania, **the legal basis** underlying welfare assessment consists of: *Law no. 205/2004 on protection of animals*, with subsequent amendments and supplements (6); *Law no. 9/2008 amending and supplementing Law no. 205/2004 on protection of animals* (7); *Order 72/2005 on Sanitary Veterinary Norm that establishes minimum standards for protection of calves* (8); *ANSVSA Order 75/2005 for approval of Sanitary Veterinary Norm on protection of farm animals* (9); *Order no. 31/2008 approving the Methodological Norms for applying Law no. 205/2004 on protection of animals* (10); *Order no. 13/2008 for approving Sanitary Veterinary Norm on minimum requirements for registration information during the inspections in farms where animals are kept for breeding purposes*.

Following the interpretation of the legal basis for assessing welfare in Romania we can conclude that welfare assessment in dairy cattle is realized after checking the following criteria:

If **staff** is trained and if this can be proved, training frequency, and if it is provided sanitary veterinary assistance.

Inspection of animals that must occur at least once a day, for those which are grown in systems in which their welfare depends on human care. Will be checked the presence of light sources, which should allow inspection of animals. There must be records showing daily inspections, to exist intervention procedures for illness or accident and if there is adequate space to isolate sick or injured animals.

Record keeping - is to verify that documents are adequatelly archived and kept for at least three years, and these contain records of treatments performed and of the number of dead animals.

Freedom of movement - demonstrates that all animals can lie, rest and move, or if animals are permanently linked and therefore the freedom of movement is restricted so that to the animal is causing suffering or injury.

Shelters and housing – it must be checked that: there were used resistant, waterproof materials, which can be easily cleaned and disinfected, if there are any sharp edges or protrusions that can cause injury, if there exist any foreign objects that may endanger animal health; the floor must be smooth, non-slip, rigid, without unevenness, the

bedding must be dry and comfortable in the areas used for isolating animals; there is an adequate and monitored microclimate, referable to air circulation, dust level, the noise, the presence and concentration of pollutants, air temperature values, humidity, ensuring natural light or artificial light; it provides protection from electrical equipment; number of available animals and useful area of the shelter.

Automated equipment necessary for health - is to check the existence of automatic equipment: feeders, drinkers, fans, etc., if these are checked daily, if there are backup systems that can be used in case of failure, and if there are alarm / warning systems in case of failure and if they are regularly inspected.

Feeding and watering – if these are done according to age, weight, physiological requirements and the expression of behavior and if they (feed and water) are readily available and appropriate in terms of quality and quantity. There must exist documents attesting the quality of water and feed, and whether drinking and feeding equipment are protected from contamination. If animals receive other substances, except those used for therapeutic, prophylactic or for treatment goal of livestock.

Mutilations – if animals are subjected to unnecessary mutilation.

Breeding methods – there are practiced natural or artificial methods that cause or may cause pain or harm to the animals involved.

References

1. Bartussek, H., Lenz, V., Ofner-Schrök, E. (2010) – *Adăposturi pentru vite,* (traducere din limba germană după ediţia a IV-a Austria de Mintici Ina), Editura M.A.S.T., Bucureşti.
2. Botreau, R., Veissier, I., Butterworth, A., Bracke M.B.M., Keeling, L.J. (2007). *Definition of criteria for overall assessment of animal welfare.* Animal Welfare: 16, 225–228.
3. Broom, D.M. (1991) – *Animal Welfare: Concepts and Measurement*. Journal of Animal Science: 69, 4167-4175.
4. Decun, M. (1997) – *Cruzimi în tranziţie*. Adevărul, nr. 2147, 14 aprilie, p. 2.
5. Decun, M. (2004) – *Etologia, bunăstarea şi protecţia animalelor*. Editura Mirton, Timişoara.
6. *** – Legea nr. 205/2004 privind protecţia animalelor, Monitorul Oficial Partea I, nr. 531 din 14.06, 2004.
7. *** – Legea 9/2008 pentru modificarea şi completarea Legii nr. 205/2004 privind protecţia animalelor, Monitorul Oficial 29 din 15 ianuarie 2008 (M. Of. 29/2008).
8. *** – Ordinul 72/2005 privind aprobarea Normei sanitare veterinare ce stabileşte standarde minime pentru protecţia viţeilor, Monitorul Oficial 759/2005.
9. *** – Ordinul 75/2005 pentru aprobarea Normei sanitare veterinare privind protecţia animalelor de fermă, Monitorul Oficial 776 din 25 august 2005 (M. Of. 776/2005).
10. *** – Ordinul 31/2008 pentru aprobarea Normelor metodologice de aplicare a Legii nr. 205/2004 privind protecţia animalelor, Monitorul Oficial 511 din 8 iulie 2008 (M. Of. 511/2008).
11. *** – Ordinul 13 din 21 februarie 2008 pentru aprobarea Normei sanitare veterinare privind cerinţele minime pentru înregistrarea de informaţii cu ocazia inspecţiilor în exploataţiile în care animalele sunt ţinute pentru scopuri zootehnice, Monitorul Oficial 219 din 21 martie 2008.
12. *** – Welfare Quality®, Assessment protocol for cattle, 2009.

SIMULTANEOUS DETECTION OF NORTH AMERICAN AND EUROPEAN PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS USING REAL-TIME RT–PCR

Leontina-Smărăndiţa MIHAI (MILEA)[1], Elena VELESCU[1], Carmen SOLCAN[1], Tomasz STADEJEK[2], Mihaela ZĂULEŢ[3], Tahar AIT-ALI[4]

[1]Universitatea de Ştiinţe Agricole şi Medicină Veterinară "Ion Ionescu de la Brad" Iaşi; [2]Institutul Veterinar de Stat Piwet Pulawy Polonia; [3]Universitatea din Bucureşti, Departamentul de Biochimie şi Biologie Moleculară; [4]Institutul Edinburgh, Scoţia UK

marutza85@yahoo.com

Abstract

Porcine reproductive and respiratory syndrome virus, an arterivirus, is the etiologic agent of an infectious disease of that name, characterized by respiratory disorders, abortion in pregnant sows and high mortality in piglets, resulting in significant economic losses in the pig industry worldwide. The disease was first reported in the United States and Canada in 1987 (Keffaber, 1989), in Japan in 1989 (Shimizu et al., 1994) and in Germany in 1990 (Lindhaus & Lindhaus, 1991). Detection of the etiologic agent, PRRS virus, represents a diagnostic challenge due to the heterogeneity of field isolates as well as the propensity for swine to develop persistent infection in which virus is difficult to detect. Since two different types of porcine reproductive and respiratory syndrome virus (PRRSV), the European (EU) and the North American (US) strain, occur or coexist in European swine herds, their rapid and reliable detection and differentiation is essential for disease surveillance. In this study we have used a Real Time reverse transcription-polymerase chain reaction (RT-PCR) for PRRSV detection and strain differentiation in piglets. We obtained positive results for the European strain of PRRSV in one farm with no clinical signs.Acording to this results we can say that the virus is circulating and it is very difficult to determinate the optimal moment for detection.

Keywords: PRRSV, european PRRSV, american PRRSV, Real –Time RT-PCR

Introduction

The clinical signs of PRRS vary with the strain of virus, the immune status of the herd and management factors. Infection may also be asymptomatic. Clinical disease in a herd is a consequence of acute viraemia in individuals and transplacental transmission of virus from viraemic dams to their foetuses, which can occur at any time, though infections in the last third of pregnancy can result in severe disease. Concurrent infections with other pathogens are also common. First step on the clinical signs is on the respiratory tract or equally may start with the reproductive failures.

On piglets, the PRRSV infection is often subclinical. It is however indirect responsible for large economic losses in finishing, due to its important role in pigs Respiratory Disease Complex. If clinical signs appears, they are: fever, sneezing, dyspnea, cough, pneumonia, lethargy, periocular edema.

Matherials and methods

Samples were collected from piglets aged between 11 days and 6 months from three farms and backyards according to the table no 1. We collected also serum samples and tissues from lung, mandibular gland, kidney and liver.

Farm 1 belongs to Moldavian area and the pigs from this farm had no clinical signs, farm 2 is an outbreak of PRRSV which belongs to Wallachia, farm 3 had the disease one year before collection and the pigs from backyard (Moldavian area) also had no clinical signs.

We collected samples from farms with no clinical sign on piglets to see if the virus is circulating and even on this piglets the virus exists. Our purpose was to collect samples from farm where are or not clinical signs of PRRS disease, because due to this diseases associated syndroms sometimes the diagnostic of PRRSV is a laboratory surprise. All samples were collected from unvaccinated pigs.

Table 1. Samples used for the experiment

Farm	No pigs	Samples	Age
1	46	Serum	6 months
2	19	Lung, kidney, liver	11 -14 days and 104-180 days
3	3	Serum, lung, mandibular gland, kidney	2-4 months
Backyard	3	Serum	3-4 months

To isolate the RNA we have used the Magna Pure LC Total Nucleic Acid Isolation Kit ROCHE (Fig 1) which can perform the simultaneous extraction of the DNA and RNA. This kit is only available for the MagnaPure LC Instruments and has the advantage that can extract both in the same tube. The volume of each sample for extraction was of 150-200µl.

After extraction, we performed the Real-Time RT-PCR using the NextGen Real-Time RT-PCR Target Specific Reagents for the identification & Differentiation of North American & European PRRSV Viral RNA kit from TETRACORE (Fig.2) according to the manufacturer instructions.

Fig. 1. Magna Pure LC Total Nucleic Acid Isolation Kit ROCHE

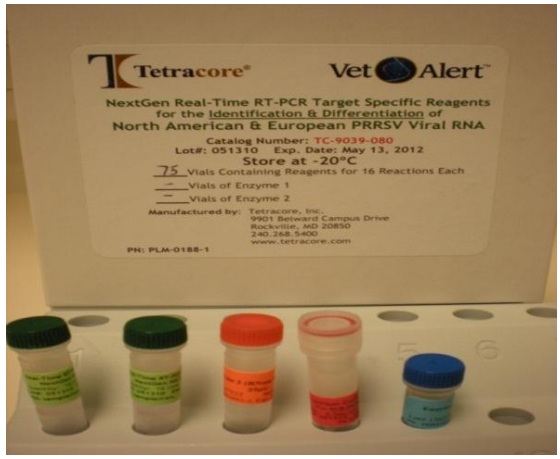


Fig. 2 NextGen Real-Time RT-PCR Target Specific Reagents for the identification & Differentiation of North American & European PRRSV Viral RNA KIT

The volume of the mix for each reaction was of 21 µl (18.25 µl-mix, 0.5µl RNase – free water, 2µl Enzyme 1 and 0.25µl Enzyme 2) and the volume of the sample was of 4 µl.

The thermal cycling protocol was: *Stage 1*: 52 °C for 30 minutes, *stage 2* : 95°C for 15 minutes , *stage 3*: 40 cycles (3-step PCR) {step 1: 95°C for 15 seonds, step 2: 50°C for 30 seconds and step 3: 61°C for 45 seconds (collection data step)}.

To perform this test we have used the Sratagene MX3005P instruments and the setting were: CY3x8, FAMx8 and CY5x1. The positive control was included in the kit.

To differentiate the Amererican vs European PRRSV, is necessary to look in the table bellow (Table no. 2) –applicable only if use IC (Inhibition control) instead of water.

Table 2. PRRSV differentiation results table

Indications	**FAM**	**CY3**	**CY5***
PRRSV not present in sample	Negative	Negative	Positive
NA PRRSV present in sample	Positive	Negative	Positive
EU PRRSV present in sample	Positive/Negative	Positive	Positive

Results and discussions

We have tested 71 piglets samples and 8 (11.26%) were positive for the European strain. The values are exposed in the table below.

From 46 pigs belongs to the Moldavian farm with no clinical sign for PRRSV 4 (8.69%) were positive. From 19 pigs samples from outbreak one (5.26%) was positive and from farm 3 also one was positive.

This results are interesting to interpret because we detected positive results were we did not expected. It is possible that the positive results detected on farm 1 to show a new beginning of another outbreak.

Table 3. Result of the test

Farm	Sample	Aged	CY3 Ct	FAM Ct
1	Serum	6 months	36.83	No ct
1	Serum	6 months	37.28	No ct
1	Serum	6 months	39.24	No ct
1	Serum	6months	37.51	No ct
2	Lung	6 months	33	30.35
3	Serum	2 months	25.6	32.25
	Lung		23.9	28.28
	Mandibular gland		26.47	32.4
Positive control EU PRRSV	-	-	23.89	25.22
Positive control NA PRRSV	-	-	-	17.56

Conclusions

1. The kit NextGen Real-Time RT-PCR Target Specific Reagents for the identification & Differentiation of North American & European PRRSV Viral RNA helped us to identify the European PRRS virus on the pigs that we have tested. The positive results from farm 1 show a beginning of a new outbreak and the positive signal from farm 3 proves that the disease have there still traces. The positive result from farm 2 is saying that the disease is there the measure of control are required.
2. We can say that the virus is circulating and is very hard to identify the moment of the viremia, but this kind of test can help us to have a clear image of the immune status of the pigs**.**

Bibliography

1. Ait-Ali, T. et al., 2007. Innate immune response to the replication of the porcine reproductive and respiratory syndrome virus in isolated Swine alveolar macrophages. Viral Immunology 20: 105-118.
2. Drolet R., R.Larochelle, M.Morin, B. Delisle R.Magar,2003- Detection Rates of Porcine Reproductive and Respiratory Syndrome Virus, Porcine Circovirus Type2,and Swine Influenza Virus in Porcine Proliferative and Necroziting Pneumonia, Vet Pathol 40: 143-148. www.vet.sagepub.com
3. Halbur PG, Paul PS, Frey ML, Landgraf J, Eernisse K, Meng XJ, Lum MS, Andrews JJ, Rathje JA, 1995 - Comparison of the pathogenicity of two US porcine and reproductive and respiratory syndrome virus isolates to that of the Lelystad virus. Vet Pathol. 32:648-660.
4. Magar R, Carman S, Thomson G, Larochelle R, 1994 – Porcine reproductive and respiratory syndrome virus identification in proliferative and necrotizing pneumonia cases from Ontario. Can Vet J 35:523–524.
5. Peijsak, Z., Stadejek, T. & Markowska-Daniela, I. (1997) Clinical signs and economic losses caused by porcine reproductive and respiratory syndrome virus in a large breeding farm. Veterinary Microbiology 55, 317-322;
6. Rossow KD, 1998 - Porcine reproductive and respiratory syndrome.Vet Pathol 35:1–20.
7. Velescu Elena Patologia bolilor infecţioase la animale, Ed. Terra Noastra, Iaşi, 2002.

USING TWO SETS OF PRIMERS TO IDENTIFY AND DIFFERENTIATE PCV1 AND PCV2 FROM THE SAME SAMPLES WITH CLASSICAL PCR ASSAY

Leontina-Smărăndiţa MIHAI (MILEA)[1], Elena VELESCU[1], Carmen SOLCAN[1], Tomasz STADEJEK[2], Mihaela ZAULEŢ[3] Tahar AIT-ALI[4]

[1]Universitatea de Ştiinţe Agricole şi Medicină Veterinară "Ion Ionescu de la Brad" Iaşi; [2]Institutul Veterinar de Stat Piwet Pulawy Polonia; [3]Universitatea din Bucureşti, Departamentul de Biochimie şi Biologie Moleculară; [4]Institutul Edinburgh, Scoţia UK; marutza85@yahoo.com

Abstract

PCV1 (non-pathogenic) and PCV2 (pathogenic) belong to the Circoviridae Family and they share less than 80% nucleotide sequence identity (Fenaux et al., 2000; Hamel et al., 1998; Meehan et al., 1998). The genomic organization of both PCV1 and PCV2 are quite similar, containing two major open reading frames (ORFs), ORF1 and ORF2. In this study, we have tested pigs samples – from two farms belong to Moldavia and Muntenia areas – for PCV1 and PCV2 viruses. We have used two sets of primers that helped us to identify and amplify the common part of PCV1 and PCV2 and after that only the PCV2 to see exactly where is the pathogenic circovirus. We obtained 49.23 % positive for PCV1 and PCV2 and 4.61% positive samples for pathogenic PCV2. All the positive results for PCV2 were also positive for PCV1, so we can say that where is PCV2 can be PCV1, but reverse is not mandatory.

Key words: PCV1, PCV2, primers , PCR

Introduction

Porcine circoviruses PCV1 and PCV2, are small, non-enveloped viruses with a single-stranded DNA genome, morphologically similar but genetically distinct one from each other. PCV1 was discovered as a non-pathogenic contaminant of the continuous PK-15 cell line while PCV has been identified in most swine producing countries worldwide. The disease has resulted in significant health challenges and economic damage to the swine industry.

The non-pathogenic PCV1 and the pathogenic PCV2 share less than 80% nucleotide sequence identity (Fenaux et al., 2000; Hamel et al., 1998; Meehan et al., 1998). The genomic organization of both PCV1 and PCV2 are quite similar, containing two major open reading frames (ORFs), ORF1 and ORF2. ORF1 of PCV1 and PCV2 encodes two replication associated proteins Rep and Rep' while ORF2 encodes a viral capsid protein. The viral capsid is a major immunogenic protein of PCV2. In addition, a third open reading frame was identified in PCV2 and may be involved in apoptosis.

Sequence analyses of the complete genomes of PCV-2 isolates showed that the complete genome of each PCV2 isolate is 1,767 to 1,768 bp in length (Fenaux et al., 2000; Mankertz et al., 2000).

Materials and methods

Samples were collected from two farms belong to Moldavia (named Farm1) and Muntenia (Farm 2) areas. From farm 1 we have collected 46 serum samples from pigs with no clinical signs for PCV2, aged 6 months. The 19 tissues samples collected from farm 2

(clinical signs presents) are from lung, liver and kidney, aged between 11 days and 6 months (Table no.1). All samples were collected from unvaccinated pigs.

Table 1. Samples used for the experiment

Farm	***No Pigs***	***Sample***	***Age***
1	*46*	*Serum*	*6 months*
2	*14*	*Lung*	*6 months*
	1	*Lung*	*14 days*
	1	*Liver*	*14 days*
	1	*Liver*	*104 days*
	1	*Kidney*	*11 days*
	1	*kidney*	*104 days*
Total	*65*	-	-

To isolate the RNA we have used the Magna Pure LC Total Nucleic Acid Isolation Kit ROCHE (Fig 1) which can perform the simultaneous extraction of the DNA and RNA. This kit is only available for the MagnaPure LC Instruments and has the advantage that can extract both DNA and RNA in the same tube. The volume of each sample for extraction was of 150-200µl.

After extraction, we performed classical PCR reaction using the MCV1, MCV2 primers to detect the porcine Circovirus, because this primers can amplify the the identic part from the genom of the two viruses. After this we tested the same samples only for PCV2 using the CORF2A, CORF2B primers. All the primers (Table no.2) were ordered from Invitrogen.

Fig.1 Magna Pure LC Total Nucleic Acid Isolation Kit ROCHE

The volume of the mix for each reaction was of 25 µl (12.3µl MilliQ, 2.5µl 10x Buffer $MgCl_2$, 2.5µl dnTP, 1.25µl primer, 1.25 µl primer,0.20 µl Taq polymerase and 5 µl of DNA sample).

The thermal cycling protocol was : Stage 1: 95 ° for 5 min, Stage 2 -35cycles-(3-step PCR) {step 1: 95°C for 30 seconds, step 2: 55°C for 30 seconds and step 3: 72°C for 45 seconds}, Stage 3 : 72° for 7 seconds and Stage 4 : 4 °- ∞.

Table 2. The name, sequences and the specificity of the primers used in this study

Primer name	Primer sequence	Specificity
MCV1°	5'- *GCT GAA CTT TTG AAA GTG AGC GGG*-3'	PCV1, PCV2
MCV2°	5'-*TCA CAC AGT CTG AGT AGA TCA TCC CA*-3'	PCV1, PCV2
CORF2A°°	5'-*ACG TAT CCA AGG AGG CGT*-3'	PCV2
CORF2B°°	5'-*ACG TAT CCA AGG AGG CGT*-3'	PCV2

° Fenaux şi col., 2000. °° Replication and transmission of porcine circovirus type 2 in mice. Cságola A, Cadar D, Tuboly T. Acta Vet Hung. 2008 ;56:421-7; nt: nucleotide

Results and discution

We tested all samples with MCV1, MCV2 primers and we obtained 32 positive results, 14 from farm no 1 and 18 from farm no 2. At that moment we couldn't say if is present PCV1 or PCV2, so we tested all this result for PCV2. The size of the amplicons is 250bp (Fig. no 2).

Table 3. Samples used and results

Farm	***No Pigs***	***Sample***	***Age***	***Results***	
				Positive PCV1/PCV2	***PCV2***
1	*46*	*Serum*	*6 months*	*14*	-
2	*14*	*Lung*	*6 months*	*13*	*1*
	1	*Lung*	*14 days*	*1*	-
	1	*Liver*	*14 days*	*1*	-
	1	*Liver*	*104 days*	*1*	*1*
	1	*Kidney*	*11 days*	*1*	-
	1	*kidney*	*104 days*	*1*	*1*
Total	*65*	-	-	*32*	*3*

From 32 positive results (49.23%) for both viruses, only 3 (4.61%) were positive for PCV2 using specific primers . The size of amplicons is about 760bp (Fig. no3). The pigs infected with pathogenic PCV2 belong to farm 2 (Table no 3).

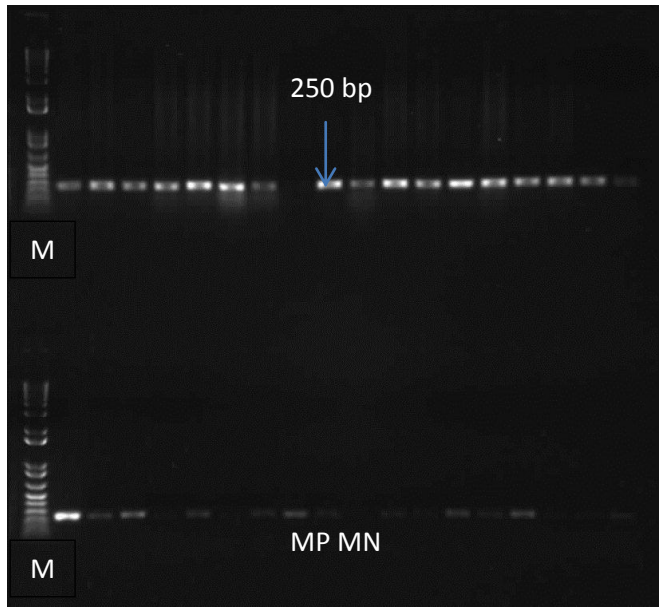


Fig. 2. Electrophoretic profile of DNA amplicons obtained using MCV1-MCV2 primer pair for detection of common sequence of PCV1 and PCV2. MP- positive control; MN – negative control; M - marker 1kb

Testing pigs belong to farm 1, we observed positive results for common part of PCV1 and PCV2, and negative results for PCV2. We can say that in that farm is present only PCV1, the non pathogenic circovirus.

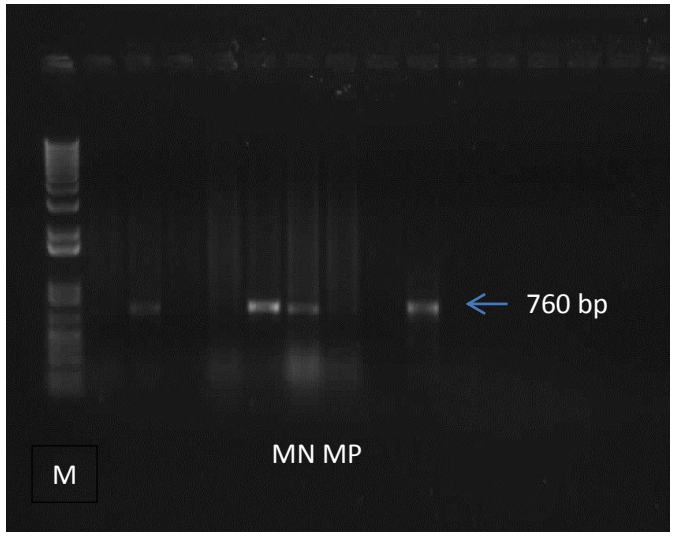


Fig. 3. Electrophoretic profile of DNA amplicons obtained using primer pair CORF2A-CORF2B for detection of specific sequence of PCV2. MP- positive control; MN – negative control; M- marker 1kb

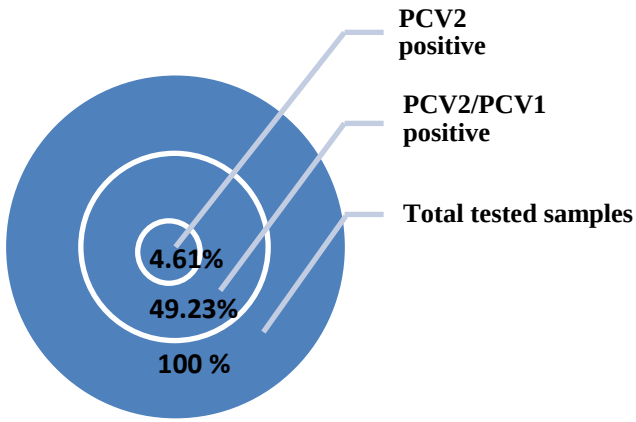


Fig. 4. Percentage representation of the positive results

Because pigs from farm no 2 with clinical signs, showed positive results, we can say that all our suspicions confirmed PCV2 infection in that farm. All positive results for PCV2 infections obtained were also positive for PCV1, so we can say that if we have PCV2 infection it is possible to find also PCV1, but not reverse.

Conclusions

1. PCV1 is common in farms were are or not PCV2, while PCV2 infection can be seen by clinical signs ;
2. The presence of PCV1 is not dependent of the presence of PCV2 ;
3. All positive results for PCV2 infections obtained were also positive for PCV1, so we can say that if we have PCV2 infection it is possible to find also PCV1, but not reverse .

Bibliography

1. Allan, G.M., Ellis, J.A., 2000. Porcine circoviruses: a review. J. Vet.Diagn. Invest. 12, 3–14.
2. Allan, G.M., McNeilly, F., Kennedy, S., Daft, B., Clark, E.G., Ellis, J.A., Haines, D.M., Meehan, B.M., Adair, B.M.1998. Isolation of porcine circovirus-like viruses from pigs with a wasting disease in the USA and Europe.J. Vet. Diagn. Invest.10,3.10.
3. Cadar D - PhD Thesis.
4. Hines RK, Lukert PD: Porcine circovirus as a cause of congenital tremors in newborn pigs. In: Proc Am Assoc Swine Pract. Chicago, Illinois. 25:344-345, 1994
5. John C.S. Harding- The clinical expression and emergence of porcine circovirus 2. Veterinary Microbiology Vol.98(2004), Pag 131-135; www.sciencedirect.com
6. Meredith, M. (2003), PMWS and Porcine Circovirus- New Research www.aasv.org/news/story.php?id=457.
7. Opriessnig, T., Anderson, M.S., Rothschild, M.F., Evans, R.B., Fenaux, M., Meng, X.J., Halbur, P.G. (2004), Proceedings Int. Pig Vet. Society, 18th Congress, 27.06-1.07, 1, 12.
8. Opriessnig T. J.R.Prickett, D.M. Madson, J.K. Lunney, D. Kuhar, J, Elsener, P. G. Halbur Effect of PCV2 vaccination on PRRSV and PCV2 coinfection, http://www.pigprogress.net.
9. Stevenson GW, Kiupel M, Mittal SK, Kanitz CL: Ultrastructure of porcine circovirus in persistently infected PK-15 cells. Vet Pathol 36:368–378, 1999
10. Velescu Elena Patologia bolilor infecţioase la animale, Ed. Terra Noastra, Iaşi, 2002.

TYPING OF C. JEJUNI ISOLATES FROM POULTRY USING MULTILOCUS SEQUENCE TYPING IN ROMANIA

Mihai OBADA, *Carmen CRETU, Alina Vlad SABIE, Mihai CARP-CARARE
Public Health Department, University of Agricultural Sciences and Veterinary Medicine, Iasi, 700489, Romania
omishud@yahoo.com, carmenccretu@yahoo.es, alinasabie@yahoo.com, mihai_carpcarare@yahoo.com

Abstract
Campylobacter jejuni may be isolated from human faeces and foods which are likely to be important in the transmission of Campylobacter. In Campylobacter jejuni isolated from humans and poultry in Romania, multilocus sequence typing (MLST) identified two sequence types : ST 21 and ST 45. All poultry isolates had groEL and hipO genes, which are optimized to detect the majority of Campylobacter spp., especially the identification of C. jejuni in humans and animals. (Wang et al., 2002). Gentic diversity was observed within the Campylobacter jejuni isolates originating from poultry, and MLST results suggested that strains were the same to strains found in humans. This suggests that there may be common sources of infection for both humans and poultry and that poultry remain a potential zoonotic risk to humans. The data implicates poultry in Romania as a potential reservoir for human infection with Campylobacter jejuni
Keywords *:* molecular epidemiology, Campylobacter, poultry

Introduction

Campylobacter spp. are amongst the most commonly reported bacterial cause of human gastroenteritis in the UK and worldwide (Adak et al., 2002; CDC., 2008c; DEFRA, 2007; Humphrey et al., 2007; Westrell et al., 2009). *Campylobacter spp.* are zoonotic bacteria that are often found in the intestine of many animal species (Brown et al., 2004; DEFRA, 2007; Wilson et al., 2008; Workman et al., 2005). In some hosts, these bacteria can cause symptoms such as diarrhoea, but in others it can remain asymptomatic (Acke et al., 2009; Feodoroff et al., 2009; Guest et al., 2007; Jenkin and Tee, 1998; Leblanc Maridor et al., 2008; Rossi et al., 2008; Smith et al., 2008). Transmission of *Campylobacter* is most frequently through contaminated food, particularly poultry meat (Humphrey et al., 2007; Hussain et al., 2007; Sheppard et al., 2009; Wilson et al., 2008). A recent report by the Food Standards Agency found that there was a *Campylobacter spp.* prevalence of 65.2% in retail chicken in the UK (FSA, 2009) and other studies have also found *C. jejuni* in chickens and/or raw poultry (Hussain et al., 2007; Little et al., 2008; Stoyanchev et al., 2007).

Although certain species, in particular *Campylobacter jejuni* and *Campylobacter coli*, are frequently associated with human foodborne campylobacteriosis throughout the world, other species vary in their prevalence from country-to country or indeed year-to-year. Although there is evidence to suggest that isolates of *Campylobacter jejuni* are pathogenic and may be isolated from human stools, it has not yet been linked to any poultry foodstuffs. Indeed in Romania, little is known about the relationship between *Campylobacter* isolated from human faecal samples and their food sources. Assessing the risk of poultry as reservoirs or vehicles of transmission of *Campylobacter* infection in all countries is key to understanding the zoonotic pathways of human campylobacteriosis and the implementation of control strategies.

MLST is a method for characterizing microbial isolates by means of sequencing housekeeping genes and has been used to determine the population structure of many bacterial pathogens, including *Campylobacter jejuni* (Colles et al., 2003; Dingle et al., 2002; French et al., 2005; Ragimbeau et al., 2008; Sopwith et al., 2008) Although it was designed primarily for global epidemiology and surveillance, MLST has the advantages that datasets are highly reproducible and can be shared over the internet without the need for exchanging live cultures. Fragments of 400-500 bp length, of usually between six and eight loci, are sequenced, with each unique fragment sequence assigned an allele marker. Each allelic combination, or profile, is then assigned a sequence type (ST) number (Jolley et al., 2004)

The aim of this study was to observe the genetic diversity of different strains of *Campylobacter jejuni* isolated from human faecal and poultry from Romania.

Material and methods

Twelve Romanian isolates of *Campylobacter jejuni* were included in this study. The isolates were collected by the Infectious Disease Hospital Iasi, Romania and the Microbiology Laboratory at the University of Agricultural Sciences and Veterinary Medicine, Iaşi (U.S.A.M.V.). All isolates were initially identified using standard biochemical tests by Institute Cantacuzino (I.C), Institute for Hygiene and Veterinary Public Health (I.I.S.P.V.), Iasi, Romania. Strain numbers 1, 2 were isolated from human stools (faeces), whilst strains 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 were isolated from food (poultry) (Table 1).

DNA extraction and MLST analysis

The 12 isolates were cultured on mCCDA agar at 42ºC overnight and chromosomal DNA was extracted using PrepMan Ultra sample preparation reagent, according to the manufacturer's instructions (PrepMan Ultra, Applied Biosystems).

MLST was performed on the DNA extracted from all 12 isolates. The sequences of the 7 selected housekeeping genes (Table 4) were amplified by PCR using primers published on the Campylobacter MLST database online (http://pubmlst.org/campylobacter//). The reaction mixture was the same for each gene and included 5µl 10x buffer, 3µl 25mM MgCl2, 1µl each of dATP, dCTP, dGTP and dTTP, 1µl each of the forward and reverse primers, 0.25µl Taq polymerase (Invitrogen) and 2µl of the DNA template to give a final reaction volume of 50µl. PCR conditions included an initial denaturation at 95°C for 2 minutes, 35 cycles of 94°C for 2 minutes, 50°C for 1 minute and 72°C for 1 minute and a final incubation at 72°C for 5 minutes. PCR products were run on a 2% agarose gel in tris-acetate buffer (TAE) buffer and visualized under ultra violet (UV) light. PCR products were sequenced and the sequence of each DNA strand was determined and assembled from the chromatograms by using Sequencer software (ChromosPro Version 1.5.). Allele numbers were assigned and converted to sequence type (ST) after the sequences were submitted to the Campylobacter MLST database .

PCR procedure

The PCR procedures were based on a protocol previously optimised, particularly for the partial groEL gene (Karenlampi et al., 2004; Westgarth et al., 2009), consisting of 22 µl Master mix 2.5mM MgCL according to the manufacturers instructions (ABgene™), with the primers as in Table 3. Primers were made to a concentration of 15 picomolars per microlitre, and added at a volume of 1µl each for all PCR assays, including groEL and hipO encoding gene, and 24 µl of Master mix with primers was added to each reaction, with 1 µl DNA, resulting in a 25 µl reaction. The species specific PCR assays, i.e. groEL and hipO each

consisted of 22 µl 1.1x reddyMix™ PCR Master Mix (1.5mM MgCl2) according to the manufacturers instructions (ABgene™), with their specific primers (1µl each) as in Table 3. The cycling parameters for all assays in Table 3 included an initial denaturation at 95°C for 2 minutes, 40 amplification cycles with denaturation at 95°C for 1 minute, annealing at 50°C for 1 minute, extension at 72°C for 3 minutes and a final incubation at 72°C for 5 minutes. The PCR products were separated by gel electrophoresis in a 2% agarose gel in TAE buffer. Ethidium bromide was added to the agarose, and the gel was visualized under UV light. Amplicon size was determined by comparison with ΦX 174 Hae III Digest DNA marker (ABgene, Epsom, UK). A previously characterized isolated *Campylobacter jejuni*, was included as a control for comparison.

Results and discussion

All poultry isolates had groEL and hipO genes, which are optimized to detect the majority of Campylobacter spp., especially the identification of C. jejuni in humans and animals. (Wang et al., 2002).

Twelve isolates were assigned to two different sequence types (Table 2). Overall, ST-45 was the most common sequence type (8 isolates) identified in the poultry (7 isolates) and humans (1 isolate), followed by sequence type ST-21 (4 isolates) identified in the poultry (3 isolates) and humans (1 isolate). The sequence types contained isolates originating from both humans (2 isolates) and poultry (10 isolates), and this similarity between human and poultry derived isolates was supported by M.L.S.T. The majority of sequence types found in poultry meat were the same as those reported in humans, including some of the most frequently isolated complexes in humans i.e. ST-45, ST-21, ST-22, ST-257 and ST-206, (Dingle et al., 2002; Duim et al., 2003; Jolley and Chan, 2004; Levesque et al., 2008; Ragimbeau et al., 2008; Sopwith et al., 2006, 2008). The most commonly identified sequence type in poultry was ST-45.

Multi-locus sequence typing, reveals that certain C. jejuni sequence types are more common than others in humans and certain animal hosts. In Humans, ST-21 and ST-45 tend to predominate. They are both found in poultry and cattle, although ST-45 is often associated with the environment (French et al., 2005; Sopwith et al., 2008).

Complexes displayed according to the frequency of isolation from humans in various studies (Colles et al., 2003; Dingle et al., 2002;; Duim et al., 2003; Karenlampi et al., 2007; Ragimbeau et al., 2008), i.e. on average ST-21 appears to be the most commonly isolated complex from humans overall.

There are various possible sources from which poultry might acquire sequence type ST-45. This sequence type has been isolated from a range of sources such as water, wild birds, cattle, sheep, rabbits, badgers, turkey chicks, broiler chicks, and soil as well as humans (Colles et al., 2003; French et al., 2005; Ragimbeau et al., 2008). Sopwith et al, (2008) found that ST-45 was the most commonly isolated sequence type from water, and suggested that it might be better adapted to survive outside a host, and thus might be crucial in the transmission of *C. jejuni* throughout the environment. Interestingly, open drains, and possibly lakes have been associated with *Campylobacter spp*. carriage in poultry (Baker et al., 1999; Wieland et al., 2005) and the ST-45 complex isolated from humans has also been significantly associated with contact with pet cats and dogs (Karenlampi et al., 2007). This may indicate common sources of infection for humans and poultry, or possibly that poultry may act as conduits of infection from the environment to humans.

Conclusion

Risk of *Campylobacter* infection in humans has been associated with many sources including poultry. This study aimed to investigate whether or not *Campylobacter jejuni* strains identified in poultry were distinguishable or not from strains identified in humans, and if there were possible common sources of *Campylobacter jejuni* infection for both humans and poultry.

MLST together with PCR virulotyping, were both used to analyse 12 *Campylobacter jejuni* isolates obtained from various poultry and humans

All poultry isolates had groEL and hipO genes, which are optimized to detect the majority of Campylobacter spp., especially the identification of C. jejuni in humans and animals. (Wang et al., 2002).

MLST data suggested that there was a large amount of genetic diversity amongst poultry *Campylobacter jejuni* isolates, and that the majority of sequence types found in poultry were the same as those reported in humans. This suggested that poultry are exposed to various sources of *Campylobacter jejuni* infection and the similarity of these sequence types to *Campylobacter jejuni* isolated from humans suggests there may be transmission, or common sources of infection for both poultry and humans. Strains of *Campylobacter jejuni* isolated from humans did not appear to group separately from poultry strains, indicating common sources of infection, or possible transmission. Although only a small number of poultry may carry *C. jejuni*, infected poultry should still be considered a potential zoonotic risk to humans.

Poultry remain a possible zoonotic risk to humans for *Campylobacter jejuni*, however, further work is needed to investigate the frequency, and severity of *C. jejuni* infection in humans.

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Table 1. Identifying virulence genes in Romanian Campylobacter strains

PCR virulotyping				**Genes screened for**	
Isolate no	Species from which derived	Pathological material	*Campylocter species*	*groEL*	*hipO*
1.	Human	faeces	Campylobacter jejuni	+	+
2.	Human	faceces	Campylobacter jejuni	+	+
3.	Chicken	Poultry meat	Campylobacter jejuni	+	+
4.	Chicken	Poultry meat	Campylobacter jejuni	+	+
5.	Chicken	Poultry meat	Campylobacter jejuni	+	+
6.	Chicken	Poultry meat	Campylobacter jejuni	+	+
7.	Chicken	Poultry meat	Campylobacter jejuni	+	+
8.	Chicken	Poultry meat	Campylobacter jejuni	+	+
9.	Chicken	Poultry meat	Campylobacter jejuni	+	+
10.	Chicken	Poultry meat	Campylobacter jejuni	+	+
11.	Chicken	Poultry meat	Campylobacter jejuni	+	+
12.	Chicken	Poultry meat	Campylobacter jejuni	+	+

Table 2. Multi-locus sequence typing (MLST) allelic profiles and sequence types (ST) of twelve *Campylobacter jejuni* strains detected in human faeces and poultry meat from Romania. Allele numbers and sequence types were assigned by comparison with those held on the public MLST profile database (http://pubmlst.org/campylobacter//). The source of the isolates is also given

Source				**Allelic profile**							**Sequence type**
Isolate no	Species from which derived	Pathological material	Serotype	aspA	glnA	gltA	glyA	pgm	tkt	uncA	
1.	Human	Faeces	*Campylobacter jejuni*	2	1	1	3	2	1	5	ST-21
2.	Human	Faeces	Campylobacter jejuni	4	7	10	4	1	7	1	ST 45
3.	Chicken	poultry meat	Campylobacter jejuni	2	1	1	3	2	1	5	ST 21
4.	Chicken	poultry meat	Campylobacter jejuni	4	7	10	4	1	7	1	ST 45
5.	Chicken	poultry meat	Campylobacter jejuni	4	7	10	4	1	7	1	ST 45
6.	Chicken	poultry meat	Campylobacter jejuni	4	7	10	4	1	7	1	ST 45
7.	Chicken	poultry meat	Campylobacter jejuni	2	1	1	3	2	1	5	ST 21
8.	Chicken	poultry meat	Campylobacter jejuni	2	1	1	3	2	1	5	ST 21
9.	Chicken	poultry meat	Campylobacter jejuni	4	7	10	4	1	7	1	ST 45
10.	Chickem	poultry meat	Campylobacter jejuni	4	7	10	4	1	7	1	ST 45
11.	Chicken	poultry meat	Campylobacter jejuni	4	7	10	4	1	7	1	ST 45
12.	Chicken	poultry meat	Campylobacter jejuni	4	7	10	4	1	7	1	ST-45

Table 3. Primers used for Campylobacter spp. Identification

Species	Genes	Primers	Sequence (5'-3')
Campylobacter Jejuni	*HipO*	HipoF (primer sens)	ACTTCTTTATTGCTTGCTGC
		HipoR (primer antisens)	GCCACAACAAGTAAAGAAGC
***Campylobacter* spp.**	*GroEL*	M13H60F (primer sens)	GGNGAYGGNACNACNACNGCNAC NGT
		T7H60R (primer antisens)	TCNCCRAANCCNGGNGCYTTNACNG

Table 4. Primers used for the amplification of C. jejuni alleles for MLST

Locus	Primer	Sequence (5'-3')
asp	aspA9(forward)	AGTACTAATGATGCTTATCC
	aspA10(reverse)	ATTTCATCAATTTGTTCTTTGC
gln	glnA1(forward)	TAGGAACTTGGCATCATATTACC
	glnA2(reverse)	TTGGACGAGCTTCTACTGGC
glt	gltA1(forward)	GGGCTTGACTTCTACAGCTACTTG
	gltA2(reverse)	CCAAATAAAGTTGTCTTGGACGG
gly	glyA1 (forward)	GAGTTAGAGCGTCAATGTGAAGG
	glyA2(reverse)	AAACCTCTGGCAGTAAGGGC
pgm	pgmA7(forward)	TACTAATAATATCTTAGTAGG
	pgmA8(reverse)	CACAACATTTTTCATTTCTTTTTC
tkt	tkt(A3)	GCAAACTCAGGACACCCAGG
	tkt(A6)	AAAGCATTGTTAATGGCTGC
unc	uncA7(forward)	ATGGACTTAAGAATATTATGGC
	uncA2(reverse)	GCTAAGCGGAGAATAAGGTGG

References

1. Acke, E., McGill, K., Golden, O., Jones, B.R., Fanning, S., Whyte, P., 2009. Prevalence of thermophilic Campylobacter species in household cats and dogs in Ireland. Vet Rec 164, 44-47.
2. Adak, G.K., Long, S.M., O'Brien, S.J., 2002. Trends in indigenous foodborne disease and deaths, England and Wales: 1992 to 2000. Gut 51, 832-841.
3. Baker, J., Barton, M.D., Lanser, J., 1999. Campylobacter species in cats and dogs in South Australia. Aust Vet J 77, 662-666.
4. Brown, P.E., Christensen, O.F., Clough, H.E., Diggle, P.J., Hart, C.A., Hazel, S., Kemp, R., Leatherbarrow, A.J., Moore, A., Sutherst, J., Turner, J., Williams, N.J., Wright, E.J., French, N.P., 2004. Frequency and spatial distribution of environmental Campylobacter spp. Appl Environ Microbiol 70, 6501-6511.
5. Bryony Nicole Parsons, 2010. The Epidemiology of Campylobacter Infection in Dogs in the Context of the Risk of Infection to Humans, Department of Veterinary Pathology, University of Liverpool.
6. Centers for Disease Control Prevention (CDC), 2008c. Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food--10 states, 2007. MMWR Morb Mortal Wkly Rep 57, 366-370.
7. Colles, F.M., Jones, K., Harding, R.M., Maiden, M.C., 2003. Genetic diversity of Campylobacter jejuni isolates from farm animals and the farm environment. Appl Environ Microbiol 69, 7409-7413.
8. DEFRA, 2007. Zoonoses Report United Kingdom 2007. http://www.defra.gov.uk/foodfarm/farmanimal/diseases/atoz/zoonoses/documents/reports/zoonoses2007.pdf
9. Dingle, K.E., Colles, F.M., Ure, R., Wagenaar, J.A., Duim, B., Bolton, F.J., Fox, A.J., Wareing, D.R., Maiden, M.C., 2002. Molecular characterization of Campylobacter jejuni clones: a basis for epidemiologic investigation. Emerg Infect Dis 8, 949-955.
10. Duim, B., Godschalk, P.C., van den Braak, N., Dingle, K.E., Dijkstra, J.R., Leyde, E., van der Plas, J., Colles, F.M., Endtz, H.P., Wagenaar, J.A., Maiden, M.C., van Belkum, A., 2003. Molecular evidence for dissemination of unique Campylobacter jejuni clones in Curacao, Netherlands Antilles. J Clin Microbiol 41, 5593-5597.
11. Feodoroff, F.B., Lauhio, A.R., Sarna, S.J., Hanninen, M.L., Rautelin, H.I., 2009. Severe diarrhoea caused by highly ciprofloxacin-susceptible Campylobacter isolates. Clin Microbiol Infect.
12. Food Standards Agency (FSA), 2009. UK Survey of Campylobacter and Salmonella in Fresh,,Chicken.http://www.thepoultrysite.com/articles/1530/uk-survey-of-Campylobacter-and-Salmonella-in-fresh-chicken.
13. French, N., Barrigas, M., Brown, P., Ribiero, P., Williams, N., Leatherbarrow, H., Birtles, R., Bolton, E., Fearnhead, P., Fox, A., 2005. Spatial epidemiology and natural population structure of Campylobacter jejuni colonizing a farmland ecosystem. Environ Microbiol 7, 1116-1126.
14. Guest, C.M., Stephen, J.M., Price, C.J., 2007. Prevalence of Campylobacter and four endoparasites in dog populations associated with Hearing Dogs. J Small Anim Pract 48, 632-637.
15. Humphrey, T., O'Brien, S., Madsen, M., 2007. Campylobacters as zoonotic pathogens: a food production perspective. Int J Food Microbiol 117, 237-257.
16. Hussain, I., Shahid Mahmood, M., Akhtar, M., Khan, A., 2007. Prevalence of Campylobacter species in meat, milk and other food commodities in Pakistan. Food Microbiol 24, 219-222.
17. Jenkin, G.A., Tee, W., 1998. Campylobacter upsaliensis-associated diarrhea in human immunodeficiency virus-infected patients. Clin Infect Dis 27, 816-821.
18. Jolley, K., Chan, M., 2004. Campylobacter jejuni and Campylobacter coli Multi Locus Sequence Typing website (http://pubmlst.org/Campylobacter /) developed by Keith Jolley and Man-Suen Chan and sited at the University of Oxford
19. Jolley, K.A., Chan, M.S., Maiden, M.C., 2004. mlstdbNet - distributed multi-locus sequence typing (MLST) databases. BMC Bioinformatics 5, 86.
20. Karenlampi, R., Rautelin, H., Schonberg-Norio, D., Paulin, L., Hanninen, M.L., 2007. Longitudinal study of Finnish Campylobacter jejuni and C. coli isolates from humans, using

multilocus sequence typing, including comparison with epidemiological data and isolates from poultry and cattle. Appl Environ Microbiol 73, 148-155.
21. Karenlampi, R.I., Tolvanen, T.P., Hanninen, M.L., 2004. Phylogenetic analysis and PCR-restriction fragment length polymorphism identification of Campylobacter species based on partial groEL gene sequences. J Clin Microbiol 42, 5731-5738.
22. Leblanc Maridor, M., Denis, M., Lalande, F., Beaurepaire, B., Cariolet, R., Fravalo, P., Federighi, M., Seegers, H., Belloc, C., 2008. Experimental infection of specific pathogen-free pigs with Campylobacter: excretion in faeces and transmission to noninoculated pigs. Vet Microbiol 131, 309-317.
23. Levesque, S., Frost, E., Arbeit, R.D., Michaud, S., 2008. Multilocus sequence typing of Campylobacter jejuni isolates from humans, chickens, raw milk, and environmental water in Quebec, Canada. J Clin Microbiol 46, 3404-3411.
24. Little, C.L., Richardson, J.F., Owen, R.J., de Pinna, E., Threlfall, E.J., 2008. Campylobacter and Salmonella in raw red meats in the United Kingdom: prevalence, characterization and antimicrobial resistance pattern, 2003-2005. Food Microbiol 25, 538-543.
25. Ragimbeau, C., Schneider, F., Losch, S., Even, J., Mossong, J., 2008. Multilocus sequence typing, pulsed-field gel electrophoresis, and fla short variable region typing of clonal complexes of Campylobacter jejuni strains of human, bovine, and poultry origins in Luxembourg. Appl Environ Microbiol 74, 7715-7722.
26. Rossi, M., Hanninen, M.L., Revez, J., Hannula, M., Zanoni, R.G., 2008. Occurrence and species level diagnostics of Campylobacter spp., enteric Helicobacter spp. and Anaerobiospirillum spp. in healthy and diarrheic dogs and cats. Vet Microbiol 129, 304-314.
27. Sheppard, S.K., Dallas, J.F., Macrae, M., McCarthy, N.D., Sproston, E.L., Gormley, F.J., Strachan, N.J., Ogden, I.D., Maiden, M.C., Ken, J.F., 2009. Campylobacter genotypes from food animals, environmental sources and clinical disease in Scotland 2005/6. Int J Food Microbiol 134, 96-103.
28. Smith, C.K., Abuoun, M., Cawthraw, S.A., Humphrey, T.J., Rothwell, L., Kaiser, P., Barrow, P.A., Jones, M.A., 2008. Campylobacter colonization of the chicken induces a proinflammatory response in mucosal tissues. FEMS Immunol Med Microbiol 54, 114-121.
29. Sopwith, W., Birtles, A., Matthews, M., Fox, A., Gee, S., Painter, M., Regan, M., Syed, Q., Bolton, E., 2006. Campylobacter jejuni multilocus sequence types in humans, northwest England, 2003-2004. Emerg Infect Dis 12, 1500-1507.
30. Sopwith, W., Birtles, A., Matthews, M., Fox, A., Gee, S., Painter, M., Regan, M., Syed, Q., Bolton, E., 2008. Identification of potential environmentally adapted Campylobacter jejuni strain, United Kingdom. Emerg Infect Dis 14, 1769-1773.
31. Stoyanchev, T., Vashin, I., Ring, C., Atanassova, V., 2007. Prevalence of Campylobacter spp. in poultry and poultry products for sale on the Bulgarian retail market. Antonie Van Leeuwenhoek 92, 285-288.
32. Waldenstrom, J., Broman, T., Carlsson, I., Hasselquist, D., Achterberg, R.P., Wagenaar, J.A., Olsen, B., 2002. Prevalence of Campylobacter jejuni, Campylobacter lari, and Campylobacter coli in different ecological guilds and taxa of migrating birds. Appl Environ Microbiol 68, 5911-5917.
33. Westgarth, C., Porter, C.J., Nicolson, L., Birtles, R.J., Williams, N.J., Hart, C.A., Pinchbeck, G.L., Gaskell, R.M., Christley, R.M., Dawson, S., 2009. Risk factors for the carriage of Campylobacter upsaliensis by dogs in a community in Cheshire. Vet Rec 165, 526-530.
34. Wieland, B., Regula, G., Danuser, J., Wittwer, M., Burnens, A.P., Wassenaar, T.M., Stark, K.D., 2005. Campylobacter spp. in dogs and cats in Switzerland: risk factor analysis and molecular characterization with AFLP. J Vet Med B Infect Dis Vet Public Health 52, 183-189.
35. Wilson, D.J., Gabriel, E., Leatherbarrow, A.J., Cheesbrough, J., Gee, S., Bolton, E., Fox, A., Fearnhead, P., Hart, C.A., Diggle, P.J., 2008. Tracing the source of campylobacteriosis. PLoS Genet 4, e1000203.
36. Workman, S.N., Mathison, G.E., Lavoie, M.C., 2005. Pet dogs and chicken meat as reservoirs of Campylobacter spp. in Barbados. J Clin Microbiol 43, 2642-2650.

ANTIMICROBIAL ACTIVITY OF NEW SYNTHESIZED [(OXADIAZOLYL) METHYL] PHENYTOIN DERIVATIVES

Omar M. ALI [a], Wael A. El-SAYED[b,*], Shorok A. EID[a], Nayera A. M. ABDELWAHED[c] Adel A.-H. ABDEL-RAHMAN[a,*]

[a]Department of Chemistry, Faculty of Science, Menoufia University, Shebin El-Koam, Egypt
[b]Department of Photochemistry, National Research Centre, Cairo, Egypt
[c]Department of Chemistry of Natural and Microbial Products, National Research Centre, Cairo, Egypt; omarmakrum@yahoo.co.uk

Abstract

A number of substituted phenytoin derivatives in addition to their sugar hydrazones were newly synthesized. Furthermore, the corresponding derived 1,3,4-oxadiazole and their thioglycoside as well as their acyclic analogs were prepared. The antimicrobial activity of the prepared compounds was evaluated against Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Aspergillus niger and Candida albicans. The dithiohydrazone as well as oxadiazole thiole derivatives, sugar hydrazones and acyclic nucleoside analogs were the highly active compounds.

Keywords: phenytoin, oxadiazole, glycosides, sugar hydrazones, antibacterial, antifungal activity

The imidazolidine-2,4-dione, or hydantoin nucleus, is a common 5-membered ring containing a reactive cyclic urea core. This heterocycle is present in a wide range of biologically active compounds including antiarrhythmics (1) anticonvulsant (2) and antitumor (3) agents. Beside the traditional usage, of hydantoin derivatives as antiepileptic (4,5), antiarrhythmics (6), antibacterial substance and sceletal muscle relaxant (7), hydantoins have been also developed as new drugs in the treatment of other diseases, for example, nilutamide, which was approved by the FDA in 1996 as a nonsteroidal, orally active antiandrogen in the therapy of metastatic prostate cancer (8). Hydantoins are structural units frequently encountered in naturally occurring substances, mostly of marine organisms, but also of bacteria. Examples for many alkaloids extracted from sponges or corals which contain a hydantoin moiety are the well-known aplysinopsins with cytotoxic properties (10-13), axinohydantoins from *Axinella* (14) *Hymeniacidon* (15) and *Stylotella* species inhibiting protein kinase C (16,17), naamidinene A, a dehydro hydantoin derivative from the genus *Leucettu* (18), and mukanadin B from *Agelus* species (19). Hydantocidin is a spiro nucleoside from *Streptomyces* hygroscopicus (20,21), which possesses herbicidal and plant growth regulatory activity due to the inhibition of adenylsuccinate synthetase (22). Among these agents, phenytoin, is a well known therapeutic drug for the treatment of epileptic seizures (23). It had been effective against electrically induced seizures in cats (24) and is still the drug of choice for the treatment of generalized tonic–clonic seizures (so-called grand mal epilepsy) and focal motor seizures (25). Phenytoin has found new applications due to the neuro-and cardioprotective properties (26,27). On the other hand, 1,3,4-oxadiazole derivatives possess a broad spectrum of biological activity in both agrochemicals and pharmaceuticals such as antibacterial (28), antimicrobial (29), insecticidal (30), herbicidal, fungicidal (31), anti-inflammatory (32), hypoglycemic (33), hypotension characteristics (34), antiviral (35), and antitumor activities (36). In view of the above facts and as continuation of

our program of identification of new candidates that may be valuable in design and synthesis of new active leads (37-42) we report in the present work the synthesis and antimicrobial activity of new phenytoin derivatives, their oxadiazolyl, glycoside and acyclic analogs.

Experimental

Chemistry

All melting points are uncorrected and were taken in open capillary tubes using silicon oil on Gallenkamp apparatus. Elemental microanalyses were performed on Elementar, Vario EL, Microanalytical Unit, National Research Centre, Cairo, Egypt. Infrared spectra were recorded on Jasco FT/IR-330E, Fourier Transform Infrared Spectrometer at cm^{-1} scale using KBr discs.

^{1}H-NMR spectra were determined by using JEOL EX-270 or JEOL ACA500 NMR spectrometers and measured in δ scale using TMS as an internal standard. Mass spectra were measured using mass spectrometer Finnigan MAT SSQ-7000 and GCMS-QP 1000EX Shimadzu Gas Chromatography MS Spectrometer.

All reactions were followed up by TLC (aluminum sheets) using $CHCl_3/CH_3OH$ (9:1, v/v) eluent and detected by UV lamp. The chemical names given to the prepared compounds are according to the IUPAC system.

Ethyl 2-(phenytoin-1-yl)acetate (**2**)

To a solution of phenytoin (5,5-diphenylhydantoin) (**1**) (2.52, 0.01 mole) in *N*,*N*-dimethyl formamide (25 mL), anhydrous potassium carbonate (0.14 g, 0.01 mole) and ethyl chloroacetate (0.12 g, 0.01 mole) was added. The solution was stirred at room temperature for 12h and poured on ice-cold water. The resulting precipitate was filtered off and recrystallized from ethanol to afford **2**.

2-(Phenytoin-1-yl)acetohydrazide (**3**)

A solution of **2** (3.38 g, 0.01 mole) and hydrazine hydrate (0.5 g, 0.01 mole) in ethanol was heated under reflux for 6 h. The mixture was cooled and the precipitate was filtered off and recrystallized from ethanol to afford **3**.

Dimethyl (phenytoin-1-yl)acetyldithiohydrazonocarbonate (**4**)

To a stirred solution of potassium hydroxide (0.56 g, 0.01 mole) in 2.5 mL of water and 1.5 mL of ethanol, compound **3** (3.24 g, 0.01 mole) was added. After stirring for 1h at room temperature, carbon disulphide (0.2 mL) and methyl iodide (0.16 mL) were added and the reaction mixture was stirred for 0.5 h. The reaction mixture was poured on ice (100 g). The yellow precipitate was filtered off and recrystallized from ethanol to afford **4**

(1,3,4-Oxadiazol-2-yl)piperidine and morpholine derivatives (**5a,b**)

Compound **4** (4.28 g, 0.01 mole) was heated under reflux in 3 mL of (morpholine or piperidine) for 2 h. The mixture was cooled, diluted with 20 mL of water and extracted with chloroform. The collected chloroform fractions were dried with magnesium sulfate, after evaporation of the solvent residual oil crystallized.

N'-Arylidine-2-(phenytoin-1-yl)acetohydrazide (**6a-c**)

To solution of compound **3** (3.24 g, 0.01 mole) in ethanol, the respective aldehyde (0.01 mole) and catalytic amount of acetic anhydride were added and the reaction mixture was refluxed for 2 h. Ethanol was removed under vacuum and the obtained solid was dried well and crystallized from ethanol.

1-(5-Mercapto-[1,3,4]oxadiazol-2-ylmethyl)-5',5'-diphenyl-imidazolidine-2',4'-dione (**7**)

To a solution of **3** (3.24 g, 0.01 mole) in absolute ethanol (50 mL) a solution of potassium hydroxide (0.56 g, 0.01 mole) in water (2 mL) and carbon disulphide (5 mL) were added. The solution was heated under reflux for 20 h. The solvent was evaporated and the residue was dissolved in water, filtered, and acidified with dilute hydrochloric acid. The precipitate was filtered off, washed with water and recrystallized from ethanol.

1-(5-Methylsulfanyl-[1,3,4]oxadiazol-2-ylmethyl)-5',5'-diphenyl-imidazolidine-2',4'-dione (**8**)

To a solution of **7** (3.66 g, 0.01 mole) and potassium hydroxide (0.56 g, 0.01 mole) in a mixture of water (30 mL) and ethanol (15 mL), methyl iodide or ethyl iodide (0.01 mole) was added. The solution was stirred at room temperature for 4h. The precipitate was filtered off and recrystallized from ethanol to afford compound **8**.

1-[(5-Hydrazino-1,3,4-oxadiazol-2-yl)methyl]-5',5'-diphenylimidazo-lidine-2',4'-dione (**9**)

A mixture of compound **8** (3.80 g, 0.01 mole) in ethanol and hydrazine hydrate (0.5 g, 0.01 mole), was refluxed for 4h. The solvent was removed under reduced pressure, the remaining precipitate was collected, dried, and recrystallized from ethanol to afford hydrazine derivative compound.

Arylaldehyde {5-[(2',4'-dioxo-5',5'-diphenylimidazolidin-1-yl)methyl]-1,3,4-oxadiazol-2-yl}hydrazone (**10a,b**)

To solution of the hydrazine derivative 9 (3.64 g, 0.01 mole) in ethanol, the respective aldehyde (0.01 mole) and catalytic amount of acetic anhydride (0.5 mL) were added and the reaction mixture was refluxed for 2h. Ethanol was removed under vacuum. The solid was dried well and recrystallized from ethanol.

Sugar {5-[(2',4'-dioxo-5',5'-diphenylimidazolidin-1-yl)methyl]-1,3,4-oxadiazol-2-yl}hydrazone (**11a,b**)

To a well stirred mixture of the respective monosaccharide [(0.01 mole) in water (1 mL)], glacial acetic acid (0.2 mL) in ethanol (10 mL) was added the hydrazine derivative **10** (3.64 g, 0.01 mole). The mixture was heated under reflux for 3h and the resulting solution was concentrated and left to cool. The precipitate formed was filtered off, washed with water and ethanol then dried and crystallized from ethanol.

O-Acetylsugar {5-[(2',4'-dioxo-5',5'-diphenylimidazolidin-1-yl)methyl]-1,3,4-oxadiazol-2-yl}hydrazone (**12a,b**)

To a solution of the hydrazinosugars **11a,b** (0.01 mole) in pyridine, acetic anhydride (0.1 mole) was added and the mixture was stirred at room temperature for 5h. The resulting solution was poured into crushed ice and the product that separated out was filtered off, washed with a solution of sodium hydrogen carbonate followed by water and then dried. The product was recrystallized from ethanol.

1-{[5-(Substitutedalkylthio)-1,3,4-oxadiazol-2-yl]methyl}-5,5-diphenylimidazolidine-2,4-dione (**13** and **14**)

General procedure: To a solution of **7** (3.66 g, 0.01 mole) in acetone or acetonitrile (15 mL), anhydrous potassium carbonate (1.38 g, 0.01 mole) was added and the mixture was stirred at room temperature for 1h. Chloromethylethyl ether or 2,3-dihydroxypropan (0.01 mole) was added and stirring was continued for 25-30 h at room temperature and then poured into cooled water. The resulting precipitate was filtered off and recrystallized from ethanol

1-{[5-[[2-(2-Hydroxyethoxy)ethyl]sulfanyl]-1,3,4-oxadiazol-2-yl]methyl}-5,5-diphenylimidazolidine-2,4-dione (**15**)

To a solution of **7** (3.66 g, 0.01 mole) in absolute *Et*OH (15 mL) potassium hydroxide (0.56 g, 0.01 mole) was added and the mixture was stirred at room temperature for 1h. 2-(2-Chloroethoxy) ethanol (1.25 g, 0.01 mole) was added and the reaction mixture was heated at reflux temperature for 6 h. The solvent was evaporated under reduced pressure and the resulting precipitate was collected and recrystallized from ethanol.

1-{[5-[(2,3,4,6-Tetra-*O*-acetyl-D-glucopyranosyl)thio]-1,3,4-oxadiazol-2-yl]methyl}-5,5-diphenylimidazolidine-2,4-dione (**17**)

To a solution of compound **7** (0.37 g, 0.001 mole) and aqueous potassium hydroxide (1.12 g, 0.01 mole) in acetone, solution of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (4.11 g, 0.01 mole) was dissolved in acetone and was added to the former solution. The reaction mixture was stirred at room temperature for 5h. The solvent was evaporated under reduced pressure at 40°C; the residue was washed with distilled water to remove potassium bromide formed.

1-{[5-[(D-Glucopyranosyl)thio]-1,3,4-oxadiazol-2-yl]methyl}-5,5-diphenylimidazolidine-2,4-dione (**18**)

A solution of **17** (0.7 g, 0.001 mole) in methanol and ammonia solution was stirred at room temperature for 4h. The solvent was evaporated under reduced pressure and the residue was dissolved in absolute ethanol (10 mL) and left over night. The solvent was removed under vacuum and the product was dried well**.**

Antimicrobial screening

The synthesized compounds were screened *in vitro* for their antimicrobial activities against *Escherichia coli* NRRL B-210 (Gram -ve bacteria), *Bacillus subtilis* NRRL B-543 and *Staphylococcus aureus* (Gram +ve bacteria), *Aspergillus niger* and *Candida albicans* NRRL Y-477 (Fungi). These microorganisms were obtained from Northern Utilisation, research and Development Division, U.S. department of agricultural Peoria, Illinois, USA.

The agar diffusion method reported by Cruickshank *et al*, 1975 (43) was used for the screening process. The bacteria and fungi were maintained on nutrient agar and Czapek's-Dox agar media, respectively.

The assay medium flasks containing 50 mL of nutrient agar for bacteria and Czapek's-Dox agar medium for fungi respectively were allowed to reach 40-50^{o}C to be inoculated with 0.5 mL of the test organism cell suspension. The flasks were mixed well and poured each into a Petri dish (15 x 2 cm) and allowed to solidify. After solidification, holes (0.6 cm diameter) were made in the agar plate by the aid of a sterile cork poorer (diameter 6 mm).

The synthesized target compounds were dissolved each in 2 mL DMSO. In these holes, 100µl of each compound was placed using an automatic micropipette. The Petri dishes were left at 5°C for 1 hour to allow diffusion of the samples through the agar medium and retard the growth of the test organism. Plates were incubated at 30^{o}C for 24 hours for bacteria and 72 h of incubation at 28^{o}C for fungi. DMSO showed no inhibition zones. The diameters of zone of inhibition were measured and compared with that of the standard, the values were tabulated. Ciprofloxacin (44,45) (50 µg/mL) and fusidic acid (46) (50 µg/mL) were used as standard for antibacterial and antifungal activity respectively. The observed zones of inhibition are presented in Table 1.

Results and discussion

Chemistry

In this investigation, when 5,5-diphenyl hydantoin **1** was allowed to react with ethyl chloroacetate in DMF and in the presence of potassium carbonate an hydrous afforded the corresponding ester derivative **2.** The acid hydrazide **3** was synthesized by refluxing its corresponding ester derivative **2** and hydrazine hydrate in ethanol. When the hydrazide **3** reacted with carbon disulphide and methyl iodide in the presence of potassium hydroxide it afforded the corresponding dithiohydrazonocarbonate derivative **4**. Its ^{1}H NMR spectra showed the signals of the methyl groups as two singlet signals at δ 2.42-2.49 ppm. Reaction of compound **4** with piperidine or morpholine resulted in the formation of *N*-substituted derivatives **5a,b**. When the hydrazide **3** was reacted with 2,5 dimethoxy benzaldehyde**,** 4-chlorobenzaldehyde or 2,4,6 trimethoxy benzaldehyde in presence of glacial acetic acid the orresponding arylidine derivatives **6a-c** were formed. The ^{1}H NMR spectrum of the **6c** showed the signals of the methyl groups as singletsat δ 3.73-3.87 ppm in addition to the disappearance of the NH_2 signal originally present in hydrazide **3** (scheme 1).

When the acid hydrazide **3** was reacted with carbon disulphide in ethanol in the presence of potassium hydroxide, it afforded the oxadiazole thiol/thione **7**. Methylation of **7** with methyl iodide in alkaline medium afforded the corresponding *S*-methyl derivative **8.** Reaction of compound **8** with hydrazine hydrate gave the hydrazine derivative **9**. Its IR spectrum showed the characteristic absorption bands at 3248 cm^{-1} corresponding to the NH_2 group and 3329 cm^{-1} corresponding to the NH group. When the hydrazine derivative **9** was reacted with *p*-fluorobenzaldehyde and 2,4,6-trimethoxybenzaldehyde in the presence of glacial acetic acid they afforded the corresponding arylidine derivatives **10a,b**. Reaction of the hydrazine derivative **9** with D-galactose and D-arabinose in an aqueous ethanolic solution and a catalytic amount of acetic acid, gave the corresponding hydrazinosugar derivatives **11a,b** respectively. The ^{1}H NMR spectrum of **11b** revealed the H-1 signal as doublet at 7.14 ppm. Acetylation of the sugar hydrazones **11a,b** with acetic anhydride in pyridine at room temperature gave the corresponding per-*O*-acetyl derivatives **12a,b**. The ^{1}H NMR spectra of **12a** showed the signals of the *O*-acetyl-methyl protons as singlet in the range δ 1.88-2.07 ppm, the rest of the sugar chain protons appeared in the range δ 3.94 – 5.49 ppm and the C-1 signal at δ 7.54 ppm (scheme 2).

Reaction of the thiole 7 with chloroethylmethyl ether, 2-(2-chloroethoxy)ethanol and 1-chloro-2,3-dihydroxy propane gave the corresponding *S*-substituted derivatives **13-15** respectively. The ^{1}H NMR spectrum of **13** showed methyl signal at 3.42 ppm in addition to the CH_2 signals each as triplet. The IR spectra of compounds **14** and **15** revealed the presence of absorption bands of the hydroxyl groups at 3416-3486 cm^{-1}. Reaction of compoud **7** with 2,3,4,6-tetra-*O*-acetyl-α-glucopyranosylbromide (**16**) in acetone afforded the thioglycoside derivative **17**. Its IR spectra showed the presence of absorption band at 1747 cm^{-1} corresponding to the *O*-acetyl carbonyl groups. Its ^{1}H NMR spectrum revealed the presence of the *O*-acetyl-methyl groups at 1.89-2.05 ppm and the anomeric proton signal appeared at 5.77 ppm with coupling constant J = 9.8 Hz indicating the *ß*-configuration of thioglucosidic linkage. The anomeric proton of β-*N*-glucosides having an adjacent C=S, was reported to appear at higher chemical shift due to the anisotropic deshielding effect of the C=S. Deacetylation of thioglycoside **17** using methanolic ammonia solution at room temperature afforded the deprotected thioglycoside **18** (scheme 3).

Antimicrobial activity

The target compounds were screened in vitro for their antimicrobial activities against *Escherichia coli* NRRL B-210 (Gram -ve bacteria), *Bacillus subtilis* NRRL B-543 and *Staphylococcus aureus* (Gram +ve bacteria), *Aspergillus niger* and *Candida albicans* NRRL Y-477 (Fungi). These microorganisms were obtained from Northern Utilisation Research and Development Division, U.S. Department of Agricultural Peoria, Illinois, USA.

The results of the preliminary antimicrobial and the antifungal activates are shown in Table I. The results revealed that compounds **4, 9, 11a** and **14** showed varying degrees of inhibition against the previously mentioned bacteria. The best antifungal activity against ***Aspergillus niger*** was displayed by compounds **6**, **7**, **11a** and **12b**. Compounds **6, 7** and **11a** showed strong antifungal activity against *Candida albicans*. Some of tested compounds showed relatively similar activities with inhibition zone values near to each other while other compounds showed little or no activity against one or more organism.

Structure-activity relationship (SAR)

The antimicrobial activity results and structure activity relationship indicated that the (oxadiazozlylmethyl)phenytoin derivatives attached acyclic arabinotetritolyl sugar moiety showed increased inhibition activities against both microorganism types. Furthermore, the hydrazinyl sugars with free hydroxyl groups showed higher activity that their corresponding acetylated analogs.

The antimicrobial activity results also proved that the attachment of hydrazinyl group to the substituted 1,3,4-oxadiazole system resulted in increased inhibition activity than hydrazides of the phenytoin moiety. This is clear as the activity increased in the oxadiazolyl hydrazine compared to the low activity of hydrazide **3**.

The acyclic nucleoside analogs with the oxadiazole ring system attached to acyclic hydroxyl oxygenated chain revealed higher inhibition activities against both microorganisms than the corresponding cyclic acetylated glucoside.

Additionally, free hydroxyl glucoside showed higher activity than its acetylated precursor. Furthermore, the dithiohydrazone and the free thiole-thione oxadiazole revealed higher inhibition activities than other synthesized mercapto derivatives.

The results revealed that the arylidine compound of the phenytoinhydrazide with phenyl ring carrying chlorine atom in the *para*-position showed higher activity that the corresponding analogs with methoxy groups.

Conclusion

New phenytoin derivatives, their oxadiazolyl, glycoside and some acyclic analogs were synthesized and evaluated for their antimicrobial activities. A number of the synthesized derivatives with sugar hydrazinyl and glucoside moieties as well as their acyclic analogs showed strong activities.

Table 1. In vitro antimicrobial activity by agar diffusion method of tested Compounds

Tested compounds	**Zone of Inhibition (mm)** **Microorganisms**				
	Escherichia coli	***Bacillus subtilis***	***Staph. aureus***	***Aspergillus niger***	***Candida albicans***
2	-	-	14	13	-
3	-	-	8	13	14-
4	19	18	16	16	17
5	17	17	16	15	-
6	19	19	18	17	19
7	19	19	18	17	18
8	-	14	15	16	51
9	19	19	18	17	17
10a	18	19	18	16	17
10b	17	17	15	16	15
11a	18	18	16	16	17
11b	19	19	18	17	18
12a	-	-	9	11	-
12b	19	18	17	16	18
13	15	15	14	13	-
14	19	18	17	16	16
15	18	17	17	15	15
17	-	15	14	13	14
18	18	17	16	15	16
Stryptomycine	22	22	21	-	-
Fusidic acid	-	-	-	17	18

Table 2. Physical and analytical data of all new compounds

Comp. no.	m.p.(^{0}C) (crystal. solvent)	Yield (%)	Mol. Formula (Mol.wt.)	Analysis (%) Calcd. / Found		
				C	H	N
2	222-223	8 7	$C_{19}H_{18}N_2O_2$ (338.36)	67.44 67.28	5.36 5.30	8.28 8.19
3	172-174	75	$C_{17}H_{16}N_4O_3$ (324.12)	62.95 62.72	4.97 4.85	7.27 7.21
4	223-225	67	$C_{20}H_{20}N_4O_3S_2$ (428.53)	56.06 55.93	4.70 4.58	13.07 12.88
5a	172-173	60	$C_{23}H_{23}N_5O_3$ 414.18) (	66.17 66.02	5.55 5.47	16.76 16.58
5b	193-194	65	$C_{22}H_{21}N_5O_4$ (419.16)	63.00 62.81	5.05 5.02	16.70 16.59
6a	173-175	86	$C_{26}H_{24}N_4O_5$ (472.49)	66.09 65.89	5.12 5.10	11.86 11.71
6b	>300	72	$C_{24}H_{19}CLN_4O_3$ (446.89)	64.50 64.32	4.29 4.14	12.54 12.29
6c	253-255	65	$C_{27}H_{26}N_4O_6$ (502.52)	64.53 64.37	5.22 5.05	11.15 10.92
7	190-192	85	$C_{18}H_{14}N_4O_3S$ (366.08)	59.01 58.88	3.85 3.69	15.29 15.15
8	160-162	67	$C_{19}H_{16}N_4O_3S$ (380.42)	59.99 59.80	4.24 4.15	14.73 14.60
9	160-161	74	$C_{18}H_{16}N_6O_3$ (364.36)	59.34 59.15	4.43 4.29	23.07 22.93
10a	160-161	90	$C_{25}H_{19}FN_6O_3$ (470.64)	63.82 63.68	4.07 4.02	17.86 17.66
10b	250-251	87	$C_{28}H_{26}N_6O_6$ (542.54)	61.99 61.68	4.83 4.70	15.49 15.28
11a	170-172	62	$C_{25}H_{29}N_6O_8$ (541.53)	55.45 55.28	5.40 5.33	15.52 15.41
11b	168-170	65	$C_{23}H_{24}N_6O_7$ (496.47)	55.64 55.50	4.87 4.69	16.93 16.77
12a	180-182	82	$C_{34}H_{36}N_6O_{13}$ (736.68)	55.34 55.11	4.93 4.72	11.41 11.19
12b	170-171	78	$C_{31}H_{32}N_6O_{11}$ (664.62)	56.02 55.85	4.85 4.59	12.64 12.55
13	168-170	55	$C_{21}H_{20}N_4O_4S$ (424.47)	59.42 59.27	4.75 4.61	13.20 13.16
14	165-166	65	$C_{21}H_{24}N_4O_4S$ (444.05)	56.74 56.66	5.44 5.28	12.60 12.49

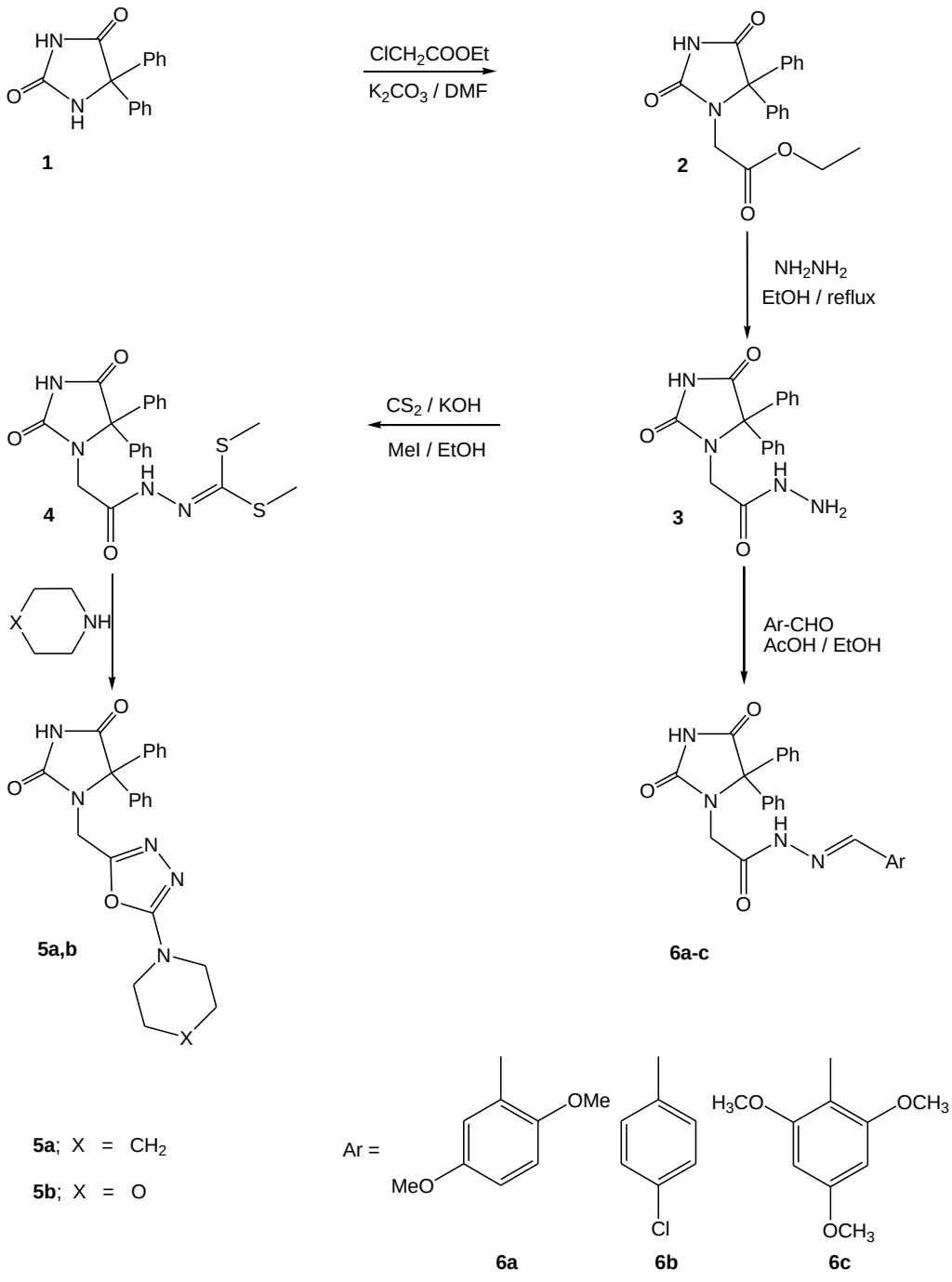
15	150-152	72	$C_{22}H_{22}N_4O_5S$ (454.50)	58.14 58.02	4.88 4.64	12.33 12.19
17	169-170	74	$C_{32}H_{32}N_4O_{12}S$ (696.68)	55.17 54.92	4.63 4.52	8.04 7.85
18	188-189	76	$C_{24}H_{24}N_4O_8S$ (528.53)	45.54 45.42	4.58 4.51	10.60 10.47

Table 3. Spectral data of the newly synthesized compounds

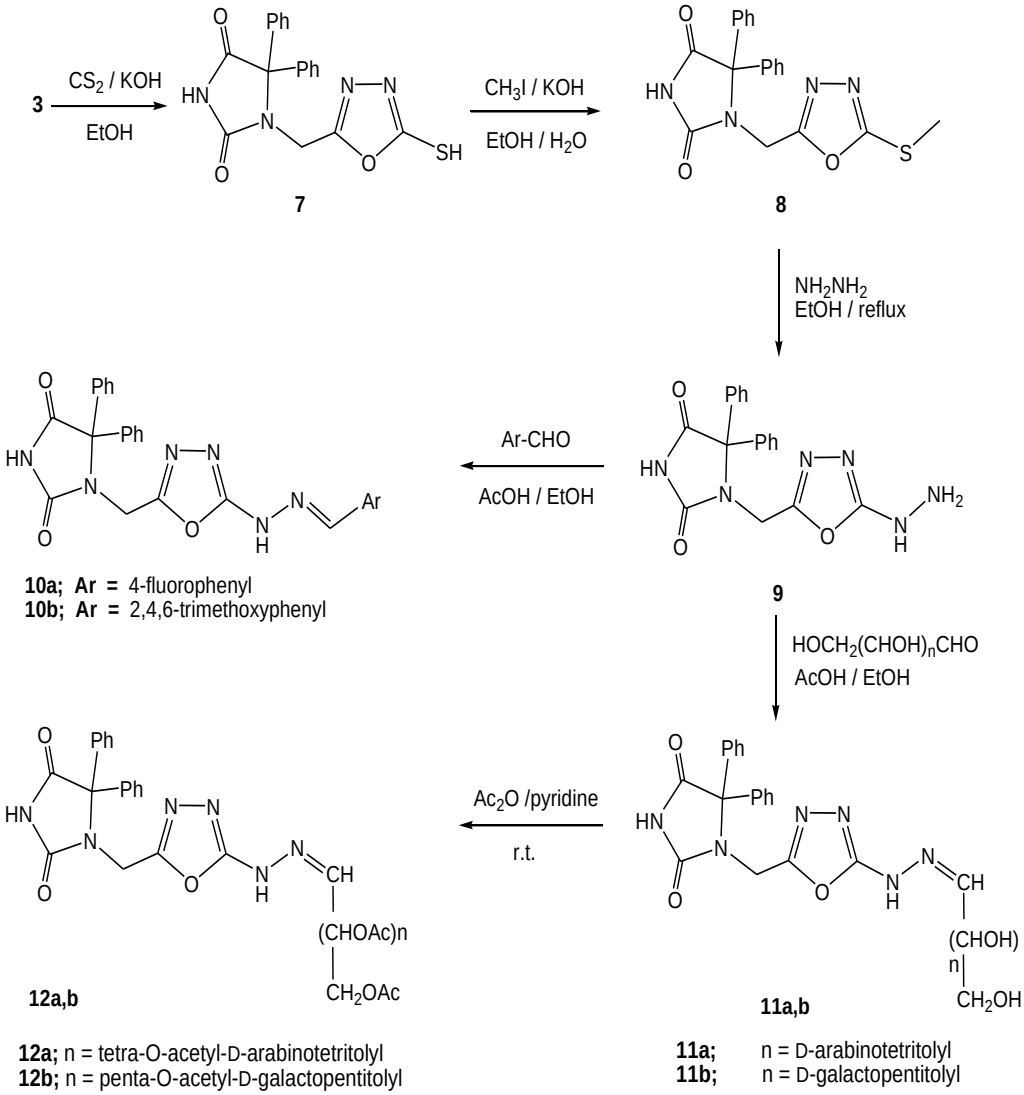
Comp. No.		IR (KBr) cm^{-1}, MS [m/z (%), ^{1}HNMR [DMSO-d_6] ppm
2	**IR** **^{1}H NMR** **MS**	1662 (C=O), 1730 (C=O), 3423 (NH). 1.22 (t, 3H, *J* = 5.6 Hz, C*H*$_3$CH$_2$), 4.21 (q, 2H, *J* = 5.6 Hz, CH$_3$C*H*$_2$), 4.57 (s, 2H, NC*H*$_2$), 7.23 (m, 4H, Ar-H), 7.30 (m, 3H, Ar-H), 7.39 (m, 3H, Ar-H), 9.92 (brs, 1H, NH); *m/z* (%) = 338 (M^+, 34).
3	**IR** **^{1}H NMR** **MS**	1695 (C=O), 3427 (NH). 4.57 (s, 2H, NC*H*$_2$), 5.80 (brs, 2H, NH$_2$), 7.23 (m, 4H, Ar-H), 7.30 (m, 3H, Ar-H), 7.43 (m, 3H, Ar-H), 8.22 (brs, 1H, NH), 9.92 (brs, 1H, NH); *m/z* (%) = 324 (M^+, 25).
4	**IR** **^{1}H NMR**	1692 (C=O), 3420 (NH). 2.43 (s, 6H, 2SCH$_3$), 4.44 (s, 2H, CH$_2$), 7.36 (m, 3H, Ar-H), 7.40 (m, 3H, Ar-H), 7.45 (m, 2H, Ar-H), 7.86 (m, 2H, Ar-H), 9.05 (bs, 1H, NH), 10.14 (bs, 1H, NH).
5a	**IR** **^{1}H NMR** **MS**	1690 (C=O), 3350 (NH). 2.95 (t, 4H, *J* = 6.4 Hz, 2CH$_2$), 3.35 (t, 4H, *J* = 6.4 Hz, 2CH$_2$), 4.33 (s, 2H, CH$_2$), 7.37 (m, 4H, Ar-H), 7.31 (m, 3H, Ar-H), 7.42 (m, 3H, Ar-H), 8.01 (s, 1H, NH). *m/z (%)* = 415 [M^++H].
5b	**IR** **^{1}H NMR**	1673 (C=O), 3341 (NH). 2.15 (m, 2H, CH$_2$), 2.24 (m, 4H, 2CH$_2$), 3.35 (t, 4H, *J* = 6.8 Hz, 2CH$_2$), 4.31 (s, 2H, CH$_2$), 7.33 (m, 4H, Ar-H), 7.37 (m, 3H, Ar-H), 7.44 (m, 3H, Ar-H), 7.97 (s, 1H, NH).
6a	**IR** **^{1}H NMR**	1666 (C=O), 3349 (NH). 3.68 (s, 3H, OCH$_3$), 3.74 (s, 3H, OCH$_3$), 4.28 (s, 2H, CH$_2$), 7.06 (m, 4H, Ar-H), 7.31-7.34 (m, 4H, Ar-H), 7.44 (m, 3H, Ar-H), 7.61 (m, 2H, Ar-H), 7.71 (s, 1H, C*H*=N), 7.99 (s, 1H, NH), 9.72 (s, 1H, NH).
6b	**IR** **^{1}H NMR** **MS**	1694 (C=O), 3405 (NH). 4.31 (s, 2H, CH$_2$), 7.05 (m, 4H, Ar-H), 7.11 (d, 2H, *J* = 7.8 Hz, Ar-H), 7.37 (m, 2H, Ar-H), 7.49 (m, 2H, Ar-H), 7.67 (d, 2H, *J* = 7.8 Hz, Ar-H), 7.70 (m, 2H, Ar-H), 7.75 (s, 1H, C*H*=N), 8.05 (s, 1H, NH), 9.80 (s, 1H, NH). *m/z (%)* = 446 [M^+].

6c	**IR** **^{1}H NMR**	1695 (C=O), 3412 (NH). 3.72 (s, 6H, 2OCH_3), 3.84 (s, 3H, OCH_3), 4.28 (s, 2H, CH_2), 7.06 (m, 4H, Ar-H), 7.31-7.34 (m, 2H, Ar-H), 7.44 (m, 3H, Ar-H), 7.60 (m, 3H, Ar-H), 7.76 (s, 1H, C*H*=N), 7.99 (s, 1H, NH), 9.77 (s, 1H, NH).
7	**IR** **^{1}H NMR** **MS**	1612 (C=N), 1692 (C=O), 3414 (NH). 4.83 (s, 2H, CH_2), 7.36 (m, 4H, Ar-H), 7.44 (m, 3H, Ar-H), 7.47 (m, 3H, Ar-H), 9.82 (s, 1H, NH), 12.52 (s, 1H, NH) *m/z (%)* = 360 [M^+].
8	**IR** **^{1}H NMR** **MS**	1612 (C=N), 1692 (C=O), 3414 (NH). 2.52 (s, 3H, CH_3), 4.94 (s, 2H, CH_2), 7.35 (m, 4H, Ar-H), 7.40 (m, 3H, Ar-H), 7.45 (m, 3H, Ar-H), 9.8 (s, 1H, NH). m/z (%) = 380 [M^+].
9	**IR** **^{1}H NMR**	3329 (NH), 3248 (NH_2), 1717(C=O), 1603 (C=O). 4.94 (s, 2H, CH_2), 5.82 (brs, 2H, NH_2), 7.32-7.36 (m, 4H, Ar-H), 7.44-7.47 (m, 3H, Ar-H), 7.52-7.55 (m, 3H, Ar-H), 9.82 (s, 1H, NH), 9.85 (s, 1H, NH).
10a	**IR** **^{1}H NMR**	1611 (C=N), 1680 (C=O), 3321 (NH). 4.92 (s, 2H, CH_2), 7.31 (m, 4H, Ar-H), 7.42 (m, 3H, Ar-H), 7.48-7.52 (m, 5H, Ar-H), 7.71 (d, 2H, *J* = 7.5 Hz, Ar-H) 9.92 (s, 1H, NH), 10.12 (s, 1H, NH).
10b	**IR** **^{1}H NMR** **MS**	1614 (C=N), 1672 (C=O), 3352 (NH). 4.94 (s, 2H, CH_2), 7.32 (m, 4H, Ar-H), 7.44 (m, 3H, Ar-H), 7.45-7.48 (m, 5H, Ar-H), 9.80 (s, 1H, NH), 9.85 (s, 1H, NH). *m/z (%)* = 471 [M^{+1}].
11a	**IR** **^{1}H NMR**	3392 (OH), 1678 (CO), 1618 (C=N). 3.37 (m, 2H, H-5,5′), 3.39 (m, 1H, H-4), 3.56 (m, 1H, H-3), 4.22 (t, 1H, *J* = 6.4 Hz, OH), 4.87 (t, 1H, *J* = 6.2 Hz, OH), 4.95 (s, 2H, CH_2), 5.15 (t, 1H, *J* = 5.4 Hz, OH), 5.29 (d, 1H, $J_{1,2}$ = 9.8 Hz, H-2), 5.39 (t, 1H, *J* = 6.6 Hz, OH), 7.12 (d, 1H, *J* = 9.8 Hz, H-1), 7.32-7.34 (m, 4H, Ar-H), 7.45-7.49 (m, 3H, Ar-H), 7.51 (m, 3H, Ar-H)
11b	**IR** **^{1}H NMR**	3261 (OH), 1676 (CO), 1615 (C=N). 3.34 (m, 2H, H-6,6′), 3.39 (m, 1H, H-5), 3.54 (m, 2H, H-3,4), 4.19 (t, 1H, *J* = 6.4 Hz, OH), 4.39 (t, 1H, OH), 4.88 (t, 1H, *J* = 6.4 Hz, OH), 4.95 (s, 2H, CH_2), 5.14 (t, 1H, *J* = 5.8 Hz, OH), 5.24 (d, 1H, $J_{1,2}$ = 9.8 Hz, H-2), 5.37 (t, 1H, *J* = 6.2 Hz, OH), 7.14 (d, 1H, *J* = 9.8 Hz, H-1), 7.30-7.34 (m, 4H, Ar-H), 7.42-7.47 (m, 3H, Ar-H), 7.50 (m, 3H, Ar-H)
12a	**IR** **^{1}H NMR**	3453 (NH), 1750 (OAc), 1633 (CO). 1.97, 2.02, 2.05, 2.07 (4s, 12H, 4CH_3), 3.97 (dd, 1H, *J* = 11.2 Hz, *J* = 2.8Hz, H-5′), 4.08 (dd, 1H, *J* = 11.2 Hz, *J* = 3.2 Hz, H-5″), 4.16 (m, 1H, H-4′), 4.62 (dd, 1H, *J* = 2.8 Hz, *J* = 6.5 Hz, H-3′), 5.27 (dd, 1H, *J* = 7.5.2 Hz, J = 9.2.5 Hz, H-2′), 5.66 (s, 2H, CH_2), 7.21 (d,1H, J = 9.5 Hz, H-1′), 7.32-7.48 (m, 4H, Ar-H), 7.42-7.47 (m, 3H, Ar-H), 7.54 (m, 3H, Ar-H), 9.95 (s, 1H, NH).

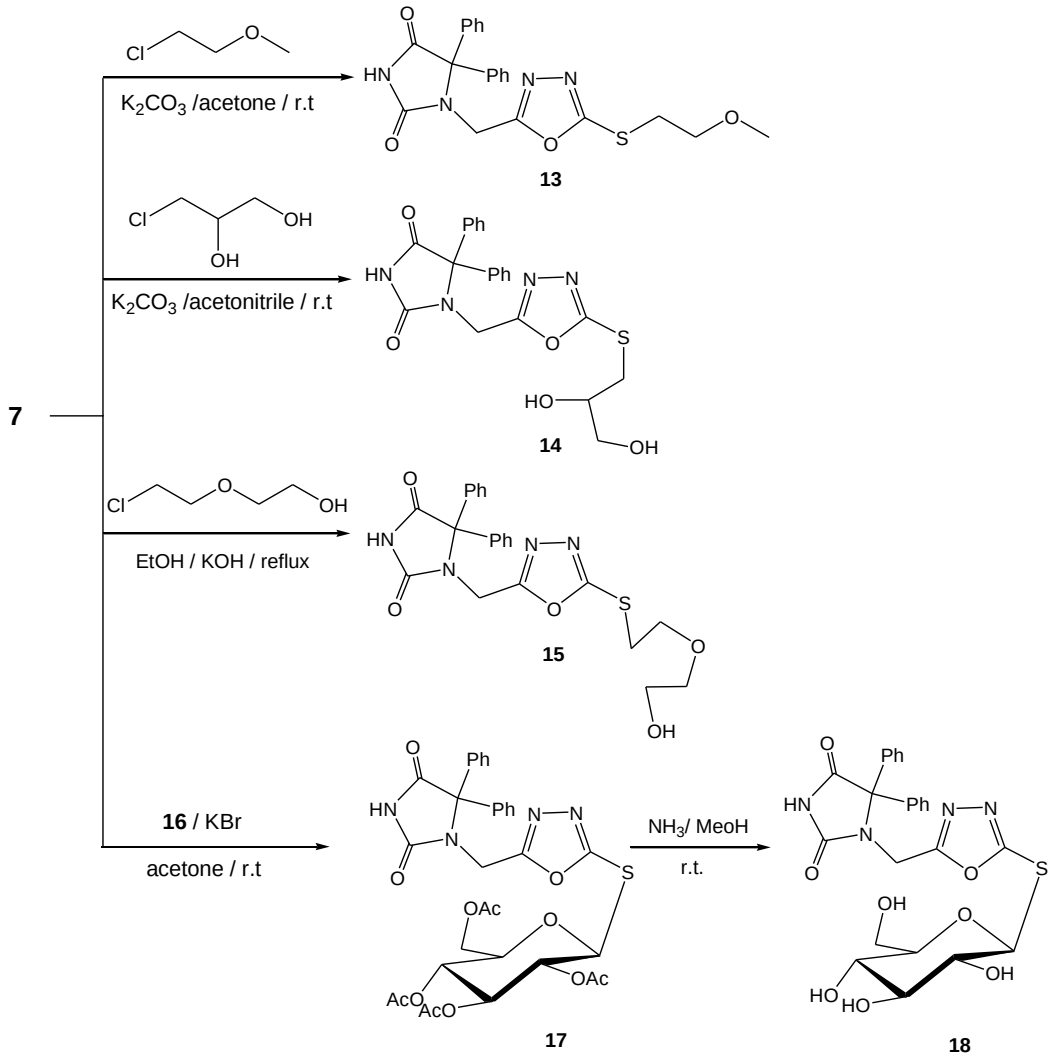
12b	**IR** **^{1}H NMR**	3428 (NH), 1748 (OAc), 1663 (CO). 1.93, 1.95, 2.03, 2.07, 2.10 (5s, 15H, 5CH_3), 3.95 (dd, 1H, *J* = 11.2 Hz, *J* = 2.8Hz, H-6′), 4.12 (dd, 1H, *J* = 11.2 Hz, *J* = 3.2 Hz, H-6″), 4.18 (m, 1H, H-5′), 4.24 (t, 1H, *J* = 7.5 Hz, H-4′), 5.20 (dd, 1H, *J* = 2.8 Hz, *J* = 6.5 Hz, H-3′), 5.27 (dd, 1H, *J* = 7.5.2 Hz, J = 9.2.5 Hz, H-2′), 5.64 (s, 2H, CH_2), 7.22 (d,1H, J = 9.5 Hz, H-1′), 7.35-7.39 (m, 4H, Ar-H), 7.42-7.45 (m, 3H, Ar-H), 7.54 (m, 3H, Ar-H), 8.92 (s, 1H, NH).
13	**IR** **^{1}H NMR**	3430 (NH), 1662 (CO). 3.42 (s, 3H, OCH_3), 4.14 (t, 2H, *J* = 5.8 Hz, CH_2), 4.93 (t, 2H, *J* = 5.8 Hz, CH_2), 4.98 (s, 2H, CH_2), 7.35-7.39 (m, 4H, Ar-H), 7.41-7.44 (m, 3H, Ar-H), 7.52 (m, 3H, Ar-H), 9.84 (s,1H, NH).
14	**IR** **^{1}H NMR**	3386 (OH), 1664 (C=O). 3.82 (m, 2H, CH_2), 4.42 (d, 2H, CH_2), 4.69 (m, 1H, OH), 4.83 (s, 2H, CH_2), 4.90 (m, 1H, CH), 5.11 (m, 1H, OH), 7.38 (m, 4H, Ar-H), 7.49 (m, 3H, Ar-H), 7.48 (m, 3H, Ar-H), 10.42 (s, 1H, NH),
15	**IR** **^{1}H NMR**	3419 (OH), 1656 (CO). 4.02 (t, 2H, *J* = 5.8 Hz, CH_2), 4.15 (t, 2H, *J* = 5.8 Hz, CH_2), 4.86 (m, 2H, CH_2), 4.92 (t, 2H, *J* = 5.8 Hz, CH_2), 505 (m, 1H, OH), 5.12 (s, 2H, CH_2), 7.37-7.40 (m, 4H, Ar-H), 7.47-7.55 (m, 3H, Ar-H), 7.66 (m, 3H, Ar-H), 9.93 (s,1H, NH).
17	**IR** **^{1}H NMR**	3431(NH), 1747 (OAc), 1639 (CO). 1.89, 1.93, 2.02, 2.05, (4s, 12H, 4CH_3), 3.90 (m, 1H, H-5), 4.05 (dd, 1H, $J_{6,6'}$ = 11.4 Hz, $J_{5,6}$ = 2.8 Hz, H-6), 4.16 (m, 1H, H-6′), 4.69 (t, 1H, *J* = 9.3 Hz, H-4), 4.73 (s, 2H, CH_2) 4.80 (dd, 1H, $J_{2,3}$ = 9.6 Hz, $J_{3,4}$ = 9.3 Hz, H-3), 5.25 (t, 1H, $J_{2,3}$ = 9.6 Hz, H-2), 5.77 (d, 1H, $J_{1,2}$ = 9.8 Hz, H-1), 7.35-7.40 (m, 4H, Ar-H), 7.46-7.53 (m, 3H, Ar-H), 7.65 (m, 3H, Ar-H), 10.31 (s,1H, NH).
18	**IR** **^{1}H NMR**	3388 (OH), 1665 (CO). 3.39 (m, 2H, H-6,6′), 3.46 (m, 1H, H-5), 3.59 (m, 2H, H-3,4), 4.26 (t, 1H, *J* = 6.4 Hz, OH), 4.48 (t, 1H, OH), 4.89 (t, 1H, OH), 4.92 (s, 2H, CH_2), 5.24 (d, 1H, $J_{1,2}$ = 9.8 Hz, H-2), 5.35 (t, 1H, OH), 5.72 (d, 1H, *J* = 9.8 Hz, H-1), 7.41-7.49 (m, 4H, Ar-H), 7.52-7.57 (m, 3H, Ar-H), 7.72 (m, 3H, Ar-H)


ClCH2COOEt
K2CO3 / DMF
NH2NH2
EtOH / reflux
CS2 / KOH
MeI / EtOH
Ar-CHO
AcOH / EtOH
1
2
3
4
5a,b
6a-c
5a; X = CH2
5b; X = O
Ar =
OMe
MeO
Cl
H3CO
OCH3
OCH3
6a
6b
6c

Scheme 1


3
CS2 / KOH
EtOH
7
CH3I / KOH
EtOH / H2O
8
NH2NH2
EtOH / reflux
9
Ar-CHO
AcOH / EtOH
10a; Ar = 4-fluorophenyl
10b; Ar = 2,4,6-trimethoxyphenyl
HOCH2(CHOH)nCHO
AcOH / EtOH
11a,b
11a; n = D-arabinotetritolyl
11b; n = D-galactopentitolyl
Ac2O /pyridine
r.t.
12a,b
12a; n = tetra-O-acetyl-D-arabinotetritolyl
12b; n = penta-O-acetyl-D-galactopentitolyl

Scheme 2



Scheme 3

References

1. Knabe J., Baldauf J., Ahlhem A.: Pharmazie 52, 912 (1997).
2. Sholl S., Koch A., Henning D., Kempter G., Kleinpeter E.: Struct. Chem. 1999, 10, 355.
3. Rodgers T.R., LaMontagne M.P., Markovac A., Ash A.B.: J. Med. Chem. 20, 591 (1977).
4. Bosch J., Roca T., Dom nech J., Suriol M.: Bioorg. Med. Chem. Lett. 9, 1859 (1999).
5. Anger T.**,** Madge D.J., Mulla M., Riddall D.: J. Med. Chem. 44, 115 (2001).
6. Bazil C.W.: Curr. Treat. Options Neurol. 6, 339 (2004).
7. Clai I.B.: Tetrahedron Lett. 44, 7475 (2003).
8. Meusel M., Gutschow M.: Organic Preparations and Procedures International. 36, 391 (2004).
9. Lamothe M., Lannuzel M., Perez M.: J. Comb. Chem. 4, 73 (2002).
10. Guella G., Mancini I., Zibrowius H., Pietra F.: Helv. Chim. Acta 71, 773 (1988).
11. Chezal J.M., Dehas G.**,** Mavel S., Elalanaoui H., MBtin J., Diez A., Blache Y., Gueffier A., Rubiralta M., Teulade J.C., Chavignon O.: J. Org. Chem. 62, 4085 (1997).

12. Selic L., Jakse R., Lampic K., Golic L., Golic-Grdadolnik S., Stanovnik B.: Helv. Chim. Acta 83, 2802 (2000).
13. Jakse R., Kroselj V., Recnik S., Sorsak G., Svete J., Stanovnik B., Grdadolnik S.G.: Z. Naturforsch. 57b, 453 (2002).
14. Pettit G.P., Herald C.L., Leet J.E, Gupta R., Schaufelberger D.E., Bates R.B., Clewlow P.J., Doubek D.L., Manfredi K. P., Riitzler K., Schmidt J.M., Tackett L.P., Ward F.B., Bruck M., Camou, F.: Can. J. Chem. 68, 1621 (1990).
15. Inaba K., Sato H., Tsuda M., Kobayashi J.: J. Nat. Prod. 61, 693 (1998).
16. Sosa A.C.B., Yakushijin K., Home D.A.: J. Org. Chem. 67, 4498 (2002).
17. Patil A.D., Freyer A.J., Killmer L., Hofmann G., Johnson R.K.: Nat. Prod. Lett. 9, 201 (1997).
18. Crews P., Clark D. P., Tenney K.: J. Nat. Prod. 66, 177 (2003).
19. Uemoto H., Tsuda M., Kobayashi J.: J. Nat. Prod. 62, 1581 (1999).
20. Nakajima N., Matsumoto M., Kirihara M., Hashimoto M., Katoh T., Terashima S.: Tetrahedron 52, 1177 (1996).
21. Mio S., Ichinose R., Goto K., Sugai S., Sato S.: Tetrahedron 47, 2111 (1991).
22. Renard A., Lhomme J., Kotera M.: J. Org. Chem. 67, 1302 (2002).
23. Bac P., Maurois P., Dupont, C., Pages N., Stables J. P., Gressens P., Evrard P.: Neurosci. J. 18, 4363 (1998).
24. Merrit H.H., Putnam, T.J.: Science 85, 525 (1937).
25. Krall R.L., Penry J.K., White B.G., Kupferberg H.J., Swinyard, E.A.: Epilepsia 19, 409 (1978).
26. Taylor C.P.: Curr. Pharm. Des. 2, 375 (1996).
27. Reagan L.P., McKittrick C.R., McEwen, B.S. Neuroscience 91, 211 (1999).
28. Ates O., Kocabalkanli A., Sanis G.O., Ekinci A.C., Vidin A.: Drug Res. 47, 1134 (1997).
29. Farghaly A.R., Chin J.: Chem. Soc. 51, 147 (2004).
30. Li X.Z.Z., Wang Y., Chen W., Huang Q.: J. Fluor. Chem. 123, 163 (2003).
31. Zou X., Zhang Z., Jin G.J.: Chem J. Res. Synop. 5, 228 (2002).
32. Palaska E., Sahin G., Kelicen P., Durlu N.T., Altinok G.: Farmaco 57, 101 (2002).
33. Mhasalkar M.Y., Shah M.H., Pilankar P.D., Nikan S.T.: J. Med. Chem. 14, 1000 (1971).
34. Tyagi M., Kumar A.: Orient. J. Chem. 18, 125 (2002).
35. El-Emam A.A., Al-Deep A.O., Al-Omar M., Lehmann J.: Bioorg. Med. Chem 12, 5107 (2004).
36. Liszkiewicz H., Kowalska M.W., Wietrzyk J., Opolski A.: J. Ind. Chem. Sec. B–Org. Chem. Including Med. Chem. 42B, 2846 (2003).
37. El-Sayed W.A., Ramiz M.M.M., Abdel-Rahman A.A.-H.: Monatsh. Chem. 139, 1499 (2008).
38. El-Sayed W.A., Fathi N.M., Gad W.A., El-Ashry E.S.H., J. Carbohyd. Chem. 27, 357 (2008).
39. El-Sayed W.A., Abdel-Rahman A.A.-H., Ramiz M.M.M.: Z. Naturforsch. 64c, 323 (2009).
40. El-Sayed W.A., Nassar I.F., Abdel-Rahman A.A.H.: Monatsh. Chem. 140, 365 (2009).
41. El-Sayed W.A., Rashad A.E., Awad S.M., Ali M.M.: Nucleosides Nucleotides and Nucleic Acids 28, 261 (2009).
42. El-Sayed W.A., Nassar I.F., Abdel Rahman A.A.-H.: J. Het. Chem. 4, 135 (2011).
43. Cruickshank R., Duguid J.P., Marion B.P., Swain R.H.A.: Medicinal Microbiology, twelfth ed., vol. II, Churchill Livingstone, London, pp. 196 (1975).
44. Dahiya R.: Sci. Pharm. 76, 217 (2008).
45. Su H. C., Ramkissoon K., Doolittle J., Clark M., Khatun J., Secrest A. Wolfgang M.C., Giddings M.C.: Antimicrob. Agents Chemother. 4626 (2010).
46. Poyner T.F., Dass B.K.: J. Eur. Acad. Dermatol. Venereol. 7, S23 (1996).

IMMUNOLOGICAL EFFICIENCY OF VACCINE STRAINS USED IN IMUNIZATION AGAINST AVIAN INFECTIOUS BRONCHITIS

Natalia OSADCI, N. STARCIUC, T. SPĂTARU, Rita GOLBAN, A. CIUCLEA, S. BUGNEAC, R. ANTOCI
State Agrarian University of Moldova

Abstract
The article includes the serological investigation about maternal and post vaccination level of specific antibodies titers against bronchitis diseases virus. For vaccination were used "H-120" strains and "Ma5+Clon30" strains, administrated by spray method separate and in combination with the the hydroalcohlic solution of propolis. The level of antibody titers was established with ELISA test. On result was established that the chickens which was vaccinated with vaccine strain "H-120" had lower level of antibody titers which were between 1: 815.727 and 1: 1189.27 comparative with of antibody level of chicken which was vaccinated with vaccine strain "Ma5+Clon30" (1: 1603.02 and 1: 2011.86).

Key words: Infectious bronchitis virus, Chickens, ELISA test, Serological investigation, Antibody titers, Vaccine strains.

Introduction

Infectious bronchitis virus, which was isolated more than 75 years ago, so far remains to be one of the most pathogenic viruses that cause respiratory infections in birds of all ages and all races. The emergence of new types of field of the pathogen further complicates diagnosis of infectious bronchitis. (Kasparieanţ S. A., Cekmariov A. D.,2009).

Avian infectious bronchitis was first introduced in Moldova in 1994 through hatching eggs and chicks aged one day. Infectious bronchitis in most cases affect the young until the age of 40 days with prominent appearance of clinical and pathological changes. Vaccination of chickens with live vaccine 25-30 days before laying period of exacerbation of this infection removed, however, has not been fully . (Scutaru I., Spataru T., Starciuc N., 2009).

Vaccines against respiratory infections such Newcastle disease and infectious bronchitis, are used successfully long times at poultry ferms. However administration of live vaccines, especially those that are intended for the same target organs, can cause negative effects. Often can be seen to decrease the activity of one or two components (interference), unexpected results and increase the response to vaccination. It is necessary to appreciate the elements compatipilitatea vaccine recommended for use associated. (Aris Malo, 2009; Muhamedşina A. R., 2009)

Anatomoclinic diagnosis is very difficult or even impossible, because the disease has many similarities with other respiratory disease of birds: avian infectious laryngo-tracheitis, Newcastle disease, avian mycoplasmosis, avian cholera and chronic difterovariola. Some primary bacterial diseases such as those produced by Mycoplasma and Ornithobacterium rhinotraheale can be included as potential differences. Other bacteria are able to infect the respiratory tract once it has been damaged primarily by a virus which can lead to difficulty establishing a clear diagnosis. (Perianu T., 2005; Grgić H.; et al., 2009; Shen C.I.; et. al., 2009).

Materials and methods

The purpose of these investigations was to ascertain the effectiveness of vaccines and immunological methods used in preventing infectious bronchitis of chickens.

This study was conducted on six experimental groups under race chicken „Hi Land" for 25 chicks in each group. Were formed 5 experimental groups of chicken and a control group. In groups of chicken vaccines were administered by drinking water and spray methods. It has been used the vaccine strain H-120 and vaccine Ma5 + Clon30, attenuated virus strain (Ma5) avian infectious bronchitis and strain (Clon30) Newcastle disease. One of the experimental groups was revaccinated at the age of 21 days with vaccine strain La-Sota (table 1).

Before vaccination and at the age 7, 10, 15, 20, 30 and 40 days from each chikens group were sacrificed 3 chicks and were collected blood serum samples to determine the antibody titres, and lungs for histological investigations. The antibody titres was assessed by immunoassay test (ELISA). All six groups of chickens were kept under analog conditions and nutrition.

The chicks were fed fodder well balanced, age appropriate, that is home to one day feed (no. 5). Of 100% corn is 65%, wheat 10%, macuh-2%, -14% soie meal, bone meal -5%, fish meal-2%, premix fortified 2%.

At the age of 3 weeks was administered combined fodder no. 6. Of 100% corn is 60%, wheat 14%, macuh -7%, -10% soie meal, bone meal -5%, fish meal-2%, premix fortified 2%.

Sampling was performed traditional conservation known.

Table 1. Vaccination of chickens against avian infectious bronchitis

No. group	Vaccine strain	No. of chickens	Age of the chickens (days)	
			I	II
1	control group	25	-	-
2	H-120	25	1	-
3	H-120	25	3	-
4	Ma5+Clon30	25	3	-
5	Ma5 + Clon30 in combination with propolis hydroalcoholic solution of 10%	25	5	-
6	H-120	25	1	21

Vaccines were administered as follows:

The first group of chicks served as control group.

Second group of chickens was vaccinated with strain H-120, once of the age one day, method - with drinking water.

The III group - with the vaccine strain H-120, at the age of 3 days, method - spray.

The Fourth group - the vaccine "Ma5 + Clon30" the age of 3 days, method - spray;

The V group - the vaccine "Ma5 + Clon30" in combination with propolis hydroalcoholic solution, at the age of 5 days, method - spray;

VI group - with the vaccine strain "H-120", at the age of 1 day, spray administration method, with revaccination chickens at the age of 21 days -Lasota strain, method - with drinking water.

After vaccination and during the examination did vaccinated chickens showed adverse reactions.

Results and discussion

The result of investigations after vaccination are presented in the table 2. In the control group the antibody titres to the first day were 1: 1304.9. In the following investigation of antibody titres were found in the report 1: 792.19 the 7-th day, in the report 1: 950.04 to the 10-th day and 1: 949.06 at the 15-th day, by failing to following examination.

After administration of strain H-120 vaccine in chickens of group II antibody titres in serum is 1: 815,72 at its 10-th day, narrowing - is up to 1: 108,82 on the 20-th day after vaccination, major-be up to 1: 437,27 at the 30-th day after vaccination with further increase of antibody titres to 1: 1189.27 on the 40-th day.

In the offspring of any third group who were vaccinated with the same strain (H-120), after the vaccine was antibody titres 1: 2894.26 on the 10-th day, narrowing to 1: 108,82 at the 20-th day, with subsequent reduction of antibody titres to other examinations.

In the offspring of the fourth group, which were vaccinated with strain Ma5 + Clon30 after vaccination antibody titres were 1: 528,45 at the 10-th day and reducing them to other examinations.

The fifth group where chickens were vaccinated with strain Ma5 + Clon30 in combination with 10% hydroalcoholic solution of propolis after vaccination antibody titres were 1: 1603.02 on the 10-th day. The highest antibody levels were set at the 30-th and 40-th day after vaccination, representing 1: 2108.51 on the 30-th and 1: 2011.86 on the 40-th day.

Offspring from this group for 5 days before vaccination and 5 days after vaccination, were administered with drinking water or hydroalcoholic solution propolius 10%. Administer each 10 ml 10% hydroalcoholic propolius per liter of drinking water.

In the offspring of Group VI, which were vaccinated with strain H-120 at the age of 1 day, with revaccination chickens at the age of 21 days after vaccination the antibody titres was 1: 1714.79 the 10-th day, narrowing to 1: 321,58 on the 20-th day, with considerable reduction of antibody titres in the 30-th and 40- th day.

The highest antibody levels were set at the 30-th and 40-th day after vaccination, representing respectively 1: 2108.51 in the group vaccinated with strain chicks Ma5 + Clon30 in combination with 10% hydroalcoholic solution of propolis. In the group of the chicks vaccinated with strain H-120 the highest antibody levels were detected at 10-th days after vaccination, representing respectively 1: 2894.26.

The results obtained allow to state that all vaccines used in experimental groups were stimulated offspring antibody titres wich satisfactory protect chikens from contamination with infectious bronchitis virus.

Table 2. The antibody titres after vaccination

No. gr.	No. of chickens	Age of vaccinated chickens (days)	Vaccine strain	Antibodi level after vaccination (days)						
				1	7	10	15	20	30	40
I	25	-	Control group	813.76578 22217.885 1304.9666	4238.4452 698.074 792.19	1037.30 950.047 845.14	139.223 419.629 949.067	-39.2176 798.079 -23.5306	-16.6675 -10,7848 12.7457	-28.4328 2.94132 -19.6088
II	25	1	H-120	-	-	2242.27 815.727 360.802	-	108.829 20.5893 -10.7848	437.277 9.80441 -38.2372	174.518 1189.27 802
III	25	3	H-120	-	-	1323.59 2894.26 121.575	-	584.343 111.77 24.511	108.829 -23.5306 -35.2959	-20.5893 0 -38.2372
IV	25	3	Ma5+Clon30	-	-	528.458 426.492 462.768	-	-35.2959 150.007 129.418	-22.5501 -29.4132 -41.1785	-27.4523 -39.2176 17.6479
V	25	5	Ma5+Clon30 in combination with propolis hydroalcoholic solution of 10%	-	-	1603.02 330.409 392.176	-	59.8069 -42.159 150.007	250.993 2108.51 375.509	696.113 996.128 2011.86
VI	25	1	H-120	-	-	390.215 1190.26 1714.79	-	321.585 23.5306	-20.5893 45.1003 1.96088	48.0416 -30.3937 -16.6675

Conclusion

1. Ma5 + Clon30 vaccine administered by spray method in combination with 10% propolis hydroalcoholic solution and drinking water showed a positive antibody titres to increase the difference in value compared to the group that was vaccinated only with vaccine.
2. The 10% propolis hydroalcoholic solution is a biological product that can be used successfully as immunity stimulator on vaccination of poultry flocks.

Reference

1. Aris, Malo. *Sovmestimosti vacţin NOBILIS ND C2 s IB 120, IB Ma5 i RHINO CV.* Intervet Internaşnal BV, Niderlandî. Veterinaria nr. 4, 2009, s 18-20.
2. Grgic, H., et. al. *Vaccine efficacy against Ontario isolates of infectious bronchitis virus.* Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada. 2009 Jul; 73(3), p. 212-216.
3. Kasparieanţ, S. A., Cekmariov, A. D. *Vacţina pulvac IB praimer protiv variantnîh ştamov virusa infecţionnovo bronhita kur.* Veterinaria nr. 1, 2009, s. 15-16.
4. Muhamedşina, A. R. *Oborudovanie dlâ provedeniâ krupnokapelinoi vakţinaţii.* ZAO „DenLen", Veterinaria nr. 4, 2009, s. 17-18.
5. Perianu, T. *Cornoviroze. Bronşita infecţioasă aviară.* Boli infecţioase ale animalelor. Viroze, vol. II, Iaşi. 2005; p. 159-164.
6. Scutaru, I., Spataru, T., Starciuc, N. *Infecţionnîe bolezni ptiţî.* Vsem OMNIBUS, 2009, s. 31-33.
7. Shen, C.I.; et. al. The infection of primary avian tracheal epithelial cells with infectious bronchitis virus. 2009; 41(1), p. 6.

COMPARATIVE STUDY OF ENZYMATIC ACTIVITY AGAINST CARBOHYDRATES AND POLYALCOHOLS OF SOME *LACTOBACILLUS SALIVARIUS* STRAINS ISOLATED FROM DENTAL ROOT CANAL WITH TWO PROBIOTIC *LACTOBACILLUS* STRAINS BY INTESTINAL ORIGIN

Anca Alexandra DOBREA (POPESCU)*, Constantin SAVU*, Bogdan DIMITRIU, Mimi DOBREA*, Ruxandra STĂNESCU**, Gabriel MURARIU*****

*Faculty of Veterinary Medicine Bucharest Splaiul Independentei nr.105, Sector 5

**Faculty of Dental Medicine Bucharest, ***University ,, Dunărea de Jos" Galaţi

andrapopescu1984@yahoo.com

Abstract

In this work we comparatively investigated the activity of some Lactobacillus salivarius strains isolated from dental root canal against 49 different substances (carbohydrates and polyalcohols) with two probiotic Lactobacillus strains by intestinal origin. The Lactobacillus salivarius strains isolated from dental root canal fermented rhamnose and raffinose. These two carbohydrates were not used by the probiotic Lactobacillus strains.

Key words: Lactobacillus salivarius, dental root canal, probiotic.

Introduction

In the last time lactic acid bacteria have gained importance regarding their positive effects on the health of humans. Major interest was focused on the *Lactobacillus* sp. and *Bifidobacterium* sp. lactic acid bacterial strains. *Lactobacillus salivarius* is part of the microbiota of the gastrointestinal tract (GIT) and oral cavity of humans and hamsters (6). The species has also been isolated from human breast milk (8) and from the intestinal tracts of swine and chickens (2). An increase of the number of studies about *L. salivarius* has been observed.

Materials and methods

In this study we examined two *Lactobacillus salivarius* strains isolated from dental root canal (G1 and G2) and two probiotic *Lactobacillus* strains by intestinal origin (*L. salivarius* probiotic and LGG). All strains were growth in MRS medium.

The enzymatic activity against carbohydrates and polyalcohols was investigated by the API System.

Results and discussions

The obtained results were presented in table 1.

The four strains have not fermented the following substances: erithriol, D and L arabinose, L- and D-xylose, menthyl-D-Xylopyranoside, inositol, menthyl-D-mannopyranoside, arbutin, esculine ferric citrat, D-celibiose, amidon (starch), glicogen, xilitol, D-turanose, D-lyxose, D- fucose, D and L-arabitol, potassium 2-ketogluconate and potassium 5-ketogluconate.

All investigated strains fermented: D-glucose, D-galactose, D-manose, D-fructose, D-mannitol, N-acetylglucoseamine, D-sucrose and D-trehalose.

Table 1. The enzymatic activity of some *Lactobacillus salivarius* strains isolated from dental root canal against different carbohydrates and polyalcohols and two probiotic *Lactobacillus* strains by intestinal origin

Sugar	*Lactobacillus salivarius* strains isolated from dental root canal		Probiotic *Lactobacillus* strains by intestinal origin	
	G1	G2	*L.salivarius* probiotic	LGG
control	-	-	-	-
glycerol	-	-	+	-
erytthriol	-	-	-	-
D-ARAbinose	-	-	-	-
L-ARAbinose	-	-	-	-
D-ribose	-	-	+	-
D-XYlose	-	-	-	-
L-XYlose	-	-	-	-
D-ADOnitol	-	-	+	-
Menthyl- D-Xylopyranoside	-	-	-	-
D-GALactose	+	+	+	+
D-GLUcose	+	+	+	+
D-FRUctose	+	+	+	+
D-MANose	+	+	+	+
L-SORbose	-	-	+	-
L-RHAmnose	+	+	-	-
DULcitol	-	-	-	+
INOsitol	-	-	-	
D-MANitol	+	+	+	+
D-SORbitol	+	+	-	+
Menthyl- D- Mannopyranoside	-	-	-	-
Menthyl- D- Glucopyranoside	-	-	+	-
N-AcetylGlucosamine	+	+	+	+
AMYgdalin	-	-	-	+
ARButin	-	-	-	-
ESCulin ferric citrate	-	-	-	-
SALicin	-	-	-	+
D-CELiobiose	-	-	-	+
D-MALtose	+	+	+	-
D-LACtose (bovine origin)	+	+	+	-
D-MELibiose	+	+	+	-
D-SACcharose (sucroze)	+	+	+	+
D-TREhalose	+	+	+	+
INUlin	-	-	+	-
D-MeLeZitose	-	-	-	+
D-RAFfinose	+	+	-	-
AmiDon (starch)	-	-	-	-
GLYcoGen	-	-	-	-
XyLiTol	-	-	-	-
GENtiobiose	-	-	-	+
D-TURanose	-	-	-	-
D-LYXose	-	-	-	-
D-TAGatose	-	-	-	+
D-FUCose	-	-	-	-
L-FUCose	-	-	-	+

D-ARabitol	-	-	-	-
L-ARabitol	-	-	-	-
potassium Gluconate	-	-	-	-
potassium 2- KetoGluconate	-	-	-	-
potassium 5- KetoGluconate	-	-	-	-

A different activity between the three *Lactobacillus salivarius* strains and *Lactobacillus rhamnosus* GG was observed for: glicerol, D-ribose, D-adonitol, D-sorbitol, amigdaline, salicin, D-lactose, melibiose, D-melesitose, methyl-D-glucopyranoside, inulin, potassium gluconate, L-fucose, D-maltose, gentibiose and D-tagatose. Of these substances, the followings were fermented only by LGG strain: dulcitol, amigdaline, salicin, D-melizitose, gentibiose, D-tagatose and L-fucose. Other substances were fermentated only by the three strains of *Lactobacillus salivarius* species: D-maltose, D-lactose and D-melibiose.

The following substances were fermented only by *L.salivarius* probiotic strain: D-adonitol, glicerol, D-ribose, L-sorbose, methyl-D-glucopiranoside and inulin. D-sorbitol was fermented by G1, G2 and LGG, but it was not used by *L. salivarius* probiotic strain.

The *Lactobacillus salivarius* strains isolated from dental root canal fermented rhamnose and raffinose, which were not used by the probiotic *Lactobacillus* strains.

Conclussions

After this study we observed that some investigated substances were fermented by all strains, while others were not used by these strains. However, some differences between strains were found:

1.Only LGG strain fermented dulcitol, amigdaline, salicin, D-melizitose, gentibiose, D-tagatose and L-fucose.

2.The *Lactobacillus salivarius* strains (G1,G2 and L. salivarius probiotic) fermented D-maltose, D-lactose and D-melibiose

3.A particular glucidolytic activity of the *Lactobacillus salivarius* strains isolated from dental root canal was observed against rhamnose and raffinose, which were not fermented by the probiotic *Lactobacillus salivarius* and LGG strains.

Acknowledgments

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References

1. Abbas Hilmi, H. T., T. Hanan, A. Surakka, J. Apajalahti, and P. E. J. Saris. 2007. Identification of the most abundant *Lactobacillus* species in the crop of 1- and 5-week-old broiler chickens. Appl. Environ. Microbiol. 73:7867–7873.
2. Casey, P. G., et al. 2004. Isolation and characterization of anti-*Salmonella* lactic acid bacteria from the porcine gastrointestinal tract. Lett. Appl. Microbiol. 39:431–438.
3. De Vuyst, Luc and Marc Vancanneyt. 2007. Biodiversity and identification of sourdough lactic acid bacteria. *Food Microbiology* 24:120-127.

4. Dolezil, L. and Kirsop, B. H. (1977), The Use of the A.P.I. Lactobacillus System for the Characterization of *Pediococci*. Journal of Applied Microbiology, 42: 213–217.
5. Hammes. W.P and Vogel. R.F. (1995). "The Genera of Lactic acid bacteria 173-234. London: Blackie Academic Press and Professional.
6. Knuuttila H. Yni, K. Kary, J.H. Meurman (2004) Sugar fermentation of probiotic *Lactobacilli* – The IADR, AADR, CADR 82nd General Session 10-13 March 2004, Honolulu.
7. Li, Y., et al. 2006. Polyphasic analysis indicates that *Lactobacillus salivarius subsp. salivarius* and *Lactobacillus salivarius subsp. salicinius* do not merit separate subspecies status. Int. J. Syst. Evol. Microbiol. 56:2397–2403.
8. Martín, R., et al. 2006. *Lactobacillus salivarius* CECT 5713, a potential probiotic strain isolated from infant feces and breast milk of a mother-child pair. Int. J. Food Microbiol. 112:35–43.
9. Manual of Systematic Bacteriology (2009) Second Edition, Vol 3 ,,The Firmicutes,,

DRIED SPICES AND VEGETABLE SEASONINGS - QUANTITATIVE STUDY ON BACTERIAL AND FUNGAL FLORA

Gina-Mihaela PRICOPE[1], Viorel FLORIŞTEAN[2], Mihai CARP-CĂRARE[2]
[1]Veterinary Directorate for Food Safety and Veterinary Diagnostic Laboratory for Food Safety Iasi, Romania; [2]University of Agricultural Sciences and Veterinary Medicine "Ion Ionescu de la Brad" Iasi, Faculty of Veterinary Medicine, ginacornelia@yahoo.com

Abstract
Dried aromatic herbs, spices and vegetable seasonings are frequently contaminated and/or infested with organisms and their metabolites which are harmful to public health; the purpose of the study is to evaluate the types of flora, bacterial and fungal, quantitatively (quantification flora) in foodstoffs control strategie. The potential public health risk of using spices and herbs as an addition to ready-to-eat foods that potentially undergo no further processing is highlighted. The experimental protocol includes: quantitative bacteriological and mycological exam: enumeration of microorganisms grow at 30^0C; detection and enumeration Enterobacteriaceeas, enumeration of sulphite -reducing bacteria in anaerobic conditions and horizontal method for enumeration of yeasts and molds. The results were unsatisfactory in 56.71% of samples examined for total bacterial count, 17.91% of all samples examined for total sulphite-reducing bacteria, 26,18% of all samples examined for Enterobacteriaceae and 52.23% for the total number of yeasts and molds.

Keywords: spices, herbs, seasonings, contamination, limits

Introduction

The term *spices*, which includes dried aromatic plants, relates to natural dried components or mixtures, used in foods for flavouring, seasoning and imparting aroma, applies equally to spices in the whole, broken or ground spice blends and products.

The ingredients used in food industry is often a source of biological risks of manufactured products: pathogenic bacteria in vegetative form, which can be present and can destroyed during manufacture, bacterial spores that can survive processes and pathogenic bacteria can recontaminate products by manufacturing, Gram positive, then Gram negative, and, yeasts and molds with a progressive development.

Objective

The purpose of the study is to evaluate the types of bacterial and fungal flora, quantitatively (flora quantification) in the categories of the most popular dried aromatic herbs, spices and vegetable seasonings.

Material and methods

67 samples were taken from 23 common spice from different types of distribution and marketing units(***fig. 1***): *Garlic (Allium sativum), Tarragon (Artemsia Dracunculus), Paprika (Capsicum annuum), Caraway (Carum carvi), Cinnamon (Cinnamomum verum), Coriander (Coriandrum sativum), Ginger (Zingiber officinale), Laurel (Laurus nobilis), Lovage (Levisticum officinale), Basil (Ocinum basilicum), Oregano (Origanum vulgare),Poppy (Papaver somniferum) Black pepper (Piper nigrum), Sage (Salvia officinalis), Susan (sesame indicum), Mustard (Sinapis alba), Clove (Syzygium aromaticum), Thyme (Thymus vulgaris), Allspice (Pimenta dioica), Parsley (Petroselinum crispum), Nutmeg (Myristica fragrans), Saffron (Crocus sativus), Rosemary (Rosmarinus officinalis).*

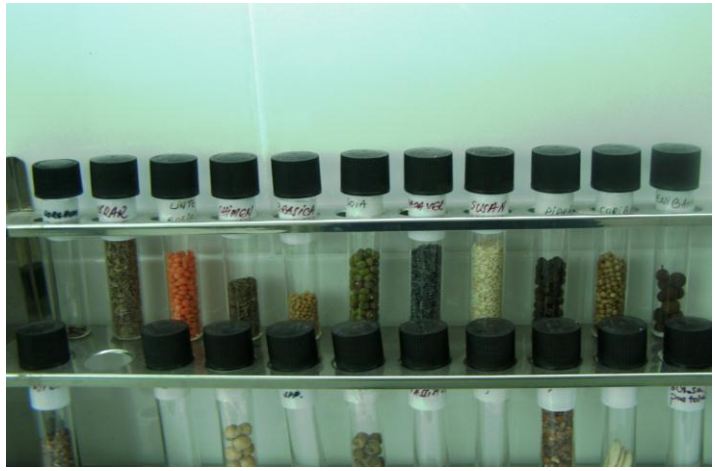


Fig. 1. Collection samples of dried spices, herb and vegetable seasonings

Working method

The experimental protocol includes: quantitative bacteriological and mycological exam:

-SR EN ISO 4833/2003-Horizontal method for enumeration of microorganisms. Colony count technique at 30^0C;

-ISO 15213/2003- Horizontal method for enumeration of sulphite-reducing bacteria in anaerobic conditions developed;

-ISO 21528-2/2007-Horizontal method for detection and enumeration Enterobacteriaceeas. Part 2: Method for enumeration of colonies;

-SR ISO 21527/2009 Microbiology. Horizontal method for enumeration of yeasts and molds. Parts 2: Colony count technique in products with water activity less than or equal to 0.95.

Results

Total aerobic mesophilic plate count (CFU/g) was performed on 67 samples of spices and vegetable seasonings (Tab.no.1).

Table 1. Total mesophilic bacteria (CFU / g) at 30^0C /g

Assortment	**CFU/g**		
	Batch 1	Batch 2	Batch 3
Garlic (Allium sativum)	$2.3x10^5$	$1.9x10^5$	-
Tarragon (Artemsia Dracunculus)	$4.7x10^6$	$4.3x10^6$	$3.9x10^6$
Paprika (Capsicum annuum)	$1.3x10^7$	$2.3x10^6$	$1.8x10^7$
Caraway (Carum carvi)	$8.1x10^6$	$7.3x10^6$	$8.2x10^6$
Cinnamon (Cinnamomum verum)	$4.5x10^5$	$3.8x10^5$	$4.9x10^5$
Coriander (Coriandrum sativum)	$5.3x10^5$	$7.4x10^5$	$6.9x10^5$
Ginger (Zingiber officinale)	$9.1x10^4$	$8.8x10^4$	$8.9x10^4$
Laurel (Laurus nobilis)	$7.7x10^6$	$8.2x10^6$	$7.9x10^6$
Lovage (Levisticum officinale)	$4.4x10^7$	$5.1x10^6$	$4.7x10^7$
Basil (Ocinum basilicum)	$5.7x10^6$	$7.3x10^6$	$5.2x10^6$
Oregano (Origanum vulgare)	$6.1x10^4$	$8.5x10^4$	$7.8x10^4$

Poppy (Papaver somniferum)	$1.1x10^7$	$1.3x10^7$	$1.9x10^7$
Black pepper (Piper nigrum)	$7.6x10^7$	$9.4x10^7$	$9.1x10^7$
Sage (Salvia officinalis)	$2.3x10^5$	$2.3x10^4$	$1.9x10^5$
Susan (sesame indicum)	$8.8x10^5$	$9.2x10^4$	$7.9x10^5$
Mustard (Sinapis alba)	$7.1x10^7$	$8.6x10^5$	$8.2x10^7$
Cloves (Syzygium aromaticum)	$5.5x10^6$	$9.1x10^6$	$8.0x10^6$
Thyme (Thymus vulgaris)	$6.8x10^6$	$8.4x10^7$	$8.7x10^6$
Allspice (Pimenta dioica)	$9.2x10^6$	$8.7x10^5$	$8.9x10^6$
Parsley (Petroselinum crispum)	$2.1x10^7$	$2.7x10^7$	$1.5x10^7$
Nutmeg (Myristica fragrans)	$7.4x10^5$	$7.1x10^5$	$8.7x10^5$
Saffron (Crocus sativus)	$8.2x10^3$	$7.7x10^3$	-
Rosemary (Rosmarinus officinalis)	$9.1x10^4$	$7.6x10^4$	$8.5x10^4$

Results of enumeration of sulphite-reducing bacteria in anaerobic conditions developed (anaerobic total number-CFU/g).

Total mesophilic anaerobic bacteria was performed on the same samples from three different lots of spices. The results are summarized in Table.no. 2.

Table 2. Total sulphite-reducing bacteria (anaerobic bacteria -CFU / g)

Assortment	**CFU/g**		
	Batch 1	Batch 2	Batch 3
Garlic (Allium sativum)	$1.9x10^3$	$1.4x10^3$	-
Tarragon (Artemsia Dracunculus)	$7.4x10^2$	$8.3x10^2$	$8.1x10^2$
Paprika (Capsicum annuum)	$3.5x10^4$	$3.8x10^4$	$4.2x10^4$
Caraway (Carum carvi)	$9.1x10^3$	$8.7x10^3$	$8.9x10^3$
Cinnamon (Cinnamomum verum)	$6.6x10^2$	$5.8x10^2$	$5.7x10^2$
Coriander (Coriandrum sativum)	$7.7x10^3$	$7.1x10^3$	$8.3x10^3$
Ginger (Zingiber officinale)	$9.1x10^2$	$8.7x10^2$	$9.0x10^2$
Laurel (Laurus nobilis)	$5.4x10^2$	$5.0x10^2$	$5.9x10^2$
Lovage (Levisticum officinale)	$7.0x10^3$	$7.6x10^3$	$6.9x10^3$
Basil (Ocinum basilicum)	$9.6x10^2$	$9.2x10^2$	$9.1x10^2$
Oregano (Origanum vulgare)	$4.3x10^2$	$4.0x10^2$	$5.2x10^2$
Poppy (Papaver somniferum)	$8.8x10^3$	$8.7x10^3$	$9.1x10^3$
Black pepper (Piper nigrum)	$3.7x10^4$	$2.9x10^4$	$3.5x10^4$
Sage (Salvia officinalis)	$5.1x10^2$	$5.6x10^2$	$5.0x10^2$
Susan (sesame indicum)	$2.9x10^2$	$2.3x10^2$	$2.3x10^2$
Mustard (Sinapis alba)	$5.8x10^3$	$6.2x10^3$	$5.5x10^3$
Cloves (Syzygium aromaticum)	$8.3x10^2$	$8.3x10^2$	$8.1x10^2$
Thyme (Thymus vulgaris)	$9.4x10^2$	$8.9x10^2$	$8.6x10^2$
Allspice (Pimenta dioica)	$6.5x10^4$	$7.1x10^4$	$7.0x10^4$
Parsley (Petroselinum crispum)	$4.8x10^4$	$4.9x10^3$	$4.6x10^4$
Nutmeg (Myristica fragrans)	$2.7x10^3$	$2.1x10^3$	$2.1x10^3$
Saffron (Crocus sativus)	$1.3x10^2$	$1.0x10^2$	-
Rosemary (Rosmarinus officinalis)	$2.1x10^2$	$2.6x10^2$	$2.3x10^2$

MPN of Enterobacteriacee(fig. no.2.) was performed according to Order no. 27 of June , 2011 on microbiological criteria for approval and food hygiene, other than those mentioned

in Regulation (EC)no. 2073/2005 of November 2005 on microbiological criteria for foodstuffs; results are summarized in Table. no.3.

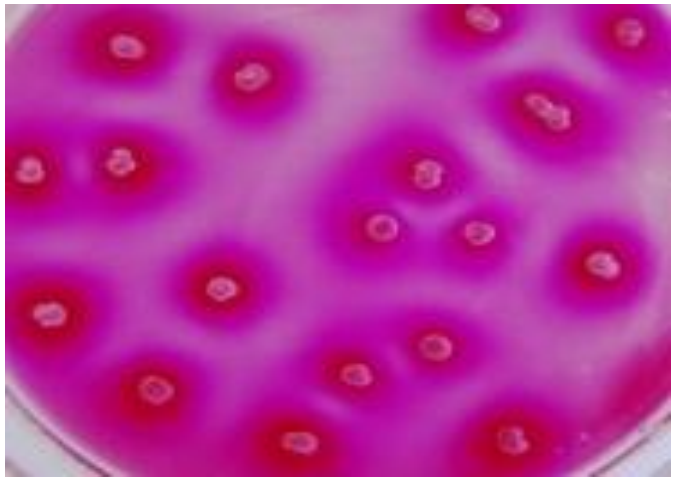


Fig.2. Enterobacteriaceae (VRBG)- Coriander (Coriandrum sativum)

Table 3. Enterobacteriaceae enumeration (CFU/g)

Assortment	***Enterobacteriaceae (CFU)/g***		
	Batch 1	Batch 2	Batch 3
Garlic (Allium sativum)	$3.1x10^2$	$2.4x10^2$	-
Tarragon (Artemsia Dracunculus)	7.2x10	2.8x10	6.1x10
Paprika (Capsicum annuum)	$4.5x10^3$	$3.0x10^3$	$3.5x10^3$
Caraway (Carum carvi)	$2.1x10^2$	$1.7x10^2$	2.3x10
Cinnamon (Cinnamomum verum)	$4.1x10^2$	$4.8x10^2$	$3.2x10^2$
Coriander (Coriandrum sativum)	$1.7x10^3$	$1.1x10^3$	$1.3x10^2$
Ginger (Zingiber officinale)	$2.1x10^2$	$5.2x10^2$	$3.4x10^2$
Laurel (Laurus nobilis)	$6.8x10^2$	$6.1x10^2$	$6.5x10^2$
Lovage (Levisticum officinale)	$4.3x10^2$	$5.6x10^2$	$6.1x10^2$
Basil (Ocinum basilicum)	$8.7x10^2$	$5.1x10^2$	$5.4x10^2$
Oregano (Origanum vulgare)	9.3x10	7.0x10	9.2x10
Poppy (Papaver somniferum)	$7.5x10^2$	$5.1x10^2$	$8.4x10^3$
Black pepper (Piper nigrum)	$6.7x10^3$	$1.9x10^2$	$7.5x10^3$
Sage (Salvia officinalis)	$3.2x10^2$	$4.1x10^2$	$3.9x10^2$
Susan (sesame indicum)	$1.9x10^2$	$2.3x10^2$	$2.3x10^2$
Mustard (Sinapis alba)	$1.8x10^2$	$1.2x10^2$	$8.5x10^2$
Cloves (Syzygium aromaticum)	$3.2x10^2$	$3.8x10^2$	$4.1x10^2$
Thyme (Thymus vulgaris)	$9.3x10^2$	$9.9x10^2$	$9.2x10^2$
Allspice (Pimenta dioica)	$7.5x10^3$	$8.1x10^3$	$5.8x10^3$
Parsley (Petroselinum crispum)	$3.1x10^2$	$4.2x10^2$	$3.6x10^2$
Nutmeg (Myristica fragrans)	$6.3x10^2$	$7.1x10^3$	$8.2x10^3$
Saffron (Crocus sativus)	1.1x10	1.4x10	-
Rosemary (Rosmarinus officinalis)	4.3x10	5.6x10	4.1x10

The total number of yeasts and molds (NTD + M)(*fig.3-4)*. Examinations were performed on the same samples and at the same time as the determinations for aerobic, anaerobic and enterobacteriaceae bacteria; results are summarized in Table.no.4.

Table 4. The total number of yeasts and molds

Assortment	NTD+M /g(UFC)/g		
	Batch 1	Batch 2	Batch 3
Garlic (Allium sativum)	$7x10^4$	$9.5x10^4$	-
Tarragon (Artemsia Dracunculus)	$1.6x10^4$	$1.3x10^4$	$1.7x10^4$
Paprika (Capsicum annuum)	$3.9x10^5$	$5.2x10^5$	$6.7x10^4$
Caraway (Carum carvi)	$8.7x10^4$	$7x10^4$	$7x10^4$
Cinnamon (Cinnamomum verum)	$6.2x10^4$	$5.5x10^4$	$4.2x10^4$
Coriander (Coriandrum sativum)	$9.3x10^5$	$4.8x10^5$	$7.7x10^5$
Ginger (Zingiber officinale)	$4.9x10^3$	$6.2x10^3$	$5.7x10^3$
Laurel (Laurus nobilis)	$1.9x10^5$	$7.2x10^5$	$9.6x10^5$
Lovage (Levisticum officinale)	$2.9x10^6$	$8.1x10^5$	$4.7x10^6$
Basil (Ocinum basilicum)	$7.7x10^5$	$1.5x10^5$	$6.5x10^5$
Oregano (Origanum vulgare)	$5.4x10^3$	$7.1x10^3$	$5.5x10^3$
Poppy (Papaver somniferum)	$3.5x10^6$	$1.8x10^6$	$6.9x10^5$
Black pepper (Piper nigrum)	$4.1x10^6$	$6.4x10^6$	$5.3x10^6$
Sage (Salvia officinalis)	$7.9x10^2$	$8.2x10^2$	$8.4x10^2$
Susan (Sesame indicum)	$9.2x10^4$	$8.7x10^5$	$9.5x10^4$
Mustard (Sinapis alba)	$9.2x10^6$	$1.9x10^6$	$4.5x10^6$
Cloves (Syzygium aromaticum)	$2.8x10^4$	$6.7x10^5$	$7.1x10^5$
Thyme (Thymus vulgaris)	$1.9x10^6$	$2.6x10^6$	$8.1x10^6$
Allspice (Pimenta dioica)	$2.2x10^6$	$5.6x10^6$	$4.9x10^6$
Parsley (Petroselinum crispum)	$4.7x10^6$	$5.9x10^6$	$5.9x10^5$
Nutmeg (Myristica fragrans)	$5.9x10^4$	$6.4x10^5$	$7x10^4$
Saffron (Crocus sativus)	$4.8x10^2$	$3.6x10^2$	-
Rosemary (Rosmarinus officinalis)	$6.9x10^4$	$5.6x10^4$	$6.1x10^4$

Fig. 3. NTD+M(medium DG18 of 5 days thermostatic)-Nutmeg(Myristica fragrans)

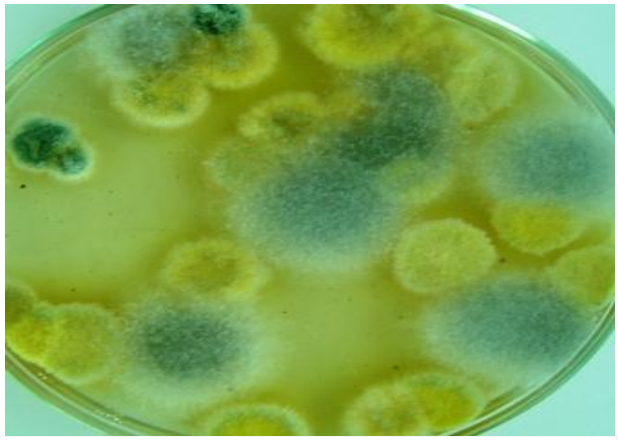


Fig. 4. NTD+ M(medium DG 18 of 5 days thermostatic)-Rosemary(Rosmarinus officinalis)

Analysis results

To 67 samples of spices and quantitative bacteriological determinations were made on:

• determine the total number of bacteria that grow at 30 ^{0}C;
• determining the total anaerobic bacteria;
• detection and enumeration enterobacteriaceae.

- The total number of bacteria that grow at 30 ^{0}C is summarized as follows: 2,99% of sample below 10^4 ; 16,42% between 10^4-10^5/g; 23,88% between 10^5-10^6 / g; 31,34% between 10^6-10^7 /g ; 25,37% above 10^7; (tab.no. 5).

Table 5. Total mesophilic bacteria (CFU / g) at 30^{0}C /g) (total sample weight percentage)

CFU/g	NUMBER OF SAMPLES	%
10^3	2	2,99%
10^4	11	16,42%
10^5	16	23,88%
10^6	21	31,34%
10^7	17	25,37%

- The total number of anaerobic bacteria is summarized as follows: 52,24% of sample below 10^3;29,85% between 10^3-10^4/g;17,91% above 10^4; (tab.no. 6).

Table 6. Total anaerobic bacteria (CFU / g)) (total sample weight percentage)

CFU/g	NUMBER OF SAMPLES	%
10^2	35	52,24%
10^3	20	29,85%
10^4	12	17,91%

- The total number of Enterobacteriaceae bacteria is summarized as follows: 16,42% of sample below 10^2 ; 56,72% between 10^2-10^3/g ; 26,18% above 10^3; (tab.no. 7).

Table 7. Enterobacteriaceae(CFU / g) (total sample weight percentage)

CFU/g	NUMBER OF SAMPLES	%
10^1	11	16,42%
10^2	38	56,72%
10^3	18	26,18%

- Results of fungal contamination(fig. no. 5-8) were located within the following limits:7.46% below 10^3/g, 8.96% between 10^3-10^4 /g, 31.34% between 10^4-10^5/g, 23.88% between 10^5-10^6/g, 28.36% above 10^6 /g (Tab.no.8).

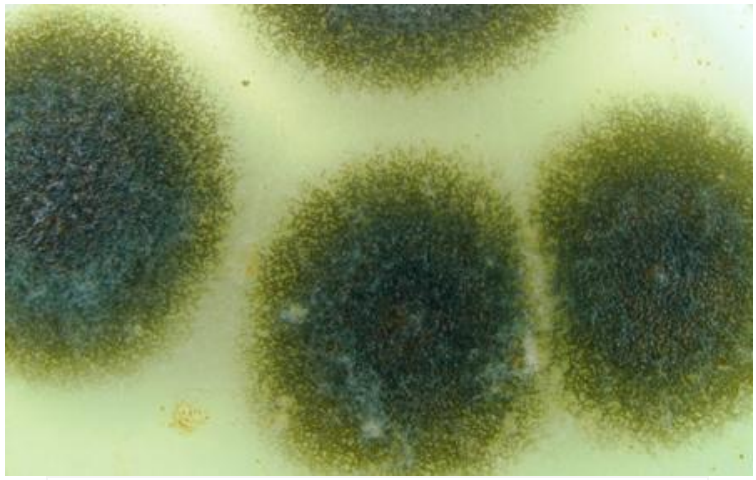


Fig. 5.NTD+M(medium DG 18 of 4 days thermostatic)-Parsley(Petroselinum crispum)

Table 8. NTD+M(total sample weight percentage)

NTD+M(CFU)/g	NUMBER OF SAMPLES	%
10^2	5	7,46%
10^3	6	8,96%
10^4	21	31,34%
10^5	16	23,88%
10^6	19	28,36%

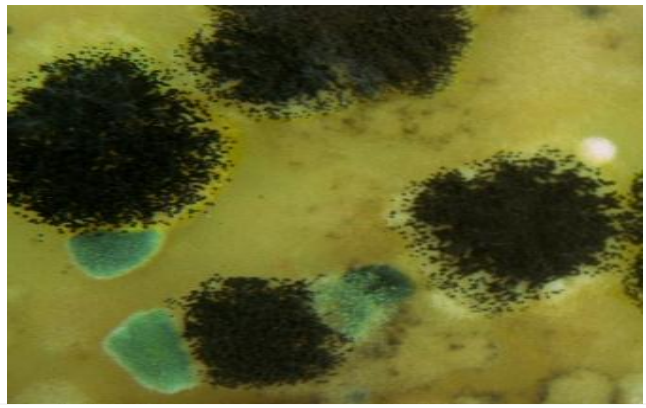


Fig. 6. NTD + M(medium DG 18 of 5 days thermostatic)-Allspice (Pimenta dioica)

Fig. 7. NTD + M(medium DG 18 of 4 days thermostatic)-Cinnamon (Cinnamomum verum)

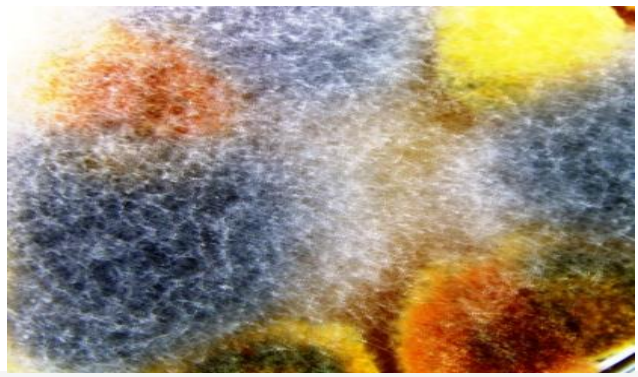


Fig. 8. NTD + M (medium DG 18 of 5 days thermostatic)-Laurel (Laurus nobilis)

Conclusions

1. Were examined 67 samples of spices from 3 manufacturers, and chose the 23 kinds of commonly used food composition; simultaneously performed quantitative bacteriological examinations: total number of bacteria that grow at 30^0C, anaerobic

bacterial flora, Enterobacteriaceae enumeration and quantitative mycological examination.

2. Maximum limits for mezophilic bacteria at 30^0C (max. 10^5) expressed in CFU /g, were exceeded in 38 samples representing 56.71% of all samples examined.
3. Maximum limits for total sulphite-reducing bacteria (max. 10^3 anaerobic bacteria - CFU/g), were exceeded in 12 samples representing 17.91% of all samples examined.
4. Maximum limits for Enterobacteriaceae (max. 10^2 expressed in CFU /g), provided ANSVSA ORD. no. 27/2011, were exceeded in 18 samples representing 26,18% of all samples examined.
5. Maximum limits for the total number of yeasts and molds (max. 10^5 expressed in CFU/g), provided ANSVSA ORD.No.27/2011 were exceeded in 35 samples representing 52.23% of all samples examined.
6. Highest values for microbiological parameters examined were recorded samples: Paprika *(Capsicum annuum)*, Lovage *(Levisticum officinale*), Mac (*Papaver somniferum*) Black pepper (*Piper nigrum*), Mustard (*Sinapis alba*), Parsley (*Petroselinum crispum*), Allspice (*Pimenta dioica*), Coriander (*Coriandrum sativum*), Laurel *(Laurus nobilis*).
7. The poor microbiological contamination had spices: Saffron (*Crocus sativus*), Sage *(Salvia officinalis*), Rosemary *(Rosmarinus officinalis*) and Oregano (*Origanum vulgare*).
8. Prevention of microbial contamination in dried herbs and spices lies in the application of good hygiene practices during growing, harvesting and processing from farm to fork, and effective decontamination. In addition, good and correct food handling practices and usage of herbs and spices by end users is essential.

References

1. ***ISO 21527/2008 - Microbiology. Horizontal method for enumeration of yeasts and molds. Partea 2: colony count technique in products with water activity less than or equal to 0.95;
2. ***ISO 7218/2009 - general guidance for microbiological examinations;
3. ***ISO 21528-2/2007 - Horizontal method for detection and enumeration Enterobacteriacee. Part 2: Method for enumeration of colonies.
4. *** SR EN ISO 4833/2003 - Horizontal method for enumeration of microorganisms. Colony count technique at 30^0C.
5. ***SR 13462-2:2002. - The risk analysis. Critical control points and guide its implementation;
6. *** World Healt Organization / Food and Agriculture Organization of the United Nations, 2006, Codex Alimentarius Commission Procedural Manual. Sixteenth edition, Codex Alimentarius, ISSN 1020-8070;
7. *** HG 924/2005- General hygiene of foodstuffs;
8. *** HG 685/2009 amending and supplementing Government Decision no. 106/2002 on food labeling;
9. *** Order no. 27 of June 6, 2011 on microbiological criteria for approval and food hygiene, other than those mentioned in Regulation (EC) no. 2.073/2005 of 15 November 2005 on microbiological criteria for foodstuffs;
10. *** Regulation 1334/2008 - on flavorings and certain food ingredients with flavoring properties for use in and on foods.
11. ***http://www.codexalimentarius.com;
12. ***http://www.fda.gov;
13. ***http://www.europa.eu.int/eur-lex.
14. *** www.doctorfungus.org
15. ***http://www.condimenteweb.ro/

MONITORING THE IMPACT OF THE DOG ORAL INFECTION ON THE IMMUNE SYSTEM HUMORAL EFFECTORS

Cristina RÎMBU[1], Eleonora GUGUIANU[1], Cristina HORHOGEA[1], Cătălin CARP-CĂRARE[1] , Ivona LAIU[1], Ramona STUPARIU[2]
[1] Facultatea de Medicină Veterinara Iaşi, Aleea Mihail Sadoveanu, nr. 8
[2]Laboratorul de analize TRITEST Iaşi
crimbu@yahoo.com

Abstract
Ignoring an outbreak of oral infection can have serious consequences on the whole body. The etiology of oral diseases is polyfactor and dependent of antiinfectious, local and general, resistance of organism. The immune response against various infectious antigens are the main way to eliminate and prevent infections caused by potentially pathogenic microorganisms. The aim of this study was to achieve an overall monitoring of the impact of oral infection on the humoral immune system effectors, local and general. The research was conducted only on dogs with various oral diseases, but healthy in general clinical examination. This selection followed the elimination of immune responses caused by other diseases than those in the mouth area.The correlation of immunological results (lysozyme, serum and salivary immunogram, serum complement C3 fraction) showed local and systemic impact of oral diseases with bacterial etiology. Most aggressive immune response to oral antigenic stimulation was found in cases with periodontitis, associated with other oral lesions or single lesion. Quantification of salivary immunoglobulins, in particular, salivary IgA as a marker of oral disease, should allow assessment of local immune system and possible risk factors on the whole body.

Introduction

Oral cavity mucosa is one of the main gates of entry for antigens in the body. Attachment of microorganisms to mucosal surface is a prerequisite for developing an infectious process. Antibacterial humoral immunity, represented by different antibodies, is the first line of attack against bacteria, both extracellular habitat and intracellularly (Târziu E., 2004).

The immune response against various infectious antigens are the main way to eliminate and prevent infections caused by potentially pathogenic microorganisms. Chronic inflammatory process occurs when acute inflammation is incomplete, and pathogen assemblages act continuously in a situation of altered immune response (Carp-Cărare M., 2002).

The immune system has the ability to respond to the action of pathogens, which interact specifically, being able to recognize and selectively eliminating foreign microorganisms and macromolecules (Carp-Cărare M,. 2002; Tîrziu E., 2004). Its inefficiency is evident when the infection becomes chronic. Mediators of inflammation can damage tissues that support teeth, leading to the formation of gum pockets, gingival recession, tooth and bone resorption (Williams CA, 1992).

Against bacteria responsible for infections localized to the entrance gate, represented by mucous surfacec, local humoral immune response is of great importance. Various antigenic stimuli, acting at the mouth, inducing a massive increase of all classes of salivary immunoglobulin (IgA, IgG, IgM).

In the mouth, there are two factors (saliva and crevicular fluid), whose composition is different, because of the structural elements they produce.

Antibacterial function of saliva is achieved through a complex mechanism, involving the salivary proteins, immunoglobulins secreted by the salivary glands (secretory IgA and IgM) and from crevicular fluid (IgM, IgG), neutrophil leukocytes.

Crevicular fluid (ditch the gum) is a liquid that contains factors of serum origin and the amount is inconsistent, depending on the inflammatory status of the production place.
Through the intercellular spaces, there is a passive output of fluid components, consisting of various proteins of serum origin among which gamma-globulins (IgG, IgA, IgM).

In these secretions the predominant immunoglobulins are IgA produced locally (secretory IgA) or from transsudat. IgAs antimicrobial function occurs by preventing adhesion and subsequent colonization of bacteria in the oral mucosal, gastrointestinal epithelial cells or enamel, interfering with attachment molecules (adhesion) on the surface of bacteria. IgAs effectively neutralizes any exotoxin produced by bacteria that multiply in the lining mucosa.

At the **systemic level**, the most important classes of immunoglobulins involved in antibacterial immune response are IgG, IgM, IgA (Carp-Cărare M., 2002).

Occurring IgG molecules in the body after secondary antigenic stimulation are the main antibody with role in neutralizing bacterial toxins, viruses, opsonic phagocytosis, antibody-dependent cytotoxicity and complement activation. IgG production increases in the secondary humoral immune response, one that takes place or is triggered in case of vaccination or repeated contact between the immune system and immunogenic (Tîrziu E., 2004).

IgM plays an important role in the defense, being the first immunoglobulin that forms after an infection or vaccination. It is also most effective in activating complement. It can be transported to mucosal secretions, provides their protection besides secretory IgA, having a secondary role of secretory immunoglobulin (Carp-Cărare M.,2002; Tîrziu E., 2004).
Serum IgA has a weak activity against antigens induced by systemic immunization, a weak opsonic effect and has no capacity to fix complement.

Elimination of absorbed antigens is very important in defending the body and thus preventing their access to immune cells and stimulating a larger response, which could divert defense resources from their normal antiinfectious protective function (Tîrziu, 2004).

Material and methods

Determination of immunoglobulin levels is one of the most commonly used methods to assess immune competence in dogs. Sampling was performed on 20 common breed dogs, identified with oral affections of bacteria origin. There were selected only animals who had mouth disease, without any organic disease, that would have changed the parameters that our study followed.

Reference values of serum immunoglobulins in dogs vary from one study to another, so that was formed and examined a control group, consisting of 10 clinically healthy dogs, corresponding as age with the patient group.

The objectives were the assessment of concentrations of lysozyme in saliva, the IgA, IgG and IgM in saliva and blood serum and serum complement to these groups.
The biological material used was represented by blood samples collected in tubes with and without sodium citrate and saliva samples collected with 1 ml of sterile syringes. Blood was collected by puncture of large vessels (cephalic vein and jugular vein) in tubes without

anticoagulant for serum expression used in serological tests. Harvesting and transport was carried out under appropriate conditions.

The samples of blood, serum and saliva, collected from the studied cases were subjected to immunological analysis of Microbiology-Immunology Laboratory of the Faculty of Veterinary Medicine Iasi and Medical Laboratory Tritest Iasi.

Determination of lysozyme was achieved by spectophotometric method which is based on the changing of a bacterial suspension degree of clarity or opacity, in contact with existing lysozyme in saliva samples. The degree of clarification is proportional to the concentration of lysozyme (Carp-Cărare M.,1998).

Qualitative and quantitative determination of immunoglobulins in serum and saliva samples were analyzed by immunoenzymatic ELISA method using automated analyzer Chemwell Manager 2902. The system has applications both for veterinary and for human.

Qualitative and quantitative determination of serum complement C3 fraction was performed using immunology automated analyzer Immulite (Siemens, Germany). This system utilizes chemiluminescence method enzyme enhanced, detecting extremely low concentrations, due to sensitivity of 10-21 mol/l (according to the manufacturer protocol overview).

Results and discussion

Results of determination of lysozyme in saliva samples

Evaluarea spectofotometrică a salivei prelevată de la cei 10 câini, care au constituit lotul mator (clinic sănătoase), a dus la obţinerea unor concentraţii variabile de lizozim.

Spectophotometric evaluation of saliva taken from the 10 dogs that were the clinically healthy group, has led to varying concentrations of lysozyme. Comparing the results obtained in the control group (average 118 mg/ml in dogs) with reference values in dogs showed varying differences. They may be the consequence of specific features related to race and age, so reporting the average values obtained in dogs with oral disease was to the control group average.

Measurements performed on saliva samples taken from dogs with diseases of the mouth indicate a high titer of lysozyme, whose average was 145,05 mg/ml, showing an expansion of activity of immune defense forces (table 1).

Table 1. Values of lysozyme in saliva

Specie	**Lysozyme values**		
	Reference values	Limit values Control group (n=10)	Limit values Stomatitis group (n=20)
Dog	113,0±8,25 µg/ml	105,8-130,2 µg/ml. **118 µg/ml.**	103,5-180,6 µg/ml **145.05 µg/ml**

We believe that this increased amount of lysozyme in saliva of dogs with oral disease in various stages of development, may be the consequence of increasing number of polymorphonuclears, being an indication of the infectious process and of favorable evolution, because the body defends itself by mobilizing pool of medullary polymorphonuclear (PMN) passing in the circulation.

Results of determinations IgA, IgG and IgM in serum and saliva

Average values obtained on control groups were compared with those of references (table 2).

In the analysis performed on samples of serum and saliva from dogs with oral diseases have emerged values oscillating around the limits set in other reference sources for IgA, IgG and IgM. Since in literature there are large variations within limits or average concentrations of serum immunoglobulins and especially salivary (limited sources), we found it necessary to report the results obtained in the group of animals with oral diseases to the ones of the control group. This aspect is supported by the possible race geographic specific from the area of study, knowing that there are variations in the concentrations of immunoglobulins, depending on the purity of breeds (HY Reynolds, 1970).

Table 2. Values of serum and salivary immunoglobulins in dogs by ELISA

Specie	**Sourse**	**Serum mg/dl**			**Saliva mg/dl**		
		IgA	IgG	IgM	IgAs	IgG	IgM
Dogs	Reference values (bibliography)	<150[b]	<2000[b]	<270[b]	<52[b]	<1,5[b]	<3,3[b]
		20-150[a]	1000-2000[a]	70-270[a]	26-47[d]	0,5-5	0,5-7
	Control group (n=10)	**112-350**	**945-2600**	**150-280**	**45-78**	**1,5-3**	**2-6**

a. Tizard, 2004,2009; b. Heddle şi Rowley, 1975; c. Yamado şi colab, 1984; d. Kikkawa A., 2003 e.Harly R. 1998 (ELISA)

Results of IgA, IgG and IgM determinations in serum and saliva collected from dogs with oral diseases

Determinations on samples of serum and saliva from dogs with oral lesions such as bacteria, showed a significant increase in most groups of immunoglobulins (table 3).

Origin of serum and salivary immunoglobulins was defined in dogs (Hedda TJ, 1975). Some recent studies showed the origin of salivary IgG as a result of transsudation from the serum, and IgMs IgAs being produced by local immune system (Stokes, 2006).

Fluctuation of values obtained in determining the quantities of serum and salivary immunoglobulins in dogs with various diseases of the mouth are presented in fig. 1 and fig. 2.

Of the 20 dogs with oral diseases, 14 were found to increase the upper limit concentration (<70 mg/dl) set by the control group. Salivary IgA titers fluctuated from 78 mg/dl (palatinitis, oral abscesses, oropharynx) to 140 mg/dl in a case of chronic periodontitis. Serum IgA titers that exceeded the upper limit ranged from 378 mg/dl (dental abscess, periodontitis - orosynus fistula) to 550 mg/dl (periodontitis, palatinitis, oropharynx).

An overall analysis of data from the group of dogs with oral diseases has led to the identification of nine cases below the detection of serum (<112 mg/dl) and salivary (<45 mg/dl) immunoglobulin A. This deficiency was identified in 4 cases with gingivitis (2 females and 2 males), a case with periodontitis (female), a case with the oropharynx (male, serum IgA deficiency) and 2 glossitis (female and male).

In dogs, the presence of serum and salivary IgA deficiency is mentioned in some specific studies to be associated with inflammation of the intestinal tract (malabsorption

syndrome), mouth inflammation (gingivitis), glossitis and oropharynx, aspects that are correlated with our survey results (Al Plechner., 2003).

Typically, detection of increased titre of serum and salivary IgA, correlates with more severe oral lesions. IgAs protects mucous membranes, blocking attachment of bacteria to receptors on mucosal (German AJ, et al. 2004).

Table 3. Values of immunogram performed in dogs with oral diseases

Sex	Case no.	Oral lesions	Saliva mg/dl			Ser mg/dl		
			IgAs	IgG	IgM	IgA	IgG	IgM
			45-70	1,5-3	2-6	112-350	945-2600	150-280
Females - Adult 5-10 years	1	Early gingivitis	**23**	**1,5**	**2**	**90**	**3600**	**400**
	2	Gingivitis with hemorrhagic inflammation	**29**	**4,8**	**6,2**	**70**	**3858**	**470**
	3	Chronic periodontitis	**30**	**6,9**	**10,4**	**86**	**3528**	**412**
	4	Periodontitis, dental abscess	**100**	**4,8**	**6,6**	**420**	2474	**336**
	5	Periodontitis and gingival recession of canine	**100**	**4,2**	**8,5**	**550**	2240	**440**
	6	Periodontitis and oro-sinus fistula	**90**	**3,8**	**7,7**	**378**	2549	**302**
	7	Tonsillitis, gingivitis	**88**	**3,7**	**7,5**	330	2636	264
	8	Palatinitis oropharynx	**78**	**3,3**	**6,6**	**550**	2240	440
	9	Palatinitis, cavities	**80**	**3,4**	**6,8**	**390**	2528	312
	10	Glossitis	12	1,5	2,5	**100**	2600	280
Males - Adult 5-10 years	1	Gingivitis	**40**	1,7	3,4	**100**	2582	288
	2	Generalized gingivitis	**40**	1,7	3,4	**92**	**3488**	329
	3	Early periodontitis	**98**	**4,1**	**8,3**	**600**	2150	**480**
	4	Chronic periodontitis. abscess	**140**	**5,9**	**11,9**	**390**	2168	**472**
	5	Chronic periodontitis, edentulism, dental caries	**132**	**5,6**	**11,3**	**490**	2348	**392**
	6	Dental abscess, oro-nasal fistula	**80**	**3,4**	**6,8**	**380**	2546	**304**
	7	Abscessed tooth (canine)	**100**	**4,2**	**8,5**	320	**2654**	256
	8	Abscess on buccigiană mucosa	**78**	**3,3**	**6,7**	**390**	2528	**312**
	9	Oropharynx, gingivitis	**80**	**3,4**	**6,9**	100	**2928**	**412**
	10	Glossitis	**35**	2,4	3	150	**2654**	**286**

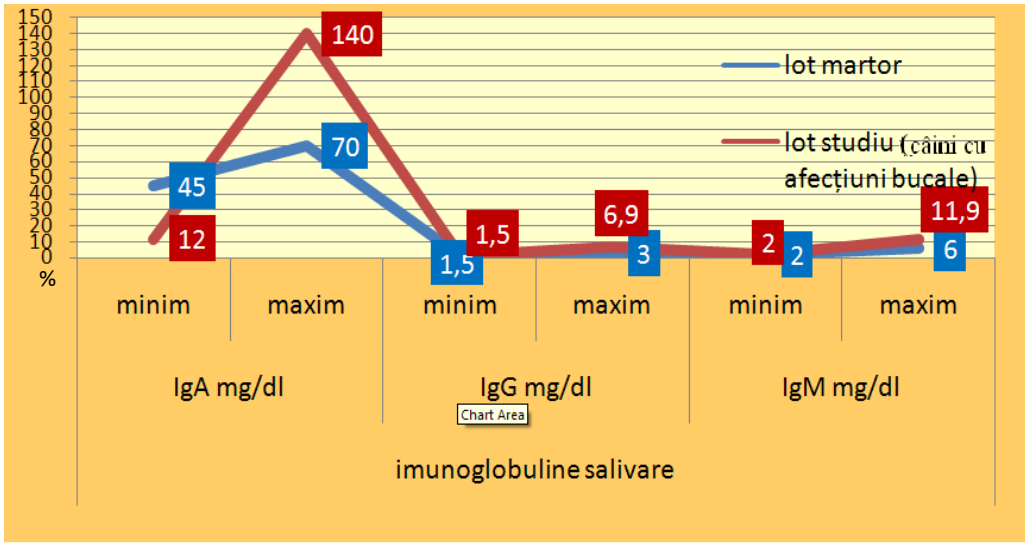


Fig. 1 - Variations of salivary immunoglobulins in dogs with oral diseases

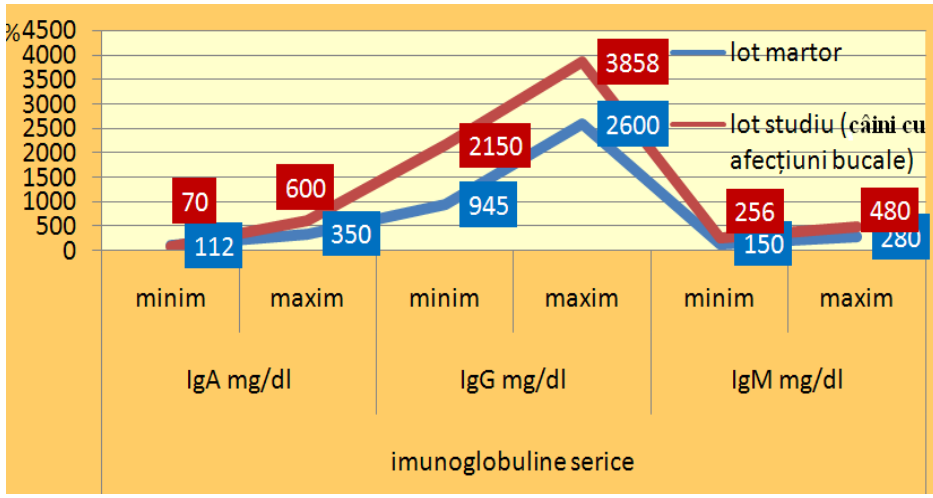


Fig. 2 - Variations of serum immunoglobulins in dogs with oral diseases

Determinations for IgG titer showed oscillating values titre in dogs with oral diseases. Salivary IgG titers, ranged between 3,3 mg/dl (abscesses, palatinitis, oropharynx) and 6,9 mg/dl (chronic periodontitis). Serum IgG titre ranged from 2636 mg/dl (tonsillitis, gingivitis) and 5250 mg/dl (chronic periodontitis), compared with the control group.

IgM immunological titration also showed values above the limits set by the control group in dogs (serum 60-280 mg/dl, saliva from 1,5 to 3,5 mg / dl). IgM synthesis precedes that of IgG in the primary immune response. Type IgM antibodies appear after a primary

antigenic stimulus, indicating the presence of an acute infectious process (Carp-Cărare M., 2002).

In dogs, evaluation of IgG in saliva led to the identification of elevated levels, with values ranging from 6,2 mg/dl (gingivitis with bleeding inflammation) and 11,9 mg/dl (chronic periodontitis). Serum IgG also suffered, quantitative changes that have fluctuated between 288 mg/dl (gingivitis) and 480 mg/dl (periodontitis).

Following investigations by serological enzyme immunoassay method, in most cases, increasing antibody titers was found. Hipergamaglobulinemia is the consequence of chronic infection. The values obtained varies from one condition to another. The strong increase in antibody titer was identified most frequently in chronic periodontitis, with or without other oral lesions. Detection of high titres of salivary antibodies could be because the epithelial barrier was destroyed by plaque, allowing penetration of the gum tissue by bacterial antigens and products of metabolism, leading to invasion by polymorphonuclear and high concentrations IgG, IgA, IgM (Carp-Cărare M., 2002).

Antigenicity of structural components of pathogenic microorganisms from the oral cavity and especially in the periodontal structures, produce, normally an intense systemic immune response, evidenced by increases in serum antibody concentrations. In this case, advanced oral lesions contribute to the complex immunopathological mechanisms.

It was found that not in all cases, humoral factors in the serum and saliva reacted to antigenic stimuli, existing a variable number of dogs with normal antibody titers. This may be due to the efficiency of local cellular immune components that interfere with phagocytosis to eliminate microorganisms. There is however a lack of correlation between serum and salivary immunoglobulins, suggesting that their combined investigation can not become a diagnostic indicator.

In conclusion, local and systemic immune response associated with oral disease remains an individual characteristic, existing individuals with an increased immune reactivity and thus, elevated levels of antibodies in both serum and saliva and individuals with reduced immune reactivity, reflected by a low titer of antibody in saliva and serum. Overall, however, most individuals with localized disease in the mouth, receive antiinfectious defense (antimicrobial), both systemic and local. We consider useful quantification of salivary immunoglobulins, in particular, salivary IgA, as a marker of oral disease that would allow assessment of local immune system and possible risk factors on the whole body.

Results of determination serum complement C3 fraction

Complement system plays an important role in body defense and inflammatory processes (Grecianu Al., 1986; Carp-Cărare M., 2002; Târziu, E.2004).

Antigen - antibody local reactions activates the complement, initiating mouth inflammatory process. Whereas it is difficult to establish the involvement of complement in local immune defense, C3 fraction levels were measured in blood serum as a reflection of oral disease on the entire immune system, correlated with antibody titers.

Evaluations of serum complement C3 in healthy dogs (control group) showed values ranging from 4,5 to 5,5 u/L. Following serological measurements, there were identified increased values in 13 from the 20 investigated dogs (table 4).

In dogs, the highest levels of complement were detected in cases with chronic periodontitis (6,5 to 6,8 u/L). In most investigated cases, the results obtained from determinations of complement C3 fraction correlates with increased antibody titer, which indicates the presence of infection progressing to chronicisation.

Complement system is the most important factor in antiinfectious nonspecific humoral defense. Some investigations have led to the idea that the complement system and antibodies of the IgG class from crevicular fluid opsonise existing bacteria, facilitating phagocytosis by polymorphonuclear (Manolescu M., Roşu L., 2000). The presence of complement promotes tissue destruction, contributing to chronic inflammation and pain and oral discomfort (M. Schaechter, 2004).

Bacterial biofilm, which is the primary cause of various diseases of the mouth and especially the gum, initially activates complement. (M. Schaechter, 2004). This explains the detection of C3 titers slightly increased above the upper limit in serum from cases of gingivitis, in which antibodies were within normal limits.

Table 4. Parameters of humoral immunity, evaluated in dogs with stomatitis

Sex	Case no.	Oral lesions	Serum			
			IgA mg/dl	IgG mg/dl	IgM mg/dl	C_3 u/L
			112-350	945-2600	150-280	4,5-5,5
Females - Adult 5-10 years	1	Early gingivitis	**90**	**3600**	**400**	4,5
	2	Gingivitis with hemorrhagic inflammation	**70**	**3858**	**470**	5,5
	3	Chronic periodontitis	**86**	**3528**	**412**	**6,8**
	4	Periodontitis, dental abscess	**420**	**2474**	**336**	**6,2**
	5	Periodontitis and gingival recession of canine	**550**	**2240**	**440**	**5,8**
	6	Periodontitis and oro-sinus fistula	**378**	**2549**	**302**	**6,2**
	7	Tonsillitis, gingivitis	**330**	**2636**	**264**	**6,5**
	8	Palatinitis oropharynx	**550**	**2240**	**440**	**6**
	9	Palatinitis, cavities	**390**	**2528**	**312**	**6,2**
	10	Glossitis	**100**	**2600**	**280**	5,5
- Adult 5-10 years	1	Gingivitis	**100**	**2582**	**288**	5,5
	2	Generalized gingivitis	**92**	**3488**	**329**	**5,9**
	3	Early periodontitis	**600**	**2150**	**480**	**6**
	4	Chronic periodontitis. abscess	**390**	**2168**	**472**	**6,2**
	5	Chronic periodontitis, edentulism, dental caries	**490**	**2348**	**392**	**6,5**
	6	Dental abscess, oro-nasal fistula	**380**	**2546**	**304**	4,2
	7	Abscessed tooth (canine)	**320**	**2654**	**256**	5,5
	8	Abscess on buccigiană mucosa	**390**	**2528**	**312**	6
	9	Oropharynx, gingivitis	**100**	**2928**	**412**	**6**
	10	Glossitis	**150**	**2654**	**286**	**5,5**

Conclusions

1. Increasing the amount of lysozyme in saliva of dogs (average 145,05 mg/ml) with oral disease, indicates an amplification of local immune defense forces.
2. Immunograma values in the control group consisting of healthy dogs were:
    a. serum-IgA 112-350 mg/dl, IgG, 945-2600 mg/dl, IgM 150-280 mg/dl
    b. saliva - IgA 45-78 mg/dl, IgG from 1,5 to 3 mg/dl, IgM, 2-6 mg/dl

3. Determinations on serum and saliva samples from dogs with oral lesions such as bacterial growth showed variable antibody
4. Evaluations of serum complement fraction C3, in the control group, showed values ranging from 4,5 to 5,5 u/L
5. The highest values of serum complement were found in dogs with chronic periodontitis (6,5 to 6,8 u/L).
6. Tie cases by sex was not relevant because there was no measurable difference
7. Correlation of immunological results (lysozyme, serum and salivary imunograma, serum complement C3 fraction) showed local and systemic impact of oral diseases with bacterial etiology
8. Most aggressive immune response to oral antigenic stimulation was found in cases with periodontitis, associated with other oral lesions or single lesions
9. Quantification of salivary immunoglobulins, in particular, salivary IgA as a marker of oral disease, should allow assessment of local immune system and possible risk factors on the whole body.

Bibliografy

1. Carp-Cărare M., 2002- *Imunologie şi Imunopatologie*, Casa de Editură Venus, Iaşi
2. German A.J., Hall E.J., Day M.J, 1998 - *Measurement of IgG, IgM and IgA conecntrations in canine serum, saliva, tears and bile.* Veterinary Immunology and Immunopatholohy, 8, 107-121.13
3. Grecianu AL., 1986 - *Microbiologie generală şi Imunologie*, Ed, Institutul Agronomic „Ion Ionescu de la Brad" Iaşi
4. Heddle R.J., Rowley D., 1975 - *Immunochemical characterization of dog serum parotid saliva, colostrum, milk and small bower fluid*, Immnology Veterinaire. 29, 185
5. Manolescu Mirela, Roşu Lucica, Ungureanu Anca, 1998 - *Aspecte generale privind flora microbiana implicată în afecţiunile stomatologice*, Editura Agora, Craiova
6. Plechner AL., 2003 - *Endocrine-Immune Mechanisms in animals and human health implications*. NewSage Press.Inc.
7. Reynolds H.Y., Johnson J.S., 1970 - *Quantitation of Canina Immunoglobulins*, Journal Immunology, v.105, 698-703
8. Schaechter M., 2004 - *The Desk Encyclopedia of Microbiology*, Academis Press Elsevier, USA
9. Stokes C., Walz Nashwa, 2006 - *Mucosal defence along the gastrointestinal tract of cat and dogs*, Veterinary Research 37; 281-293
10. Tîrziu E., 2004 - *Imunologie*, Ed. Brumar, Timişoara
11. Tizard I.R., 2009 - *Introduccion a la Inmunologia Veterinaria*, octava edicion, Ed. Elsevier, Spania
12. Williams C.A., Aller M. S.,1992 - *Gingivitis/stomatitis in cats.* Veterinary Clinics of North America Small Animal Practice, 22 (6), 1361-1383

STAPHYLOCOCCUS AUREUS – IMPLICATIONS OF THE ORAL CAVITY DISEASE AT THE DOG AND CAT

Cristina RÎMBU, Eleonora GUGUIANU, Cristina HORHOGEA, Mihai CARP-CĂRARE
Facultatea de Medicină Veterinară Iaşi, UŞAMV Iaşi,
crimbu@yahoo.com

Abstract
Microbiological investigations performed on different biological taken from the mouth to 200 dogs and 135 cats identify Staphylococcus aureus into a significant share. In dogs, were isolated and identified 13 (82,1%) strains of Staphylococcus aureus from abscesses, 4 (25%) strains of palatine, 10 (20.8%) strains from the oro-pharyngitis and tonsillitis, 13 (18,7%) strains of oro-sinus fistulas, 9 (15,5%) of gingivitis, and 2 (2,6%) strains of periodontitis. At cats, were isolated and identified 21 (53,8%) strains of Staphylococcus aureus from abscesses, 4 (26,6%) strains of oro-sinus fistulas, 8 (16%) strains from the oro-pharyngitis and tonsillitis, 8 (12,1%) strains of gingivitis, 1(10%) strains of palatinate and 7 (8,6%) strains of periodontitis. Coexistence into a common habitat led to transfer the risk of infection between humans and pets. The major implications of this species are given by the pathogenic factors, but also by the posibility to select some MRSA strains, which are strains with major health risk for the public.

Keywords: cat, dog, oral cavity, Staphylococcus

Introduction

Oral ecosystem is diverse, estimated about 500 commensal bacterial species, spread across various oral niches, with salivary microbiota (Marshall Z. J., 2010).

Most studies related to commensal and pathogenic microbiota of the mouth in humans were well detailed, while in dogs and cats are limited or poorly described studies (Elliott D.R., 2005).

The diversity of bacterial species emphasizes the importance of the habitat in the formation of local microbiota.

Allochthonous microorganism to organism, has a transient, sometimes disease character and implantes only in conditions in which normal symbiotic system is disrupted by the disappearance of a local micro.The balance reinstalls when the allochthonous microorganism will retake his niche, excluding the allochthonous species (Guguianu E, 2002). The diverse polymicrobian pathology of the mouth has been attributed to an impressive number of commesal and also transient bacteria.

In the mouth, the infection can develop as focal infection (dental abscess) or as a bacterial invasive nature (bacteremia) (O'Reilly P.G., 2000). It is known that, because saliva, all surfaces maintain a constant humidity that fosters the creation and development of a bacterial biofilm (Pavlika Z., 2006).

Staphylococcus aureus is considered an opportunistic pathogen, commonly found in humans, involved in various skin localized infections, sepsis and toxic syndromes shape (Gyles C.L., 2004). This species is rarely identified in mouth disease at pet carnivores (Harvey C.E., 1990; Guérin-Faublée V., 2000; Euzéby J.P., 2002; Elliott D. R, 2005).

In dogs and cats confined normal bacterial microflora in the mouth is divided into a variety of aerobic, facultative or strictly anaerobic (Pavlika Z., 2006).

In cats there however, in 1999, Lilenbaum W. et al. isolates from 150 samples of saliva, 100 strains of staphylococci of which 71% were coagulase negative (*Staphylococcus felis*, *Staphylococcus haemolyticus*, *Staphylococcus simulans*, *Staphylococcus saprophyticus*, *Staphylococcus epidemidis*) and 29% coagulase positive (*Staphylococcus intermedius*) without isolating *Staphylococcus aureus* too.

Guérin-Faublée V. and Euzby J.P. (2000), classifyed isolated bacteria from various oral diseases in dogs and cats but the implications of coagulase-positive mentions only in case of pharyngotonsillitis.

Materials and methods

Between 2007-2010, were investigated 200 dogs and 135 cats of mixed breeds, which show different mouth disease: gingivitis, periodontitis, abscesses, adenoids, glossitis, tonsils, adenoids, dental caries. The animals were part of casuistry Faculty of Veterinary Medicine and Veterinary of Iasi.

Sampling was carried aseptic conditions and varied, depending on location in infection and suspected etiologic agent properties. Pathological material were subjected to microbiological examination in the laboratory of Microbiology of the University Center for Veterinary Medicine, Faculty Medicine Veterinary Iasi.

In clinical microbiology, bacterial cultures of isolates aims pathological isolation, identification and susceptibility testing of bacterial strains to various antimicrobial substances, in order to initiate and monitor anti-infective.

For isolation and identification of staphylococci were used have used the usual culture media for aerobic bacteria (nutrient agar, nutrient broth), plain or supplemented with 10% sheep blood and special media, agar Chapmann to test the ability to ferment mannitol, agar Baird Parker favoring the multiplication of coagulase-positive (*Staph. aureus, Staph. intermedius*).

To determine pathogenicity in vitro, haemolysis test has been carried (as beta-hemolytic *Staphylococcus aureus*) and citrate plasma coagulation test to differentiate coagulase positive staphylococci (*S. aureus, S. intermedius*) of the coagulase negative (*S. epidemidis*) and streptococci.

Identification of *Staphylococcus aureus* was performed using API Staph identification systems, consisting of 20 microtest dehydrated form. Identification of bacterial species was based on interpretation of the code number, obtained after processing in biochemical results, as the Catalogue of interpretation (*API Staphy Analytical Profile Index*).

Senbility antibiotic profile of strains of *Staphylococcus aureus* has been shown by antibiogram, diffusion method. The purpose of these measurements was to identify the most effective antimicrobials that can be applied in veterinary dentistry.

Results and discussions

Identification of *Staphylococcus aures* corroboration was based on cultural aspects (fig. 1), morphology (fig. 2), biochemical (table 1) and pathogenicity.

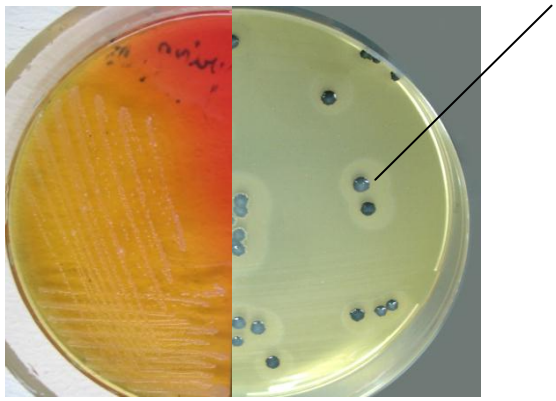


Fig. 1 Strain of *Staphylococcus aureus* on Baird Parker medium (left) and Chapmann environment

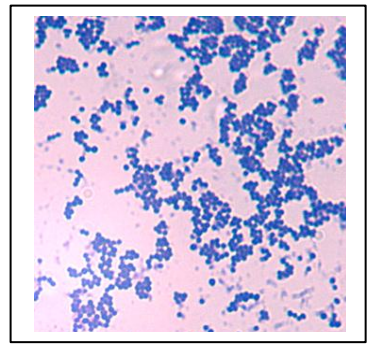


Fig.2 *Staphylococcus aureus*, Gram stain, MO 10x100

Table 1. Biochemical profil of the species *Staphylococcus aureus*

API STAPH	0	GLU	FRU	MNE	MAL	LAC	TRE	MAN	XLT	MEL
	M-	+	+	+	+	+	+	+	-	-
	NIT	PAL	VP	RAF	XyL	SAC	MDG	NAG	ADH	URE
	+	+	+	-	-	+	+	+	+	V

There are different clinical entities that vary with the severity of inflammation. The presence of bacteria in favorable conditions lead to different clinical forms in relation to several factors: the virulence of bacterial strains, the bacteria concentration and stamina (M. Manolescu, 1998).

The most common oral clinical entities who are gingivitis and periodontitis and cats. Appearance of other clinical forms (abscess, palatinitis, glossitis, fistulas, etc.), it seems, are favored by intolerance to dental plaque (bacterial) or due to exaggerated response of the local defense system, a mechanism that induces inflammation, which can graft pathogenic microorganisms that have complex equipment. Other causes are favorable genetic predisposition, environmental stress and diet (C. Gorell, J. Bellows, 2010).

Generally, tonsillitis and oro-pharyngitis are considered and treated as upper respiratory disease, and only tangentially, the effect of some oral diseases. For this reason, we selected cases of oro-pharyngitis and tonsillitis that were associated only with oral lesions.

The etiology of these clinical entities identified in dogs and cats of our study was polifactorială, which is not consistent with the literature data (C. Gorell, 2010; J. Bellows, 2010). Therefore, *Staphylococcus aureus* was isolated from mixed cultures, isolates obtained from oral pathology.

Analysis of data obtained show a significant incidence of *Staphylococcus aureus* strains isolated from various oral lesions, identified in dogs and cats.

Microbiological research conducted for isolates obtained from the 200 dogs with various oral diseases have led to the isolation and identification of 242 bacterial strains of which 41 (16,94%) strains of *Staph. aureus* (table 2, fig.3).

Table 2. The incidence of Staphylococcus aureus, isolated from oral diseases

	dogs			cats		
Mouth disease	Totally bacterial strains isolated	Strains *Staphy. aureus*		Totally bacterial strains isolated	Strains *Staphy. aureus*	
		Nr.	%		Nr.	%
Gingivitis	58	9	15,5	66	8	12,1
Periodontitis	76	2	2,6	81	7	8,6
Abscesses	28	13	82,1	39	21	53,8
Oro-sinus fistulas	16	3	18,7	15	4	26,6
Palatinitis	16	4	25	10	1	10
Oro-pharyngitis, tonsillis	48	10	20,8	50	8	16
All strains	242	41	16,94	261	49	18,7

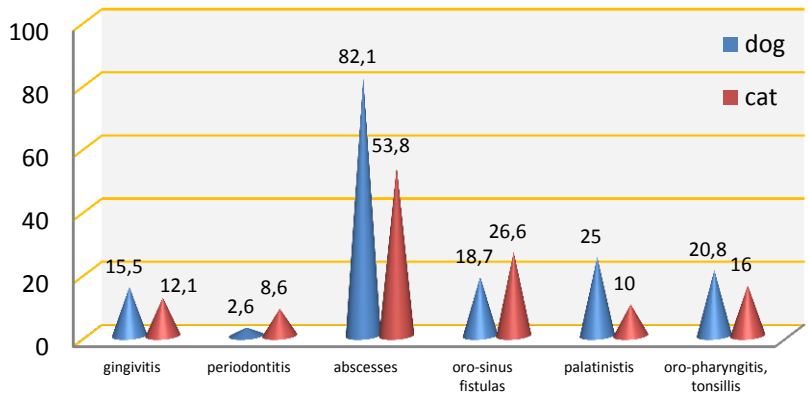


Fig. 3 Incidence of *Staphylococcus aureus* strains isolated from oral sampled

From the 135 cats with oral disease have been isolated and identified 261 bacterial strains of which 49 (18,7%) strains of *Staph. aureus*.

The roots were isolated and identified 13 (82.1%) strains of *Staphylococcus aureus* from abscesses (fig 5), 4 (25%) strains of palatinate, 10 (20,8%) strains from the oro-pharyngitis and tonsillitis, 13 (18,7%) strains of orosinus fistulas (fig.6), 9 (15,5%) of gingivitis, and 2 (2,6%) strains of periodontitis.

In cats, were isolated and identified 21 (53,8%) strains of *Staphylococcus aureus* from abscesses (fig.7), 4 (26,6%) strains of orosinus fistulas (fig.8), 8 (16%) strains from the oro-pharyngitis and tonsillitis, 8 (12,1%) strains of gingivitis, 1(10%) strains of palatinitis and 7 (8,6%) strains of periodontitis.

It is recognized the pyogenic action of *Staphylococcus aureus* species, look emphasized by isolation, into a high percentage of oral abscesses, at dogs and also at cats.

Ideally, there should be tested all strains isolated from an outbreak.

The results obtained by testing antibiotics are necessary for antimicrobial therapy and may be useful in identifying mechanisms of resistance and identification of epidemiological trends.

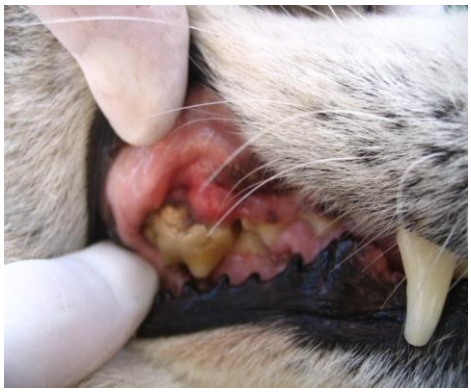


Fig. 4. Gingival abscess, right molar, dog, 8 years

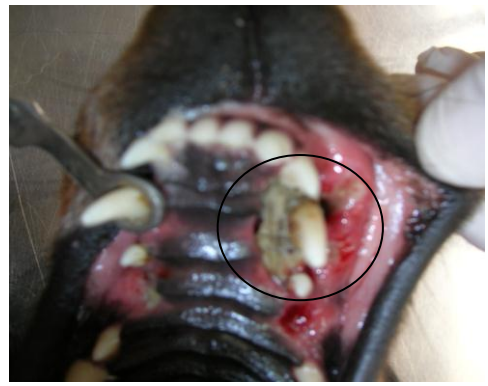


Fig. 5. Oral fistula, dog 10 years

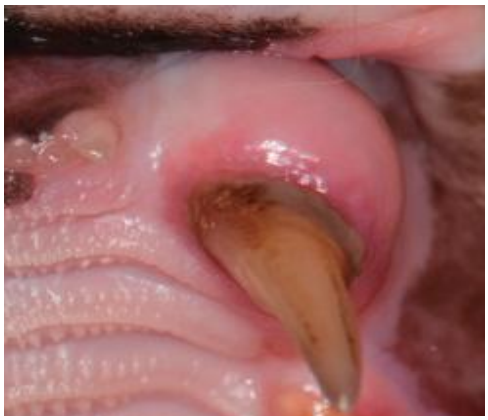


Fig. 6. Abscessed tooth (endodontic disease), cat 7 years

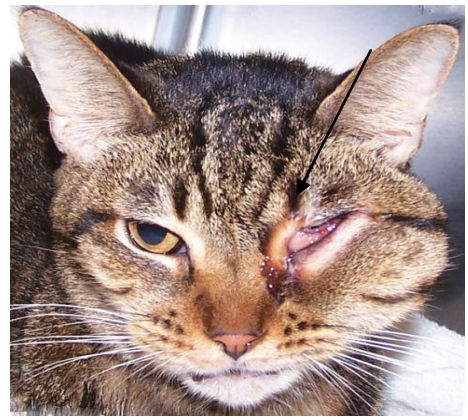


Fig. 7. Oro-sinus fistula, under his left eye, cat 3 years

It is recognized that the percentage of strains with gained resistance to antibiotics increases in direct proportion with the intensity of their use (Buic D., 2010).

The results of investigations performed antimicrobial *Staphylococcus aureus* strains isolated from dogs and cats are shown in table 3.

In dogs, the analysis of data obtained, shows that, 37(90,2%) strains of *S. aureus* were susceptible to gentamicin, 36(87,8%) to amoxicillin clavulanic acid, 35(85,3%) in clindamycin and erythromycin, 33(80,5%) to doxycycline, 30 (73,1%) to amoxicillin, 29(70,7%) to cefoperazone. Antimicrobial resistance of *S. aureus* strains was insignificant and doxycycline was observed in 4(9,7%), enrofloxacin 4 (9.7%) and amoxicillin 3(7,3%).

Table 3. Antimicrobial test results of *Staph. aureus* strains, isolated from mouth samples

Antibiotic tested	Dogs 41 strains *S. aureus*						Cats 49 strains *S. aureus*					
	S		MS		R		S		MS		R	
	nr	%	nr	%	nr	%	nr	%	nr	%	nr	%
Amoxicillin	30	73,1	8	19,5	3	7,3	30	61,2	12	24,5	7	14,3
Amoxicillin+ clavulanic acid	36	87,8	3	7,3	2	4,8	38	77,5	9	18,3	2	4,0
Ampicillin	33	80,5	8	19,5	1	2,4	33	67,3	11	22,4	5	10,2
Erythromycin	35	85,3	5	12,2	1	2,4	27	55,1	22	44,9	-	0
Gentamicin	37	90,2	2	4,8	2	4,8	33	67,3	12	24,5	4	8,1
Doxycycline	33	80,5	4	9,7	4	9,7	32	65,3	7	14,3	10	20,4
Clindamycin	35	85,3	5	12,2	1	2,4	33	67,3	9	18,3	7	14,3
Enrofloxacin	27	65,8	10	24,4	4	9,7	30	61,2	14	28,5	4	8,1
Cefoperazone	29	70,7	10	24,4	2	4,8	17	34,7	19	38,7	13	26,5
Ceftiofur	27	65,8	12	29,3	2	4,8	21	42,8	22	44,9	6	12,2
Cefadroxil	27	65,8	13	31,7	1	2,4	36	73,5	9	18,3	4	8,1
Metronidazole-spiramycin	19	46,3	19	46,3	3	7,3	22	44,9	21	42,8	6	12,2

S- sensibility; MS- moderate sensibility; R- rezistance

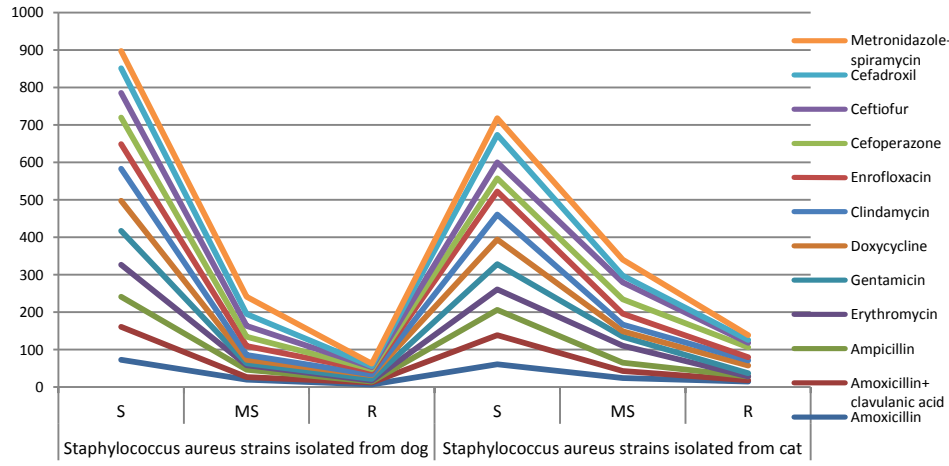


Fig. 8 The share of bacterial strains isolated from dogs, sensitivity to antibiotics which manifest

In cats, the proportion of *S. aureus* strains susceptible to antibiotics was low. Of all strains tested, 38(77,5%) strains were susceptible to amoxicillin clavulanic acid, 36(73,5%) strains to cefadroxil, 33(67,3%) strains to ampicillin, gentamicin, clindamycin, 32(65,3%) strains to doxycycline, 30(61,2%) to ampicillin. Antimicrobial resistance was there however, manifested in a significant number of *S. aureus* strains. There is resistance, 13(26,5%) strains of *S. aureus* to cefoperazone, 10(20,4%) strains resistant to doxycycline, 7(14,3%) to amoxicillin and clindamycin, 6(12,2 %) to ceftiofur and spiramycin metronidazole, 5(10,2%) to ampicillin, 4 (8,1%) to gentamicin and enrofloxacin.

Although antibiotics used have a broad spectrum of action, it appears however that there is wide variation limits for strains that show sensitivity to them.

Irrational use of antibiotics is the main cause of antibioresistance, which explains the presence of bacterial strains resistant to broad-spectrum antibiotics. Therefore, it is necessary, the need for a susceptibility to establish accurately the antimicrobial treatment.

Another consequence, the administration of oral antibiotics is destruction oro-pharyngeal microbiota, followed by prompt colonization of pathogenic bacteria including species *Stapylococcus aureus*.

Conclusions

1. *Staphylococcus aureus* is an opportunistic pathogen status in etiology stomatitis to dogs and cats.
2. In national and international context, the study complements the few existing information on the scope and implication of this pathogen in oral pathology to dog and cats.
3. We believe that the main cause of the presence of *S. aureus* species in the oral cavity of these species is due coexistence into a common habitat and lead to infection risk transfer between humans and pets.

Bibliography

1. Bellows J., 2010 - *Feline dentistry - oral assessment, treatment and preventative care*, Ed. Wiley-Blackwell, USA
2. Buic D., 2010 – *Tratat de Microbiologie clinică*, Ed. Medicală Iaşi
3. Elliott D. R., Wilson M., Buckley C. M., Spratt D.A., 2005 - *Cultivable oral microbiota of domestic dogs.* Journal of Clinical Microbiology 43:5470-5476
4. Euzéby J.P., 2002 - *Flores normales et pathologiques du chein et du chat* (*www.bacterio.cict.fr/bacdico/site*)
5. Gorrel Cecilia, 2010 - *Odontologia de pequeños animales*. Elsevier Saunder Espana
6. Guérin-Faublée V., Euzéby J.P., 2000.- *Étude de quelquer bactéries pathogénes pour le cheval et ou les carnivores domestiques,* 456-500. In: Freney J, Renaud F, Hansen W, Bollet C. *Precis de Bacteriologie Clinique*. Eska, Paris.
7. Guguianu Eleonora, 2002 - *Bacteriologie generală*, Casa de Editură Venus, Iaşi
8. Gyles C.L., Prescott J.F., Songer J.G., Thoen C.O., 2004 - *Pathogenesis of bacterial infections in animals*, third edition, Pb. Blackwell
9. Harvey C.E., 1990 - *Feline oral pathology, diagnosis and management.* Crossley DA & Penman S (eds) Manual of Small Animal Dentistry, British Small Animal Veterinary Association, Gloucestershire, UK,
10. Lilenbaum W., Esteves A.L., Souza G.N., 1999 - *Prevalence and antimicrobial susceptibilitz of stafilococci isolated from saliva of clinically normal cats*, Letters in Applied Microbiology, 28, 448-452
11. Manolescu Mirela, Roşu Lucica, Ungureanu Anca, 1998 - *Aspecte generale privind flora microbiana implicată în afecţiunile stomatologice*, Editura Agora, Craiova
12. Marshall-Jones Z., 2010 - *The microflora and periodontal health in dogs*. Companion Animals Symposium:Microbes and Health, Journal Animal Science, vol.88
13. O'reilly P.G., Claffey N.M., 2000 - *A history of oral sepsis as a cause of disease.* Periodontology ;23:13–8
14. Pavlica Z., 2006 - *Biofilm: Microbial communities and periodontal disease,* World Congress World Small Animal Veterinary Association

USE SEVERAL MULTITEST SYSTEMS IN PRACTICE FOR CONDITIONAL PATHOGENIC AND PATHOGENIC FISH BACTERIA IDENTIFICATION

Liliana ROŞCA[1], Elena IŞAN[1], Felicia ŢÂRCA[1], Ionela Miki SĂLCEANU[1], Petru ROŞCA[2]
1.DSVSA Iaşi, 2. Facultatea de Medicină Veterinară Iaşi

Abstract
Choosing a multitest system for identification of bacteria is recommended especially for identifying bacterial samples that conventional systems can not properly recognized. After obtaining pure cultures, a variety of multitest systems for identification of bacterial genera and species could be used successfully. The material consisted of several test samples represented by conditional pathogenic/ pathogenic bacterial suspensions / to fish, received in the laboratory for identification by biochemical tests in 2009 and 2010. To identify bacterial genera and species have been working through several steps: o*btaining pure bacterial cultures, examination of cultural and tinctorial characters, oxidase and catalase reaction test performance, determining the respiratory type. Depending on the bacterial genera identified presumably, biochemical tests were chosen for API 20E and 20NE galleries. Identification of taxa was performed using Analytical Catalogue searching numeric index profile. Of the 13 bacterial strains tested using API 20E galleries, for 11 (84.61%) the identify quality was excellent (99.9% id and T> 0.75), very good (99.0% id and T> 0.5) and good (90.0% id and T> 0.25) and only for 2 strains (15.38%) the identify quality was poor (only genus identification) (% id 80.0 and T> 0). Of the 7 bacterial strains tested using API 20NE galleries, for 6 (85.71%) the identify quality for genera and species was excellent(% id 99.9 and T> 0.75), very good identification (% id 99.0 and T> 0.5), and good identification (90.0% id and T> 0.25) and only for one bacterial strain (14,28%) the identify quality was poor (only genus identification) (% id 80.0 and T> 0). Combining several conventional biochemical tests with biochemical tests performed with API 20E and 20NE galleries, can be easily identified some bacterial genera and species of fish involved in the morbid episodes, shortening the analytical time and eliminating the use of culture media. Very good identification was observed for genera and species of nonfermentative bacteria (Shewanella putrefaciens, Pseudomonas fluorescens) testing using the API 20NE galleries.*

Key words: multitest system, identification of bacteria, conditional pathogenic/ pathogenic bacterial for fish

Many of conventional microbiology methods have remained over time and applicable to today and irreplaceable, such as use of media with agar to obtain isolated colonies is a method that has not yet been replaced. (O'Hara C., M., et al, 2003).

The emergence of media in Petri dishes ready to use and then bacterial identification test in miniature tubes, were the first forms automation for detailed identification of bacterial species and genera (Darbandi Fazel, 2010). Using these test in miniature tubes is cheap and time is long.

Although some authors (Topic Popovic N, 2007) states that using API 20E and API 20NE galleries the diagnosis of bacterial infections in fish may be used only for presumptive diagnosis because the database includes not all genera and species of concern, but notes that can be used if biochemical tests are appropriate, we used these products for this purpose obtaining satisfactory results.

Materials and methods

The material consisted of several test sample represented by pathogenic/ conditional pathogenic bacterial suspensions to fish, received in laboratory for identification by biochemical tests in 2009 and 2010.

To identify bacterial genera and species have been working through several steps:

Obtaining pure bacterial cultures:

Bacterial suspensions were sown on solid culture media (TSA - Tryptone soy agar, nutrient agar, brain heart infusion agar) - the ribbed, to obtain isolated colonies as recommended by Rocco, C., (2001), (see foto 1) and incubated at 25 ° C for 48 hours, (OIE, 2003).

Examination of cultural and tinctorial characters:

The examination of cultural characters was done visually on nonselective solid culture media after their development and the examinations of tinctorial characters by Gram stained smears performed.

Oxidase and catalase test reaction:

These are mandatory tests to identify bacteria of the family *Enterobacteriaceae* and non-fermentative Gram-negative bacteria. Bacteria of the family *Enterobacteriaceae* give negative oxidase reaction test.

Type of metabolism (respiratory type):

Bacterial metabolism may be: oxidative, fermentative, or inactive

Correlating the above test results, the test strains were presumptively assigned to bacterial genera.

Bacterial genera and species identification by rapid tests using API 20 E and 20NE galleries:

Depending on the bacterial genera identified presumably biochemical tests were chosen as API 20E and 20NE galleries. API 20E and 20NE galleries were inoculated with test strains standard protocol and the use recommended by the manufacturer, and the interpretation of results was done manually, using the Analytical Catalog.

Identification of taxons was performed using analytical Catalogue searching numeric index profile.

Numeric index is accompanied by two important information:

I. The two indexes for each talon

- ***Percentage of identification*** (% id) - estimate how much corresponds to the taxed profile database.
- ***Index T*** - estimate how much corresponds to the typical reaction profile for each talon. The value can be between 0 and 1 and is inversely proportional to the number of atypical tests.

II. Comments about the quality of identification:

i. Excellent identification -% id = 99.9 and T> 0.75;
ii. Very good identification -% id = 99.0 and T> 0.5;
iii. Good identification -% id = 90.0 and T> 0.25;
iv. Acceptable identification% id = 80.0 and T> 0 http://www.apiweb.biomerieux.com).

Results and discussion

Cultural characteristics examination of bacterial colonies was performed visually on nonselective solid culture media after their development.

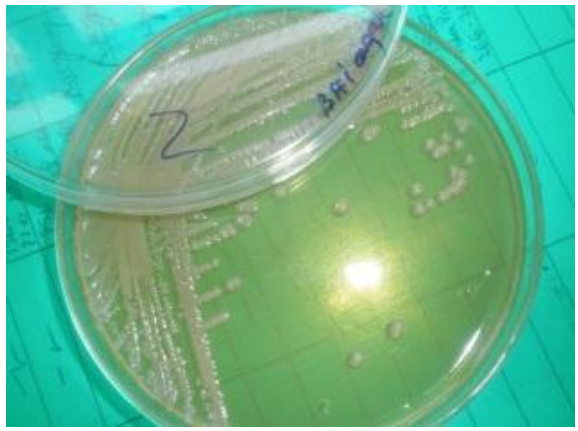


Fig.1 Geting isolated colonies on nonselective culture media

In examining the dyeing characters have Gram stained smears performed, resulting in 100% of cases cocobacili and /or bacili Gram negative with uncharacteristic settlement.

Of the 13 bacterial strains isolated, 9 (69.23%) were oxidase positive and only 4 (30.76%) were oxidase negative, 11 (84.61%) were catalase positive and only 2 (15.38 %) showed a weak positive reaction for catalase

Of the 13 bacterial strains isolated, 10 (76.92%) had fermentative metabolism and oxidative (see foto.2B), are presumably assigned to fam. *Enterobacteriaceae*, 2 (15.38%) showed oxidative metabolism and 1 (7.69%) had inactive metabolism (see foto 2A).

A B

Fig. 2. Inactive (A) and fermentative (B) metabolism

Correlating the above test results, test strains were assigned to bacterial genera such alleged (see table 1).

With the API 20 E and 20NE galleries were tested 13 bacterial strains After incubation, color reactions and/or disturbing the culture medium (indicating bacterial growth) were read from the wells, resulting in a numerical code for each strain. This number was searched in the catalog to identify the bacterial strain from the database.

Table 1. Correlation of biochemical tests for presumptive identification of bacterial strains

Ox P O P F P C P	**%**	**Ox N O P F P C P**	**%**	**Ox P O P F N C P**	**%**	**Ox N O N F N C P/N**	**%**	**Ox P O P F P C P/N**	%
6	**46,15**	**3**	**23,07**	**2**	**15,38**	**1**	**7,69**	**1**	**7,69**
Presumptive family or genus									
Aeromonas spp.		***Fam. Enterobacteriaceae***		***Pseudomonas spp.***		***Shewanella spp.***		***Aeromonas spp***	

Ox –oxidase reacţion test; O –oxidative metabolism; F fermentative– metabolism;
C - *catalse reaction test; N negative; P pozitive; P/N –weak positive reaction*

Of the 13 bacterial strains tested using Api 20E galleries, for 11 (84.61%) the identify quality was excellent (99.9% id and T> 0.75), very good (99.0% id and T> 0.5) and good (90.0% id and T> 0.25) and only for 2 strains (15.38%) the identify quality was poor (only genus identification) (% id 80.0 and T> 0) (see table 2).

Yersinia ruckeri is not specified as a taxa in the database for Api 20E, so is all that *Hafnia alvei*, which otherwise resembles phenotypically. The difference between the two bacterial strains required an additional test execution: xylose fermentation for only *Hafnia* is positive (N. Buller, 2004).

The differentiation between *Aeromonas hydrophila* and *Aeromonas caviae* strains was based on glucose fermentation test. *Aeromonas caviae* ferment glucose without gas production. The test was performed in tubes with liquid medium with 1% glucose, fermentation tube and indicator light see foto. 3).

Fig. 3. Fermentation of glucose without gas production (*Aeromonas caviae*)

Table 2. Identify quality of the bacterial strains using API 20E galleries

NC	Strain No.	Identify taxa	%id	T	Ei	Vgi	Gi	Ai	Ig
5305112	1	*Hafnia alvei 1*	99,9	1,0	X				
2006104	2	*Aeromonas salmonicida salmonicida*	99,6	1,0		X			
5105100	3	*Hafnia alvei 2 posib Yersinia ruckeri*	99,8	0,84		X			
2207006	4	*Pseudomonas fluorescens/putida Pseudomonas aeruginosa*	50,9 48,7	0,59 0,6					X
2046137	5	*Aeromonas hydrophila gr.1*	98,1	0,54			X		
3247705	6	*Aeromonas hydrophila gr.1*	98,2	0,52			X		
5105100	7	*Hafnia alvei 2 posib Yersinia ruckeri*	99,8	0,84		X			
2207046	8	*Pseudomonas fluorescens/putida Pseudomonas aeruginosa*	75,5 24,2	0,51 044					X
6047125	9	*Aeromonas hydrophila gr.1 Aeromonas hydrophila gr.2*	85,3 14,5	0,62 0,51		X			
0402006	10	*Shewanella putrefaciens gr.*	99,1	0,55		X			
3047127	11	*Aeromonas hydrophila gr.1 Pos. V. fluviatilis*	98,4	0,92			X		
4305112	12	*Hafnia alvei 1*	99,9	0,92	X				
7247125	13	*Aeromonas hydrophila gr.2*	99,0	1,0		X			
TOTAL					2	6	3	0	2
%					15,38	46,15	23,07	0	15,38
						84,61		0	15,38

NC - numerical code, Strain No. – number of strains identification, Ei- excellent identification; Vgi – very good identification; Gi- good identification, Ai-acceptable identification; Ig – identification of the genus

With the API 20 NE galleries were tested seven bacterial strains (Table 3). After incubation, the tests were performed by the manufacturer, achieving - the numerical profiles that identify bacterial genus and species database.

Table 3. Identify quality of the bacterial strains using API 20NE galleries

NC	Strain No.	Identify taxa	%id	T	Ei	Vgi	Gi	Ai	Ig
1157155	**1**	***Pseudomonas fluorescens***	**99,9**	**0,61**		**X**			
1057555	**2**	***Pseudomonas fluorescens***	**99,6**	**0,8**		**X**			
7176745	**3**	***Aeromonas sobria***	**97,5**	**0,82**			**X**		
7176755	**4**	***Aeromonas sobria***	**99,2**	**1,0**		**X**			
1011114	**5**	***Shewanella putrefaciens***	**99,9**	**0,63**		**X**			
7577754	**6**	***Aeromonas hidro/caviae***	**99,9**	**1,0**	**X**				
7175754	**7**	***Aeromonas hidro/caviae Aeromonas sobria***	**95,6 4,3**	**0,76 0,47**					**X**
TOTAL					**1**	**4**	**1**	**0**	**1**
%					**14,28**	**57,14**	**14,28**	**0**	**14,28**
						85,71		**0**	**14,28**

NC - numerical code, Strain No. – number of strains identification, Ei- excellent identification; Vgi – very good identification; Gi- good identification, Ai-acceptable identification; Ig – identification of the genus

Of the 7 bacterial strains tested using API 20NE galleries, for 6 (85.71%) the identify quality for genera and species was excellent (% id 99.9 and T> 0.75), very good identification (% id 99.0 and T> 0.5), and good identification (90.0% id and T> 0.25) and only for one bacterial strain (14,28%) the identify quality was poor (% id 80.0 and T> 0).

Very good identification was observed for genera and species of nonfermentative bacteria (*Shewanella putrefaciens*, *Pseudomonas fluorescens*) testing using the API 20NE galleries.

Conclusions

In conclusion, strains were identified correctly and timely with API 20E galleries and 20and but with some specifications:

1. Temperature and incubation time for bacterial strains tested were selected according to the OIE Manual, 2003, at 25 ° C for at least 48 h;
2. Identification of any bacterial genera and species requiring additional tests;
3. To identify bacterial genera and species test was properly required purification of cultures, a step which required time, given that the development of pathogenic bacterial colonies in fish requires at least 48 hours.

However it was noted that:

a. Using rapid tests was much easier especially considering microbiological technique that has not required the preparation of all culture media (including carbohydrates culture media , differential or specific culture media).
b. Galleries sowing technique is easy and fast;
c. How to interpret the results easily and quickly.

The results presented show that both types of galleries largely lends itself to identify pathogenic and /or conditional pathogenic bacteria to fish, resulting a good or a very good identification of bacterial genera and species (84,61% of strains tested with API 20E galleries and that to 85,71% of strains tested API 20 NE galleries).

References

1. Buller, Nicky B., 2004, *Bacteria from fish and other aquatic animals : a practical identification manual*, ISBN 0-85199-738-4, CABI Publishing
2. Fazel Darbandi, 2010, *Parallel Comparison of Accuracy in Vitek2 Auto-analyzer and API 20 E / API 20 NE Microsystems* - Master thesis University College of Borås
3. O'Hara, C. M., E. G. Sowers, C. A. Bopp, S. B. Duda, and N. A. Strockbine., 2003, *Accuracy of six commercially available systems for identification of members of the family Vibrionaceae.,* J. Clin. Microbiol. 41:5654–5659.
4. Topic Popovic N, R. Coz-Rakovac, I. Strunjak-Perovic, 2007, *Commercial phenotypic tests (API 20E) in diagnosis of fish bacteria:* a review *Veterinarni Medicina, 52, 2007 (2): 49–53*
5. OIE (Office International des Epizooties), 2003 : *Manual of Diagnostic Tests for Aquatic Animals,* available at: http://www.oie.int/eng/normes/fmanual/A_00002.htm
6. http:// apiweb.biomerieux.com

EVALUATION OF *SALMONELLA* INFANTIS SEROTYPE CIRCULATION IN POULTRY POPULATIONS IN ROMANIA

[1]Elena ROTARU, [1]Stelian BARAITAREANU, [2]Mihail CARTOJAN, [2]Sorin PARVU, [1,3]Doina DANES

[1]University of Agronomic Sciences and Veterinary Medicine – Faculty of Veterinary Medicine Bucharest, Romania; [2]DSVSA – Giurgiu County Laboratory Veterinary and Food Safety Giurgiu, Romania; [3]University of Agronomic Sciences and Veterinary Medicine, Institute of Comparative Medicine, Bucharest, Romania

doruvet@yahoo.com

Abstract

Present study aimed to investigate the incidence and prevalence of S. Infantis in chicken flocks from Giurgiu County, Romania, in period 2008-2010. A (total) sum of 3,106 biological samples from seven poultry holdings have been submitted to bacteriological exam (carried out) using standardized methods (SR EN ISO 6579:2003). Salmonella Infantis was present in 71.43% (5/7) poultry holdings, representing 51.80% (273/527) from all Salmonella strains, from those identified in period 2008-2010 in the investigated poultry populations. Prevalence of Salmonella Infantis in all samples researched was 8.79%. Incidence of Salmonella ifantis has been significantly increased in 2009, comparatively with 2008, from 9 isolates to 140, but the proportion of infected holdings was constant 42.85% (3/7). The incidence trend of Salmonella Infantis strains decreased in 2010, comparatively with 2009, from 140 isolates to 124, but the average of the infected poultry holdings increased 71.43% (5/7).

Keywords: poultry salmonellosis, salmonella incidence, salmonella surveillance

Salmonella history begins in 1880 when Eberth and Koch revealed in people died of typhoid fever a bacillus which they considered the etiologic agent of this disease. Twenty-five years later, Babes described the first Romanian case of salmonellosis [10]. Today more than 2,600 *Salmonella* serotypes are classified. The actual taxonomy system used for members of the genus *Salmonella* is based on the WHO Collaborating Centre recommendations, being accepted and used by several national institutes of diagnostic [1, 3, 8, 9, 14, 15, 18, 28].

The genus *Salmonella* contains two species: *S. enterica* and *S. bongori*. Subspecies have been biochemical differentiated [1, 9, 27, 28] and by genomic relatedness [3, 9, 14, 27, 28]. The type strain of S. enterica is CIP 60.62 (a H2S-producing clone of strain LT2). S. enterica is divided into six subspecies [1, 8, 9], which are referred to by a Roman numeral and a name (I, S. enterica subsp.enterica; II, S. enterica subsp. salamae; IIIa, S. enterica subsp. arizonae; IIIb, S. enterica subsp. diarizonae; IV, S. enterica subsp. houtenae; and VI, S. enterica subsp. indica). S. bongori was formerly subspecies V [8, 9, 14, 15].

The prevalence of specific *Salmonella* serotypes differs from up to the geographical region, and their involvement in human infections is widely variable. It is considered that all Salmonella serotypes can be pathogenic for humans, but in Salmonella food poisoning are mainly involved *Salmonella* Enteritidis and *Salmonella* Typhimurium, and more rarelly *Salmonella* Infantis, *Salmonella* Virchow and *Salmonella* Hadar [15].

Since the late 1970s *Salmonella enterica* subsp. *enterica* serotype Infantis has been increasingly recorded worldwide. Since 2001 in Germany and Europe this serovar has been involved in several human infections [5]. A survey of the European Food Safety Authority (EFSA) identified poultry products as the main source of *S.* Infantis infection for humans.

The highest frequency of serovar S. Infantis in layer and broiler farms has been reported in Eastern Europe (87% in Hungary, 19% Poland, 13% Czech Republic) [12, 24, 25].

The paper's objective is assessment of the incidence and prevalence of *Salmonella enterica* subsp. *enterica* serotype Infantis in layer and broiler holding in Giurgiu County. Data used in this study were supplied by Giurgiu Veterinary and Food Safety Laboratory, and are part of national program of Salmonella surveillance in the period 2008-2010.

Materials and method

A total of 3,106 biological samples from seven layer and broiler holdings were submitted: 1,532 samples in 2008, 833 samples in 2009 and 741 samples in 2010. The populations of layers and broilers in all investigated holding counted 8,860,612 subjects in 2008, 8,852,478 subjects in 2009, and 7,104,428 subjects in 2010.

Bacteriological exam has been carried out using standardized methods [15, 19, 20, 21, 22, 23, 26]. Samples were pre-enriched in buffered peptone water (BPW) followed by enrichment in Modified Rappaport-Vassiliadis (RV) Enrichment broth and Selenite Cystine Broth. Resulting cultures have been plated onto XLD, Istrate Meitert and Wilson-Blair agar plates, incubated at 37°C for 24h. Presumptive Salmonella isolates have been biochemically and serologically confirmed.

Results and discussion

During 2008 – 2010, have been identified 527 *Salmonella* isolates, of which 51.80% (273/527) *Salmonella enterica* subsp. *enterica* serotype Infantis. Six holding were *Salmonella* positive in 2008, four in 2009 and six in 2010. In holding 1, 2 and 7 *Salmonella spp.* were isolated each year, in holding 3 and 4 *Salmonella spp.* were isolated in 2008 and 2010, in holding 5 in 2008 and 2009, and in holding 6 in 2009 and 2010.

Five of seven holding were contaminated with *S.* Infantis, but only holding 1 and 7 were continuously positive for this over the three years of the study (table 1). In 2008 *Salmonella spp.* Were present in 12.98% (199/1532) of samples, 4.52% (9/199) from this being *Salmonella* Infantis. In 2009 the sample with *Salmonella spp.* represented 19.68% (164/833), of which 85.36% (140/164) *Salmonella* Infantis. In 2010 *Salmonella spp.* was present in 21.86% (162/741) samples, of which 76.54% (124/162) *Salmonella* Infantis.

A similar situation has been reported in Israel, when serotype *S.* Infantis became predominant in poultry holdings during the same period (2007–2009), and the prevalence of serotypes Enteritidis, Typhimurium, Virchow, Bredeney, Newport, and Paratyphi B var. Java decreased [6]. In other countries serotype Enteritidis is still predominant [2], and in other countries *S.* Infantis cannot be found or have low ratio in Salmonella strains isolated in poultry [4, 11].

Table 1. Number of *Salmonella enterica* subsp. *enterica* serotype Infantis isolates in seven poultry holdings

Year	Holding 1	Holding 2	Holding 3	Holding 4	Holding 5	Holding 6	Holding 7	Total
2008	4	0	0	1	0	0	4	9
2009	67	38	0	0	0	0	35	140
2010	47	68	2	5	0	0	2	124
Total	**118**	**106**	**2**	**6**	**0**	**0**	**41**	**273**

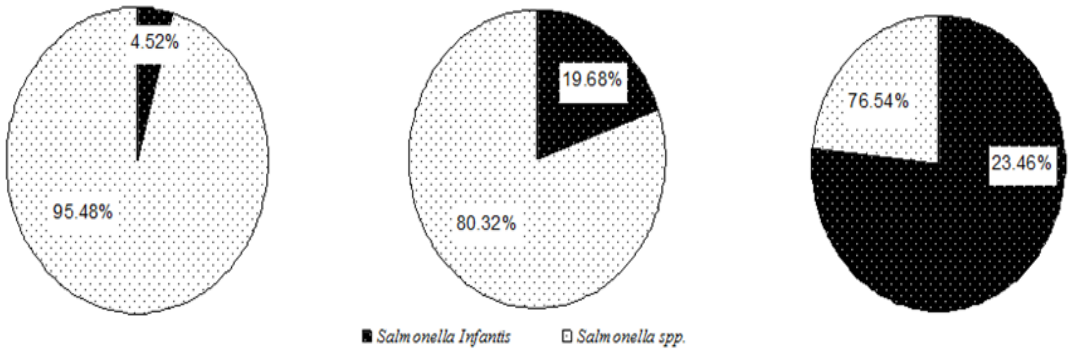


Fig. 1. Incidence of *Salmonella enterica* subsp. *enterica* serotype Infantis isolates in 2008, 2009 and 2010, in seven poultry holdings. *S.* Infantis became more frequent than all isolates of *Salmonella spp* in last two years

Salmonella Infantis is constantly isolated from chickens and humans worldwide. Shahada *et al.* conducted a *S.* Infantis study in 44 poultry holdings in Southern Japan and, for the first time, all S. Infantis isolates showed resistance to at least three classes of antimicrobial agents. In this study, it was observed that the intestinal tract of healthy poultry has been a reservoir of the extended-spectrum cephalosporin-resistant isolates of serovar Infantis [16]. In Japan has been obtained similarly results: 22.6% prevalence of serotype *S.* Infantis in poultry populations [7, 16]. Nogrady *et al.* reported in 2008 a high incidence of S. Infantis serotype in broiler flocks in Hungary: 43% of the fecal samples and up to 100% in abattoir carcass and retail raw meat samples [12]. At that time in Romanian poultry holdings, the number of *S.* Infantis isolates had a low incidence 0.58% (9/1532), but the next two years revealed a increased incidence (16.81%, 140/833 in 2009 and 16.73%, 124/741 in 2010), becoming the prevalent Salmonella serotype in 2010. Similar status was reported in Serbia where *S.* Infantis incidence increased from 1.46% (20/1745) in 2009 to 2.40% (44/1832) in 2010, and *S.* Infantis became more frequent than *S.* enteritidis [17].

Conclusions

1. *Salmonella* Infantis was present in 71.43% (5/7) poultry holdings, and represented 51.80% (273/527) from all *Salmonella* strains identified in period 2008-2010 in these poultry populations. Prevalence of *Salmonella* Infantis in all studied samples was 8.79%.
2. Incidence of *Salmonella* Infantis increased significantly in 2009, comparatively with 2008, from 9 isolates to 140, but the rate of infected holdings was constant 42.85% (3/7). Incidence of *Salmonella* Infantis strains decreased slightly in 2010, comparatively with 2009, from 140 isolates to 124, but the rate of the infected poultry holdings increased 71.43% (5/7).

Bibliography

1. Brenner, F.W., McWhorter-Murlin, A.C., (1998) Identification and serotyping of Salmonella. (Centers for Disease Control and Prevention,Atlanta, Ga).
2. Carraminana, J.J., Rota, C., Agustin, I., Herrera, A., High prevalence of multiple resistance to antibiotics in Salmonella serovars isolated from a poultry slaughterhouse in Spain. Veterinary Microbiology, 2004; 104:133-139
3. Crosa, J. H., Brenner, D.J., Ewing, W. H., Falkow, S., Molecular relationships among the salmonellae.J. Bacteriol. 1973; 115:307–315.
4. Frye, G.J., Fedorka-Cray, P.J., Prevalence, distribution and characterisation of ceftiofur resistance in Salmonella enterica isolated from animals in the USA from 1999 to 2003. International Journal of Antimicrobial Agents. 2007; 30:134-142.
5. Galanis, E., Lo Fo Wong, D. M., Patrick, M. E., Binsztein, N., Cieslik, A., Chalermchikit, T., Aidara-Kane, A., Ellis, A., Angulo, F. J., Wegener, H. C., Web-based surveillance and global Salmonella distribution, 2000–2002. Emerg Infect Dis. 2006. 12:381–388.
6. Gal-Mor, O., Valinsky, L, Weinberger, M., Guy, S., Jaffe, J., Schorr, Y.I., Raisfeld, A., Agmon, V., Nissan, I., Multidrug-Resistant Salmonella enterica Serovar Infantis. Israel, Emerging Infectious Diseases, 2010; 16(11):1754-1757.
7. Ishihara, K., Takahashi, T., Morioka, A., Kojima, A., Kijima1, M., Asai, T., Tamura, Y., 2009. National surveillance of Salmonella enteric in food-producing animals in Japan. Acta Vet. Scand. 51:35
8. Janda JM, Abbott SL (2006). "The Enterobacteria" Second Edition., ASM Press Washington, USA
9. Le Minor, L., Popoff, M.Y., Request for an opinion. Designation of Salmonella enteric sp. nov., nom. rev., as the type and only species of the genus Salmonella. Int. J. Syst. Bacteriol. 1987; 37:465–468
10. Manzat, R. M. (2001), Boli infectioase ale animalelor - Bacterioze, Ed. Brumar, Timisoara, 26, 53
11. Muhammada, M., Lawal U.M., Abdul-Ganiyu, A., Aliyu, U.M., Samuel, A., Lisa, B. Prevalence of Salmonella associated with chick mortality at hatching and their susceptibility to antimicrobial agents. Veterinary Microbiology. 2010; 140:131-135.
12. Nógrády N., Kardos G., Bistyák A., Turcsányi I., Mészáros J., Galántai Z.S., Juhász Á., Samu P., Kaszanyitzky J.É., Pászti J., Kiss I. (2008): Prevalence and characterization of Salmonella infantis isolates originating from different points of the broiler chicken–human food chain in Hungary. International Journal of Food Microbiology, 127, 162-167
13. Popoff, M.Y., Le Minor, L., (1997) Antigenic formulas of the Salmonella serovars, 7th revision. World Health Organization Collaborating Centre for Reference and Research on Salmonella (Pasteur Institute,Paris, France).
14. Reeves, M.W., Evins, G. M., Heiba, A. A., Plikaytis, B.D., Farmer, J.J., Clonal nature of Salmonella typhi and its genetic relatedness to other salmonellae as shown by multilocus enzyme electrophoresis and proposal of Salmonella bongori comb. nov. J. Clin. Microbiol. 1989; 27:313–320.
15. Sandu, I. (2008), Manual de diagnostic al salmonelozelor, Institutul de Diagnostic si Sanatate Animala – Laboratorul National de Referinta pentru Salmoneloze animale, (IDSA Bucuresti, Romania). 3-5, 15-24
16. Shahada F, Sugiyama H, Chuma T, Sueyoshi M, Okamoto K. Genetic analysis of multi-drug resistance and the clonal dissemination of beta-lactam resistance in Salmonella Infantis isolated from broilers. Vet Microbiol. 2010; 140:136–41. Epub 2009 Jul 10.
17. Stojanov, I., Kapetanov, M., Prodanov-Radulović, J., Pušić, I., Petrović, J., Baloš-Živkov, M., The resistency of Salmonella serovar. enteritidis/infantins isolated in poultry against nalidixic acid. Biotechnology in Animal Husbandry, 2011; 27(3):751-758.
18. Wayne, L.G., Judicial Commission of the International Committee on Systematic Bacteriology. Int. J. Syst. Bacteriol. 1991; 41:185–187.
19. ***Commission Regulation (EC) No 1003/2005 of 30 June 2005 implementing Regulation (EC) No 2160/2003 as regards a Community target for the reduction of the prevalence of certain salmonella serotypes in breeding flocks of Gallus gallus and amending Regulation (EC) No 2160/2003.

20. ***Commission Regulation (EC) No 1168/2006 of 31 July 2006 implementing Regulation (EC) No 2160/2003 as regards a Community target for the reduction of the prevalence of certain salmonella serotypes in laying hens of Gallus gallus and amending Regulation (EC) No 1003/2005.
21. ***Commission Regulation (EC) No 213/2009 of 18 March 2009 amending Regulation (EC) No 2160/2003 of the European Parliament and of the Council and Regulation (EC) No 1003/2005 as regards the control and testing of Salmonella in breeding flocks of Gallus gallus and turkeys
22. ***Commission Regulation (EC) No 646/2007 of 12 June 2007 implementing Regulation (EC) No 2160/2003 of the European Parliament and of the Council as regards a Community target for the reduction of the prevalence of Salmonella enteritidis and Salmonella typhimurium in broilers and repealing Regulation (EC) No 1091/2005
23. ***Commission Regulation (EU) No 200/2010 of 10 March 2010 implementing Regulation (EC) No 2160/2003 of the European Parliament and of the Council as regards a Union target for the reduction of the prevalence of Salmonella serotypes in adult breeding flocks of Gallus gallus
24. ***EFSA, Report of the Task Force on Zoonoses. Data Collection on the Analysis of the baseline study on the prevalence of Salmonella in holdings of laying hen flock. The EFSA Journal. 2007a; 97:1-85.
25. ***EFSA, Report of the Task Force on Zoonoses. Data Collection on the Analysis of the baseline survey on the prevalence of Salmonella in broiler flocks Part A. The EFSA Journal. 2007b; 98:1-85.
26. ***SR EN ISO 6579:2003/Amendament 1:2007 – Microbiology of food and animal feeding stuffs. Horizontal method for the detection of Salmonella spp. (ISO 6579:2002) 8-Feb-2003 / 34 pages Amendments:UE-EN ISO 6579:2003/A1:2007
27. ***The Foodborne Outbreak Online Database, CDC. Foodborne diseases active surveillance network (FoodNet).
28. ***World Organisation for Animal Health Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2011, chapter 2.9.9., 2, 5-8.

IMMUNOPROPHYLAXIS OF AVIAN INFECTIOUS BRONCHITIS IN INDUSTRIAL CONDITIONS

N. STARCIUC, Natalia OSADCI, T. SPATARU, Rita GOLBAN
State Agrarian University of Moldova

Abstract
The article includes the serological investigation about maternal and post vaccination level of specific antibodies titres against bronchitis diseases virus. For vaccination were used following strains: "H-120", and "4/91", which were administrated with drenching water, by aerosol method and by spray method. The blood serum samples were collected from poultry enterprises in the country such as: poultry enterprise SRL "Puiu de Aur", village Stoianovca, district Cantemir, SRL "Pasărea Argintie", village Ciorăscu, poultry enterprise from Ialoveni, Orhei, Anenii-Noi. After that the blood serum samples were sent to the Veterinary Diagnostic Center. The level of antibodies titres was established with ELISA test. The level of antibodies titres varies between positive and negative limits.

Key words: Antibodies titres, ELISA test, Diseases virus, Strains vaccine, increased antibody levels, low level of antibodies

Introduction

Infectious bronchitis virus (IBV) causing a respiratory disease of poultry, affecting poultry of all ages, causing economic problems for the poultry industry worldwide. Although infectious bronchitis virus is controlled by using live attenuated and inactivated vaccines remains a major problem due to the existence of many serotypes. Live attenuated vaccines are produced by repeated passages in embryonated eggs spontaneous mutations resulting. As a result weakened viruses have only a few mutations responsible for loss of virulence, to be different between vaccines affect virulence and immunogenicity and restores the virulence. Was obtained by reverse genetics a new generation of vaccines in order to obtain an optimal balance between loss of virulence and ability to induce immunity. (Charlton R., Bermudez A. J., Boulianne M., 2006; Jean – Luc Guerin, Cyril Boissieu, 2008; Britton P, Armesto M, Cavanagh D, Keep S., 2012).

Infectious bronchitis virus (IBV) is stopped in principle the use of attenuated vaccines, which are known vaccine strains with a limited range of protection. Alternative vaccines against new strains developing can improve the disease control. A study was conducted to evaluate the immunogenic potential of two recombinant viral proteins, which were administered by drops in the eye, without the presence of a vector. S1 and N recombinant proteins of strain M41 were tested in the presence of E. coli and live attenuated vaccine H-120 was used as a positive control. Following three immunizations S1 glycoprotein induced 40% protection, whereas vaccination with N protein have not induce protection. (Meir R, Krispel S, Simanov L., 2012).

Materials and methods

As research material served chicken flocks of poultry enterprises in the country, where intensified cases of disease in chickens. Experimental investigations were made to farms poultry: SRL „Puiul de Aur", Stoianovca village, district Cantemir, SRL "Pasărea Argintie", village Ciorăscu, poultry enterprise from Ialoveni, Orhei, Anenii-Noi. Chickens affected by infectious bronchitis virus exhibited breathing difficulties, sneezing, coughing, tracheal rales and nasal jetaj. Often clusters can be observed from heat sources.

In some cases was suspected and contamination with Newcastle disease, which was diagnosed after necropsy chicken carcasses, based on changes in the glandular stomach mucosa namely inflammation and bleeding detection and in some cases were detection button aboves at bifurcation caecums.

Sometimes was suspected the contamination with avian colibacillosis, which was diagnosed by laboratory examination, necropsy from chicken carcasses, namely the method bacterioscopic (make smears, Gram staining method and microscopic examination) and bacteriological (cultivation of microorganisms on universal and differential nutrient media).

Parallel to these pathological changes become swollen established pulmonary, bleeding red-purple, fibrin films lungs, fibrinous pericarditis and inflammation of the air sacs, tumefierea, enlarged and pale kidneys, ureters are filled with clusters of urate.

For this reason samples were collected blood serum samples of lung and were investigated by laboratory investigations at Center of Veterinary Diagnostic and Preventive Medicine in Chisinau. Following laboratory investigations was diagnosed - "avian infectious bronchitis".

Serum samples were examined by serological investigations by ELISA using the indirect method known, using diagnostic kit produced by IDEXX Laboratory, USA. Absorption value was 650 nm. For valid test is necessary to consider the difference between positive control and negative control should not exceed 0,075.

Absorption parameters of the negative control must be less than the 0,150. Serum samples absorption rate of less than 0.20 are considered negative.

Results and discusion

Serological investigation results are presented in table no. 1-5. In the table no. 1 are the results of serological investigations serum samples collected from chickens aged 21 days, which were maintained in farm poultry SRL „Puiul de Aur", Stoianovca village, district Cantemir. Chicken flock was vaccinated with live attenuated vaccine strain of "H-120", which was administered once at age 2 days , method of administration - with drinking water. All 10 samples examined were positive. Antibody titres ranged from 1:3014,43 up to 1:10769,14. Optimal after vaccination titres normally varies within 1:1000 or 1:2000. In this situation more than 80% of the samples examined had much higher titers 1:3000. Based on the table it can be stated that the number examined is held and the use of a virulent virus after the virus action is the formation of an antibody level to achieve the level of 1: 7385,26 and more.

Table 1. The post vaccine antibody titres in chickens vaccinated against infectious bronchitis with strain "H-120", administered with drinking water

No. sample	Vaccine strain	Age of vaccinated chickens (days)	The age of examined chickens (days)	Wavelength (650 nm)	S/P Absorption rate	The post vaccine antibody level(days)
1				0,1044	PC	PC
2				0,1021	PC	PC
3				0,0418	NC	NC
4				0,0451	NC	NC
5	"H-120"	2 days	21 days	0,2338	3,18311037	6302,55853
6				0,1096	1,52243959	3014,43038
7				0,2059	2,71655618	5378,77926

8				0,2373	3,2416388	6418,44482
9				0,212	2,81856187	5580,75251
10				0,3687	5,43896321	10769,1472
11				0,2665	3,72993311	7385,26756
12				0,1814	2,30685619	4567,57525
13				0,1387	1,59280936	3153,76254
14				0,3658	5,39046823	10673,1271

Table 2. The post vaccine antibody titres in chickens vaccinated against infectious bronchitis with strain "H-120", administered by spray method

No. sample	Vaccine strain	Age of vaccinated chickens (days)	The age of examined chickens (days)	Wavelength (650 nm)	S/P Absorption rate	The post vaccine antibody level(days)
1	-	-	-	0,2352	PC	PC
2	-	-	-	0,2519	PC	PC
3	-	-	-	0,0403	NC	NC
4	-	-	-	0,0429	NC	NC
5	“H-120”	2 days	21 days	0.5001	-0.45137	-893.715
6				0.0693	-2.29713	-4548.32
7				0.0637	-2.32112	-4595.82
8				0.0527	-2.36825	-4689.14
9				0.0825	-2.24057	-4436.34
10				0.0788	-2.25643	-4467.72
11				0.2631	-1.4668	-2904.25
12				0.4876	-0.50493	-999.756
13				1.0807	2.0362	4031.68
14				0.5176	-0.37639	-745.257
15				0.1013	-2.16003	-4276.85
16				0.0829	-2.23886	-4432.94
17				0.0438	-2.40638	-4764.64
18				0.1082	-2.13046	-4218.32

In the table no. 2 are the results of serological investigations serum samples collected from chickens aged 21 days, which were maintained in farm poultry SRL „Pasărea Argintie”, village Ciorăscu, municipality Chişinău. Chicken flock was vaccinated with live attenuated vaccine strain of "H-120", which was administered once at age 2 days , method of administration - spray. Of the 14 samples examined 13 samples were negative and only one sample was positive. Antibody titres ranged from 1: -745.25 up to 1: 4031.68.

Analyzing data table can be noted that the titres of specific antibodies to the virus BIA in more than 95% of the samples examined were negative. But later, after these results, there were clinical signs of disease and after countless necropsy were found pathological changes characteristic of avian infectious bronchitis.

In the table no. 2 are the results of serological investigations serum samples collected from chickens aged 21 days, which were maintained in farm poultry „Ialoveni”. Chicken flock was vaccinated with the same vaccine strain („H-120”), was administered once at age 2 days , method of administration - aerosol. Of the 19 samples examined 10 saplas were positive and 9 samplas were negative. 52% of samples are positive and 48% of samplas are

negative. Titres of antibodies in positive samples ranged from 1: 76.47 up to 1: 3798.45. Titres of antibodies in negative samples ranged from 1: -20.58 up to 1: -4612.78. More than 80% of the samples examined had much higher titers 1:3000. After analyzing the results of the table it can be stated that the staff considered to be revaccinated as soon to be avoided an outbreak of infection and huge economic losses among birds.

Table 3. The post vaccine antibody titres in chickens vaccinated against infectious bronchitis with strain "H-120", administered by aerosol method

No. sample	Vaccine strain	Age of vaccinated chickens (days)	The age of examined chickens (days)	Wavelength (650 nm)	S/P Absorption rate	The post vaccine antibody level(days)
1	-	-	-	0,2352	PC	PC
2	-	-	-	0,2519	PC	PC
3	-	-	-	0,0403	NC	NC
4	-	-	-	0,0429	NC	NC
5	"H-120"	2 days	21 days	0.0495	0.03912	77.4548
6				0.0557	0.06982	138.242
7				0.0395	-0.0104	-20.5893
8				0.1592	0.58232	1153
9				0.0764	0.17232	341.193
10				0.0802	0.19114	3798.45
11				0.0616	0.09903	196.088
12				0.0494	0.03862	76.4744
13				0.066	0.12082	239.228
14				0.0678	0.12974	256.875
15				0.1227	0.40158	795.137
16				0.1668	-1.8793916	-3721.1954
17				0.1083	-0.8211248	-1625.8271
18				0.0617	-2.3296915	-4612.7892
19				0.2714	-1.4312339	-2833.8432
20				0.2032	-1.72344	-3412.4
21				0.459	-0.62746	-1242.38
22				0.0695	-2.29627	-4546.62
23				0.272	-1.42866	-2828.75

The data in table no. 4 shows the results of serological investigations samples collected from chickens aged 21 days, which were maintained in farm poultry „Anenii-Noi". Chicken flock was vaccinated with strain "H-120" at the age of one day and strain "4/91" at the age of 14 days. Both vaccine strains were administered by spray method.

The vaccine strain "4/91" is a live attenuated, freeze-dried, which was administered by means of large spray droplets under instruction. The water used in the vaccine may vary depending on the age of birds, generally using between 250-400 ml per 1000 doses. Suspend vaccine was sprayed evenly over the birds at a distance of 30-40 cm. Aerosol spray was used with high particle.

Of 10 samples examined were all negative. Antibody titres ranged from 1: -379,37 up to -677,76. After analyzing the data we can say that vaccines have moderate efficiency is the danger of infectious bronchitis.

Table 4. The post vaccine antibody titres in chickens vaccinated against infectious bronchitis with strains "H-120" and "4/91", administered by spray method

No. sample	Vaccine strain	Age of vaccinated chickens (days)	The age of examined chickens (days)	Wavelength (650 nm)	S/P Absorption rate	The post vaccine antibody level(days)
1				0,3521	PC	PC
2				0,1373	PC	PC
3				0,0934	NC	NC
4				0,2102	NC	NC
5	"H-120", "4/91"	I vac. – one day II vac. -14 days	21 days	0,134	-0,1916039	-379,37567
6				0,1207	-0,2048748	-405,65217
7				0,1267	-0,270183	-534,96233
8				0,12	-0,3423036	-677,76103
9				0,129	-0,24543	-485,942
10				0,132	-0,21313	-422,002
11				0,123	-0,31001	-613,821
12				0,131	-0,2239	-443,315
13				0,1217	-0,324	-641,529
14				0,138	-0,14855	-294,123

Table 5. The post vaccine antibody titres in chickens vaccinated against infectious bronchitis with strains "H-120" and "4/91", administered with drinking water

No. sample	Vaccine strain	Age of vaccinated chickens (days)	The age of examined chickens (days)	Wavelength (650 nm)	S/P Absorption rate	The post vaccine antibody level(days)
1				0,0872	PC	PC
2				0,1061	PC	PC
3				0,0428	NC	NC
4				0,0639	NC	NC
5	"H-120", "4/91"	I vac. – one day II vac. -14 days	21 days	0,0684	0,3475751	688,19861
6				0,0422	-0,208997	-413,8144
7				0,0556	0,051963	102,88684
8				0,0455	-0,181293	-358,9607
9				0,0442	-0,21132	-418,406
10				0,0459	-0,17206	-340,67
11				0,0478	-0,12818	-253,788
12				0,0481	-0,12125	-240,069
13				0,058	0,10739	212,633
14				0,0496	-0,08661	-171,478
15				0,0485	-0,11201	-221,778
16				0,0458	-0,17436	-345,242

17				0,0443	-0,20901	-413,834
18				0,0817	0,65473	1296,37
19				0,0416	-0,27136	-537,298
20				0,0535	0,00346	6,85912
21				0,0564	0,07044	139,469
22				0,0694	0,37067	733,926

In the table no. 5 are the results of serological investigations serum samples collected from chickens aged 21 days, which were maintained in farm poultry „Orhei". Chicken flock was vaccinated with live attenuated vaccine strain of "H-120", which was administered once a day old, method of administration - with drinking water and live attenuated vaccine, lyophilized from strain "4/91" which was given at the age of 14 days, method of administration - with drinking water. Of all 18 blood serum samples which were investigated 7 samples were positive and 11 samples were negative. Around 60% of the examined samples the antibody titers were negative. Positive antibody titres ranged from 1: 102,88 up to 1: 1296,37, and ranged from negative 1: -171,47 till 1: -537,29. Samples examined had much lower titers than 1:2000.

Conclusions

1. The results in table 1 allows us to say that chicken flocks of farm poultry SRL „Puiul de Aur" there is movement of a virulent virus and this virus from the action takes place forming an antibody level to achieve the level of 1: 7385,26 and more.
2. At various farms aviclole immunological effectiveness of vaccines in serological investigations, is interpreted differently. In some cases the antibody titres far exceeds the optimum, while in other cases is much lower than the optimum, even negative.
3. Analyzing the immunoassay test results can mention that some poultry companies in the country is required to review schemes and methods to vaccinate chickens against avian infectious bronchitis.

References

1. Abdel-Moneim A.S.; et. al. *S1 gene sequence analysis of a nephropathogenic strain of avian infectious bronchitis virus in Egypt*. Department of Virology, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef 62511, Egypt. Virol J. 2006 Sep; 20(3), p. 78.
2. Bart van Leerdam, et al. *Interpretaţia rezulitatov ELISA dlia virusa bronhita (IBV)*. Informaţionîi biulleteni. Vîpusc 2009. E-mail: mail @arriah.ru.
3. Britton P, Armesto M, Cavanagh D, Keep S. *Modification of the avian coronavirus infectious bronchitis virus for vaccine development.* 2012 Mar 1; 3(2).
4. Charlton R., Bermudez A. J., Boulianne M., et. all. *„Manual al Bolilor aviare".* Asociaţia Americană a Patologilor Aviari. Ediţia a şasea. 2006, p. 241.
5. Elena Potecea. *Bronşita infecţioasă.* Boli infecţioase ale animalelor. Universitatea Spiru Haret. Editura Fundaţiei România de Mâine, Bucureşti, 2002, p. 204-205.
6. Jean – Luc Guerin, Cyril Boissieu. *La bronchite infectieuse.* AVI campus. Ecole Nationale Veterinaire, Toulouse, 2008.
7. Manuel terrestre de L OIE. *Bronchite infectieuse aviare.* 2005, p. 969-981.
8. Meir R., Krispel S., Simanov L., et al. *Immune Responses to Mucosal Vaccination by the Recombinant S1 and N Proteins of Infectious Bronchitis Virus.* Viral Immunol. 2012 Jan 6.
9. Perianu, T. *Cornoviroze. Bronşita infecţioasă aviară.* Boli infecţioase ale animalelor. Viroze, vol. II, Iaşi. 2005; p. 159-164.

REQUIREMENTS REGARDING THE MICROBIOLOGICAL PARAMETERS OF NONSTERILE MEDICINAL PRODUCTS

Simona STURZU, Daniela TIRSINOAGA, Ioana DIACONU, Alina DRAGHICI
Institute for Control of Veterinary Biological Products and Medicines Bucharest
sturzu.simona@icbmv.ro

Keywords: European Pharmacopoea, nonsterile medicinal products, microbiological requirements

The European Pharmacopoeia is a collection of quality standards for the medicinal substances. It is used by individuals and organizations involved in pharmaceutical research, development, manufacture and testing. Pharmacopoeia standards are public available and legally enforceable standards of quality for medicinal products and their constituents. The Pharmacopoeia is an important statutory component in the control of medicines which complements and assists the licensing and inspection processes from EU. The European Pharmacopoeia is developed by the European Directorate for the Quality of Medicines (EDQM) and is a part of the Council of Europe, Strasbourg, France. It has been created by the Convention on the elaboration of a European Pharmacopoeia in 1964.

The *Convention on the elaboration of a European Pharmacopoeia* was adopted and signed by 36 countries. All medicines sold in the 36 Member States of the European Pharmacopoeia must comply with these quality standards so that consumers have a guarantee of products obtained from pharmacies and other legal suppliers. For the European Union, the monographs of the European Pharmacopoeia have mandatory applicability.

This Convention entered into force in Romania by Law nr. 98/2002 for Romania's accession to the Convention on the Elaboration of a European Pharmacopoeia.

The convention is open for signature by all European countries. Other countries can get observer status.

From January 2011, the 7th Edition European Pharmacopoeia entered into force and replaced the 6th Edition which was legally binding until 31 December 2010. The 7th Edition is comprised of two initial volumes published in July 2010, which are updated by non-cumulative supplements issued three times a year following the decisions taken at each session of the European Pharmacopoeia Commission.

All draft monographs are first published in Pharmeuropa and available to the public for comments.

The mandatory character of Pharmacopoeia is seated also by Directive 2001/82/CE (Order nr. 187/2007 regarding Code on veterinary medicinal products)

The tests on the finished products will be performs on basis of the requirements from relevant monographs and general chapters of the European Pharmacopoeia, for all products, according to requirements of the Order nr. 57/2009.

For non sterile medicinal products the European Pharmacopoeia provided a set of criteria depending on dosage form.

The presence of certain microorganisms in nonsterile preparations may have the potential to reduce or inactivate the therapeutic activity of the product and has a potential to

adversely affect the health of the patient. Manufacturers have therefore to ensure a low bioburden of finished dosage forms by implementing current guidelines on Good Manufacturing Practice during the manufacture, storage, and distribution of pharmaceutical preparations.

Microbial examination of nonsterile products is performed according to the methods given in the chapters for microbial examination of non sterile products:

- microbial enumeration tests – Chapter 2.6.12
- tests for specified micro-organisms - Chapter 2.6.13
- microbiological quality of non sterile pharmaceutical preparations and substances for pharmaceutical use - Chapter 5.1.4

I. Microbial Enumeration Tests

The tests allow quantitative enumeration of mesophilic bacteria and fungi which may grow under aerobic conditions. The tests are designed primarily to determine whether a substance or preparation complies with an established specification for microbiological quality. The methods are not applicable to products containing viable micro-organisms as active ingredients.

The test is perform taking into account that it should be avoiding the contamination such that they do not affect any micro-organisms which are to be revealed in the test. If the product to be examined has antimicrobial activity, this is insofar as possible removed or neutralized. If inactivators are used for this purpose their efficacy and their absence of toxicity for micro-organisms must be demonstrated. If surface-active substances are used for sample preparation, their absence of toxicity for micro-organisms and their compatibility with inactivators used must be demonstrated.

II. Tests for Specified Micro-organisms The tests allow determination of the absence of, or limited occurrence of specified microorganisms which may be detected under the conditions described. If the product to be examined has antimicrobial activity, this is insofar as possible removed or neutralized. If surface-active substances are used for sample preparation, their absence of toxicity for micro-organisms and their compatibility with inactivators used must be demonstrated.

The 5.1.4 Chapter on microbiological quality of non sterile pharmaceutical preparations includes the acceptance criteria for pharmaceutical preparations and substances for pharmaceutical use

Acceptance criteria for nonsterile pharmaceutical products based upon the total aerobic microbial count (TAMC) and the total combined yeasts and molds count (TYMC) are given bellow.

Table 1. Acceptance Criteria for Microbiological Quality of Nonsterile Dosage Forms

Route of Administration	**TAMC (cfu/g or cfu/mL)**	**TYMC (cfu/g or cfu/mL)**	**Specified Microorganism(s)**
Nonaqueous preparations for oral use	10^3	10^2	Absence of *Escherichia coli* (1 g or 1 mL)
Aqueous preparations for oral use	10^2	10^1	Absence of *Escherichia coli* (1 g or 1 mL)
Rectal use	10^3	10^2	—

Route of Administration	**TAMC (cfu/g or cfu/mL)**	**TYMC (cfu/g or cfu/mL)**	**Specified Microorganism(s)**
Oromucosal use	10^2	10^1	Absence of *Staphylococcus aureus* (1 g or 1 mL)
			Absence of *Pseudomonas aeruginosa*(1 g or 1 mL)
Gingival use	10^2	10^1	Absence of *Staphylococcus aureus* (1 g or 1 mL)
			Absence of *Pseudomonas aeruginosa*(1 g or 1 mL)
Cutaneous use	10^2	10^1	Absence of *Staphylococcus aureus* (1 g or 1 mL)
			Absence of *Pseudomonas aeruginosa*(1 g or 1 mL)
Nasal use	10^2	10^1	Absence of *Staphylococcus aureus* (1 g or 1 mL)
			Absence of *Pseudomonas aeruginosa*(1 g or 1 mL)
Auricular use	10^2	10^1	Absence of *Staphylococcus aureus* (1 g or 1 mL)
			Absence of *Pseudomonas aeruginosa*(1 g or 1 mL)
Vaginal use	10^2	10^1	Absence of *Pseudomonas aeruginosa*(1 g or 1 mL)
			Absence of *Staphylococcus aureus* (1 g or 1 mL)
			Absence of *Candida albicans* (1 g or 1 mL)
Transdermal patches (limits for one patch including adhesive layer and backing)	10^2	10^1	Absence of *Staphylococcus aureus* (1 patch)
			Absence of *Pseudomonas aeruginosa*(1 patch)
Inhalation use (special requirements apply to liquid preparations for nebulization)	10^2	10^1	Absence of *Staphylococcus aureus* (1 g or 1 mL)
			Absence of *Pseudomonas aeruginosa*(1 g or 1 mL)
			Absence of bile-tolerant Gram-negative bacteria (1 g or 1 mL)

The laboratory for microbiological control of pharmaceuticals product from ICBMV is part of OMCL.

It performs all the tests for microbiological control according to requirements of European Pharmacopoeia.

The Microbiology laboratory obtained the accreditation by RENAR for all methods for microbiological control of pharmaceutical products.

Since 2008 also it participated to the network OMCL activities and is involved in the collaborative studies, PTS, sampling CAP Programme and mutual joint audit (MJA).

The staff from microbiology laboratory has been trained in the Germany on following methods and subjects: sterility test, microbial enumeration tests, tests for specified micro-organisms, microbiological validation methods, grow promotion of culture media.

Refference

1. European Pharmacopoeia
2. Directive 2001/82/EC on the Community code relating to veterinary medicinal products
3. Directive 2004/28/EC amending Directive 2001/82/EC on the Community code relating to veterinary medicinal products
4. Directive 2009/9/EC of 10 February 2009 amending Directive 2001/82/EC of the European Parliament and of the Council on the Community code relating to medicinal products for veterinary use
5. Ordin nr. 187/2007 din 31/10/2007 pentru aprobarea Normei sanitare veterinare privind Codul produselor medicinale veterinare
6. Ordin nr. 57/2009 *din 03/09/2009* privind modificarea Ordinului preşedintelui Autorităţii Naţionale Sanitare Veterinare şi pentru Siguranţa Alimentelor nr. 187/2007 pentru aprobarea Normei sanitare veterinare privind Codul produselor medicinale veterinare

STATUTE AND CRITERIA FOR THE OFFICIAL MEDICINES CONTROL LABORATORIES

Simona STURZU, Simona STAN, Mirela MARINESCU
Institute for Control of Veterinary Biological Products and Medicines Bucharest
sturzu.simona@icbmv.ro

Keywords: OMCL, medicinal products, quality control, veterinary code

The Commission of the European Union (EU) and the Council of Europe decided on 26 May 1994 to create a network of official medicines control laboratories (OMCLs). This network amounted to a new collaboration in the area of quality control of marketed medicinal products for human and veterinary use. Collaboration within this network saves public money thanks to resource pooling and for the competent national authorities, it shares, thus avoids duplication of, work. The Terms of Reference (TOR) and its annexes define the rules and roles of the Network and its members. They specify the duties and benefits arising from membership and are publicly on the website of European Directorate for Quality Medicines (http://www.edqm.eu).

Official Medicines Control Laboratories (OMCLs) are the public institution that supports regulatory authorities in controlling the quality of medicinal products for human and veterinary use available on the market. For EU member states, the respective rules for medicinal products are laid down in the "human code" (Directive 2001/83/EC as amended) and the "veterinary code" (Directive 2001/82/EC as amended).

Independently from manufacturers, and without any conflict of interest, OMCLs test these products, depending on the activity, in either:

- Member and Observer States of the Convention on the Elaboration of a European Pharmacopoeia involved in the general activities of the network; or
- Member States of the European Union (EU) and the European Economic Area (EEA) and Switzerland where appropriate.

There are two levels of collaboration within the network.

The first one refers to the general activities which are open to all the member and observer states of the European Convention on the Elaboration of a European Pharmacopoeia. These activities are as follows:

- the Quality Assurance (QA) Programme;
- the Proficiency Testing Scheme (PTS) studies;
- the general Market Surveillance Scheme (MSS);
- educational activities; and
- applied analytical research and standardization development.

Secondly, certain activities involve countries from the European Union (EU) and the European Economic Area (EEA) only. These are as follows:

- the Centrally Authorised Products (CAP) Sampling & Testing Programme;
- the Mutual Recognition Procedure (MRP) and the Decentralised Procedure (DCP) Post Marketing Surveillance Scheme;

- the Official Control Authority Batch Release (OCABR) for human biological medicinal products, and OCABR and Official Batch Protocol Review (OBPR) for immunological veterinary medicinal products (IVMPs).

Status of an OMCL within the Network may be given provided that:

1. The laboratory is part of the Ministry of Health/Agriculture or the National Medicines Agency or is an independent governmental body.

2. The Competent Authority transfers the responsibility for the given field of activity to that laboratory and the activity is not of an occasional nature or restricted to a specific analytical technique or product.

According to TOR, the National Competent Authority must be assured that a laboratory which acts as its OMCL should fulfill the following criteria to enable the laboratory full access to the OMCL Network and thus to confirm its status as OMCL within the Network:

- The control laboratory should be organised in such a way, which confirms independence regarding the testing and control of medicinal products.
- Scientific judgement and conclusions on results of control activities need to be independent.
- The control activities of the laboratory should be publicly funded. Where fees for official activities are received these arrangements should be organised such that the payments do not affect the independence of the laboratory.
- Any possible conflicts of interest should be clearly stated and assessed and where necessary a register of interests should be prepared.
- The member state for which the control laboratory is performing testing shall have implemented the Ph.Eur. as a common standard in the national rules governing medicinal products.
- The Competent Authority should provide the control laboratories within the OMCL Network with a clear mandate as to their responsibilities and duties; in this context some Competent Authorities may wish to retain within the regulatory organisation certain specific duties.
- Any testing activities, which are subcontracted should be clearly defined and subject to technical, impartiality and confidentiality agreements (in line with the requirements of ISO/IEC 17025) and where appropriate approved by the relevant authority.
- Implementation of the ISO/IEC 17025 standard in relation to OMCL activities.

The laboratories from the Institute for Control of Veterinary Biological Products and Medicines are nominated as laboratories for quality control of veterinary medicinal products by the National Sanitary Veterinary Authority and for Food Safety and it are included in the OMCL network since 2006. They are structured in four main sections: Physical-chemical pharmaceutical control laboratory, microbiological pharmaceutical control laboratories, biological products control laboratories and biochemical control laboratories.

Until now the laboratories participated in many activities of OMCL network such as:

- Proficiency Testing Scheme (PTS) studies;
- Collaborative studies (CS);
- Educational activities;
- Centrally Authorised Products (CAP) Sampling Programme;

- Centrally Authorised Products (CAP) Testing Programme;

The Institute for Control of Veterinary Biological Products and Medicines as veterinary OMCL is involved in a continuous improvement process of their performance.

The performance of institute is on the one hand materialized through the judicious efforts of the management team to coordinate its activity with the existing resources, with aim to maintain certification of the quality management system granted by SRAC (ISO 9001) and another hand for increase of its technical competence with aim to conduct the laboratories tests in order to maintain and extend the scope of accreditation granted by RENAR (ISO 17025).

Reference

1. Terms of Reference for the General European OMCL Network (GEON) of the Council of Europe (PA/PH/OMCL (07) 79 18Rcorr)
2. Annex 1 to the GEON Terms of Reference: Definition of an OMCL and OMCL Status within the GEON(PA/PH/OMCL (07) 89 8R)
3. Annex 2 to GEON Terms of Reference: Factors for determining OMCL status within the GEON(PA/PH/OMCL (07) 90 4R)

SAMPLING AND TESTING PLAN BY A RISK-BASED APPROACH FOR VETERINARY MEDICINAL PRODUCTS NATIONAL AUTHORIZED

Simona STURZU, Mihaela SCRIPCARIU, Ileana MUSAN, Mirela MARINESCU
Institute for Control of Veterinary Biological Products and Medicines Bucharest
sturzu.simona@icbmv.ro

Abstract
Market surveillance is performed to confirm that the registered quality of a medicinal product is met and maintained throughout its shelf-life. The surveillance includes various types of activity within the Inspection Services and in the Official Medicinal Control Laboratories. Since 2010, the Institute for Control of Veterinary Biological Products and Medicines (ICVBPM) co-ordinate the Sampling and Testing Plan for veterinary medicinal products national authorized by a risk-based approach (RBA). The list of products to be included in the annual plan is prepared by the ICVBPM and approved the National Sanitary Veterinary Authority and for Food Safety (NSVAFS). Samples are collected, throughout the entire medicines distribution chain by inspectors from the nominated Sanitary Veterinary Direction and for Food Safety (SVDFS). These are send to the ICVBPM for testing in accordance with well-established procedure derived from marketing authorization (MA) dossiers and European Pharmacopoeia. The ICVBPM collects the results in an analysis report and inform the NSVAFS and MAH.
Keywords: sampling, testing, national authorized products, risk approach

The legal requirements for the sampling and testing of medicinal products are laid down in the Directive 2001/82 Art. 80, as amended by Directives 2004/28 and the Government Decision no. 341/2012 of for approval of veterinary actions contained in the program of supervision, prevention, control and eradication of animal diseases, zoonotic diseases and environmental protection, identification and registration of bovine, swine, sheep and goats, actions included in surveillance and control program on food safety and also related to the maximum tariffs for 2012.

The objectives of the post-marketing sampling and testing Plan are derived from the legal requirements:

- to supervise the quality of medicinal products placed on the market;
- to check compliance of the medicinal product placed on the market with its authorized specifications and Pharmacopoeia.

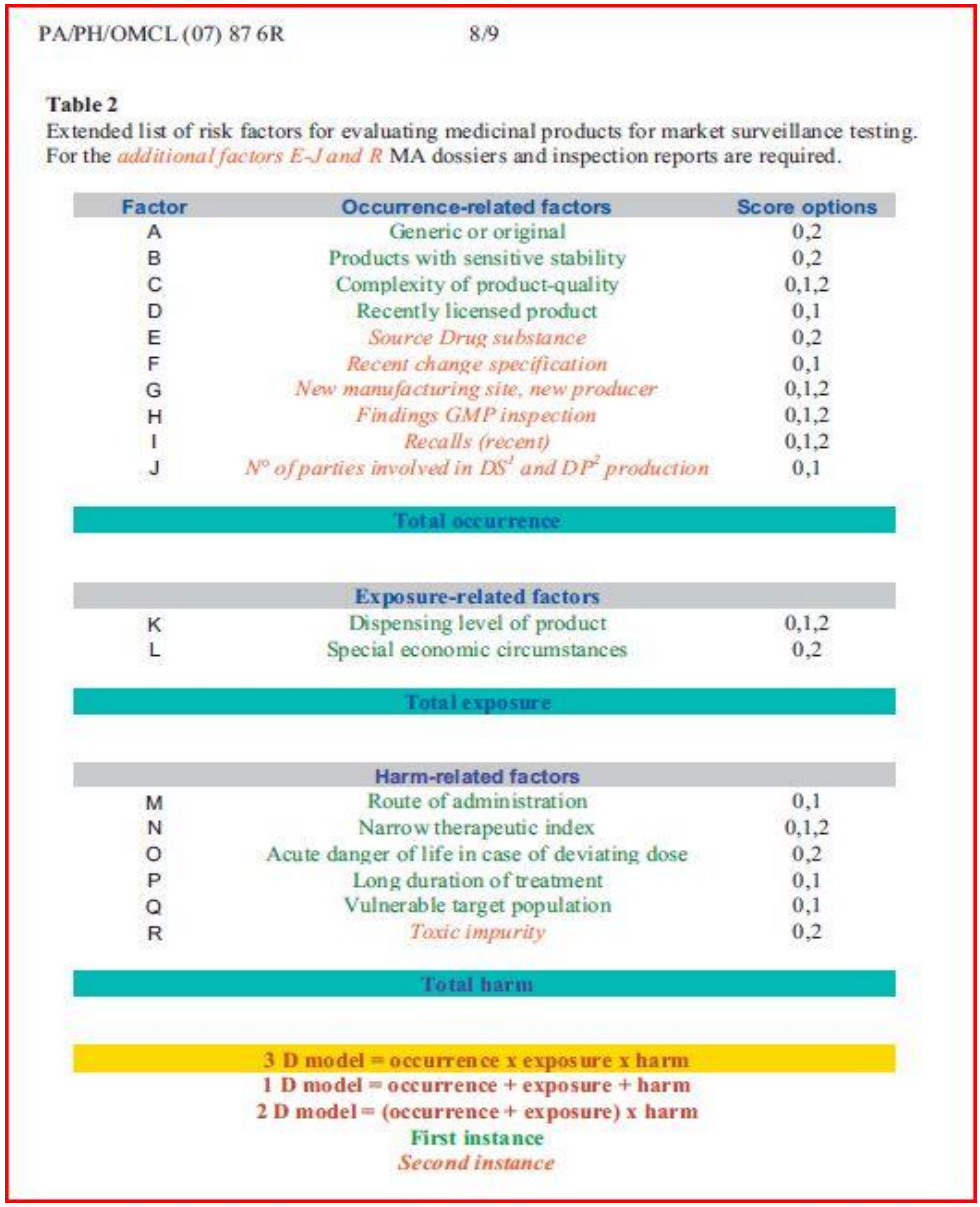
The sampling and testing Plan involve four stages:

- selection of VMP national authorized;
- sampling of selected VMP national authorized from the Romania market;
- testing of these VMP national authorized for parameters identified and;
- reporting of results to NSVAFS.

I. Selection of VMP national authorized

The national authorized products are selected for testing usually three years after the product get the a National Marketing Authorisation. In addition to this annual testing scheme, in the future, products may be selected based on a risk analysis.

Such risk analysis would be based on findings at assessment, GMP inspections, submitted variations, findings in similar products or presentations, etc (Figura 1).



PA/PH/OMCL (07) 87 6R 8/9

Table 2

Extended list of risk factors for evaluating medicinal products for market surveillance testing. For the *additional factors E-J and R* MA dossiers and inspection reports are required.

Factor	Occurrence-related factors	Score options
A	Generic or original	0,2
B	Products with sensitive stability	0,2
C	Complexity of product-quality	0,1,2
D	Recently licensed product	0,1
E	*Source Drug substance*	0,2
F	*Recent change specification*	0,1
G	*New manufacturing site, new producer*	0,1,2
H	*Findings GMP inspection*	0,1,2
I	*Recalls (recent)*	0,1,2
J	*N° of parties involved in DS[1] and DP[2] production*	0,1
	Total occurrence	
	Exposure-related factors	
K	Dispensing level of product	0,1,2
L	Special economic circumstances	0,2
	Total exposure	
	Harm-related factors	
M	Route of administration	0,1
N	Narrow therapeutic index	0,1,2
O	Acute danger of life in case of deviating dose	0,2
P	Long duration of treatment	0,1
Q	Vulnerable target population	0,1
R	*Toxic impurity*	0,2
	Total harm	

3 D model = occurrence x exposure x harm
1 D model = occurrence + exposure + harm
2 D model = (occurrence + exposure) x harm
First instance
Second instance

Selection criteria could include risks identified for active substance, species, poor stability of product, production process, pharmaceutical form and data from previous controls. The design and timing of any re-testing will not be identical for every product.

Also, it will be necessary to conduct a filtering step to avoid the same products being tested in consecutive years.

Products tested in the previous year will be filtered out unless specifically requested for reinclusion in plan. Products subject to "Official Control Authority Batch Release" (OCABR) will also filtered out as is current practice.

II. Sampling of selected VMP national authorized from the Romania market

An operational procedure for sampling and testing of VMP national authorized has been developed based on collaboration between ICVBPM and SVDFS. Inspections Sector performs the sampling of VMP. Product sampling is allocated randomly to the Inspection Services based on the marketing volume in different counties. The sample amount is calculated based on the test methods and the standard operating procedures. It is recommended that each set of samples should originate from the same batch, so that results can be related to given batch numbers.

III. Testing of VMP national authorized

The Testing stage is responsibility of the ICVBPM. The test is pertormed according to the well-established procedure derived from marketing authorization (MA) dossiers and European Pharmacopoeia.

IV. Reporting of results to NSVAFS

ICVBPM prepares and sends to NSVAFS detailing reports on the results of the test. An analysis report (copy) is provided also to MAHs. The detailed reports are available on the Database of Sampling and testing Plan (for regulators only) and annual summary reports on the sampling and testing plan is drawer.

In this year the Sampling and Testing Plan involved a number of 102 veterinary medicinal products national authorized: 30 from Romanian manufacturers and 72 from non Romanian manufacturers.

Reference

1. Directive 2001/82
2. Directives 2004/28
3. Government Decision no. 341/2012 of for approval of veterinary actions contained in the program of supervision, prevention, control and eradication of animal diseases, those transmitted from animals to humans, animals and environmental protection, identification and registration of bovine animals, swine, sheep and goats, of actions in surveillance and control program on food safety and related maximum tariffs for 2012.
4. Ghid OMLC: atribuirea factorilor de risc - PA/PH/OMLC (07) 87 6 R

ALTERNATIVE METHOD OF *SALMONELLA SPP.* IDENTIFICATION

I. ŢIBRU, Zoriţa Maria COCORA, Gyöngyi DOBAI
The Faculty of Veterinary Medicine Timisoara, The Department of Hygiene,
cod 300645, Calea Aradului No. 119, Timisoara, Romania
tibru_ioan@yahoo.com

Abstract
Food infections caused by ingestion of products contaminated with Salmonella spp. represent a major issue worldwide. Such epidemia can be associated with consumption of pork products. The reduction in the Salmonella spp. contamination level of pigs is based on the use of fast and economical methods which allow the early discovery of germs at animals in farms. The objective of this work is comparing two different methods, which are the ISO 6579/2002, which allows asessment of results in approximately 5 days, and a second faster method, using impedance. The latter generates results in 2 days, by using the μ-Trac device. The comparative analysis of 2000 samples revealed the similarity between the two methods. This fact allows us to recommend using the inductance method. However, the cost analysis showed a 1 to 5 difference of the ISO method – the supplies needed for one sample cost 125 lei, while μ-Trac needed an expense of 25 lei.
Keywords: Salmonella spp., alternative method, pork.

Salmonellosis is one of the most common causes of food poisoning worldwide (13). Well documented information from Europe from the past 20 years (11) shows that *Salmonella spp.* remains the second etiologic agent in zoonoses reported in the European Union, in spite of its descending trend in the last few years (14) by 13,5% between 2007 and 2008 and also adding the continuous descent in the last 5 years. The notification rate in the EU was of 26,4 cases reported per 100 000 population, varying from 2,9 cases in Romania up to 126.8 confirmed cases in Slovakia. Germany, The United Kingdom and the Czech Republic represented half the cases (49,5%) in 2008 (14).

To be mentioned that these cases represent only a small part of the total number of existing cases in human population (1), given that 9 EU member states as well as Iceland registered a 30% raise in the number of confirmed cases. Most cases were reported in Denmark, where, in 2008, the number of cases doubled, comparing to 2007, from 1662 to 3669 cases (14).

In 2009 a total of 108.614 salmonellosis cases were confirmed in the EU (according to TESSY – The European Surveillance System), representing a decrease by 17,4% compared to 2008. The notification rate for confirmed cases in EU was of 23.7 cases per 100000 inhabitants, varying from 2,1 cases in Portugal to 100.1 cases in the Czech Republic.

Germany, The United Kingdom and Poland represented half of all the confirmed cases (56,0%) in 2009 (15), although in Germany a reduction of 45,7% of reported and confirmed cases was registered.

A significant reduction, of 77,1% confirmed cases was reported in Romania (624 cases in 2009, from 1,105 cases in 2008). This fact is an evidence in the improvement of surveillance system in Romania, so 2009 was the first year Romania was able to report data on *Salmonella spp* (15).

The conclusion of the several studies implemented was that the economic consequences of the *Salmonella spp.* infection are substantial (2, 6, 8, 9). In developed

countries, mortality is low, while morbidity is high; most episodes are short and self-limiting, so most cases do not require medical care; incidence is socially and economically significant (5, 6).

The costs of every human salmonellosis case is estimated from 40 $ for uncomplicated cases up to 400 $ for cases which require hospitalisation or end in death (18). ERS – USDA estimated the annual costs of *Salmonella spp.* infections to 3 billion $ in the USA (16).

These numbers include direct and indirect medical costs, such as absence from school or work (10).

Taking into account the high incidence of *Salmonella spp.* infections as well as the fact that most cases are caused by pork and pork-derived products consumption(3), it is essential to have a close watch at every stage of production in order to prevent contamination of pork (11).

Pigs shedding *Salmonella* (without clinical signs) are of great importance for public health. Pigs affected by subclinical infections represent an important issue for food safety because of the salmonellosis transmission path to humans (7).

Several studies showed that pigs coming from Salmonella-negative herds during and after slaughter do not keep their *Salmonella spp.* negative status (4). On the other hand, carcasses coming from *Salmonella spp.* free herds may be contaminated during slaughter (12), contamination may come from pigs shedding *Salmonella spp.*, which are slaughtered the same day or from equipment used in slaughter (4).

The cross contamination risk can be reduced by identifying contamination sources and processes and by modifying slaughter procedures according to these findings (4).

Materials and methods

The ISO 6579/2002 has been used, according to instructions, as well as an alternative method, using the μ-Trac analyzer (impedance).

The principle of the method consists in the fact that microorganisms' activity in the sample to be analyzed generates an electrical measurement signal (induction). This signal is perceived by a sensor when the chosen limit value is exceeded. The induction's detection duration represents the main parameter and it consists on the period of time from the beginning of the measurement until the moment microorganisms are detected in the samples. When the measurement signal exceeds the limit value having been set, the sample is considered to be positive. This method can express the quantity of germs identified in the sample. This quantity is influenced by microorganisms' activity and by the specific medium. The bigger the degree of contamination of the sample, the faster the limit value is exceeded and the shorter the duration of the impedance detection is.

The analyzer includes an incubator assuring microorganisms growth, an aluminum device equiped with 21 positions (cells), a temperature reading device, as well as a cooling system (fig.1). Containers are being introduced into every cell. They include the medium, in which the sample to be analyzed has been introduced. Each container is set with two electrodes in the lower part, coming in contact with two resistors. The reading is performed by a computer programme which allows interpretation of results.

The samples' preparation was the same, except that out of the 25 g of sample placed in 225 ml of water and incubated for 24 hours at 37˚C, 0.1 ml have been taken away and

afterwards placed on the sterile containers holding 9.9 ml of BiMedia 205A sterile broth. The next step was homogenizing the container and introducing it in the analyzer's incubator.

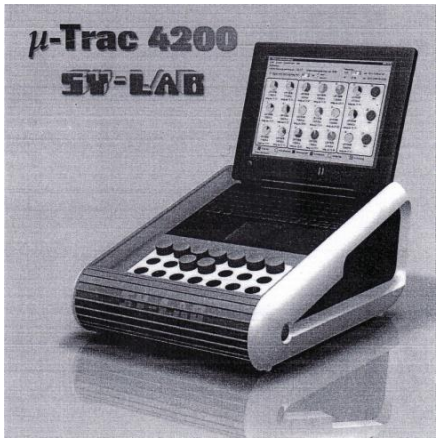


Fig. 1. The µ-trac analyzer

Further away, parameters were set in the analyzer's software, in order to perform the specific measurements. Positive samples were identified by noticing the emergence of the red colour in 22,5 hours.

2000 faeces samples gatheres from different slaughter pig farms were used for testing.

Results and discussions

The comparative analysis of the results obtained from samples examined by using the two methods generated similar outcomes. Out of 2000 analyzed samples, 25 (12.5%) were positive, and the rest were Salmonella-negative samples. This correlation allows us to recommend using this method for identifying indicator microorganisms in food industry.

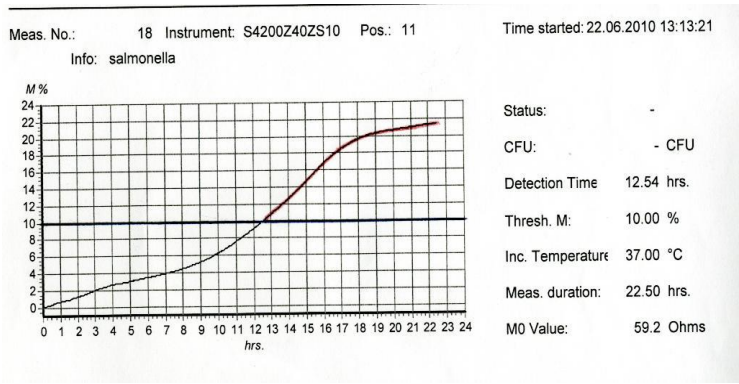


Fig. 2. Express the resuls

Since the duration needed in order to obtain results from gatheres samples was consistent with the technological flow, the economic aspect of using this alternative method is to be discussed (fig. 2).

A cost analysis on the supplies needed for the two different methods showed an 1 to 5 ratio, which means that the supplies needed for one sample analyzed using the ISO 6579/2002 method cost 125 lei, while one cell (container) for µ-Trac cost 25 lei.

Conclusion

The first, microbiological method allows us to determine the carrier status of pigs within a week, while the alternative method allows us to obtain the same result in only 2 days.

The alternative method was also profitable from the economic point of view.

References

1. Anon., 2008e., Scientific Opinion of the Panel on Biological Hazards on a request from the European Commission on a quantitative microbiological risk assessment on *Salmonella* in meat: Source attribution for human salmonellosis from meat. The EFSA Journal 625, 1-32.
2. Buzby, JC., Roberts, T., (2009) "The Economics of Enteric Infections: Human Foodborne Disease Costs", Gastroenterology 136:1851-1862.
3. Cray Fedorka, P.J., GRAY, T.J. and WRAY, C., (2000b): *Salmonella* Infections in Pigs. In: Wray, C., and A. Wray (eds); *Salmonella* in Domestic Animals. CABI Publishing, Wallingford, UK 191-207.
4. Lo Fo Wong, D. M., Halt, T., : Salmonella in Pork (SALINPORK): Pre-harvest and Harvest Control Options based on Epidemiologic, Diagnostic and Economic Research, 2000.
5. De Wit Mas, Koopmans, MPG., Kortbeek, LM., van Leeuwen, NJ., Bartelds, AIM., şi van Duynhoven, YTH.P., (2001b) "Gastroenteritis in Sentinel General Practices, the Netherlands," *Emerging Infectious Diseases* 82 *Vol. 7, No. 1, January–February 2001.*
6. Hellard, ME., Sinclair, MI., Harris, AH., Kirk, M., Fairley, CK., (2003), "Cost of Community Gastroenteritis", Journal of Gastroenterology and Hepatology 18, 322-328.
7. Malorny, B., and Hoorfar J., (2005): Toward Standardization of Diagnostic PCR Testing of Fecal Samples: Lessons from the Detection of *Salmonellae* in Pigs. Journal of Clinical Microbiology 43, 3033–3037.
8. Maki, DG., (2009), "Coming to Grips with Foodborne Infection — Peanut Butter, Peppers, and Nationwide *Salmonella* Outbreaks", New England Journal of Medicine, 360;10: 949-953.
9. McNamara, PE., Liu, X., Miller, GY., (2003), "The Costs of Human Salmonellosis Attributable to Pork: A Stochastic Farm-to-Form Analysis", American Agricultural Economics Association Annual Meeting 2003.
10. Roberts, J., Cumberland, P., Sockkett, PN., Wheeler, J., Rodrigues, LC., Sethi, D., Roderick, PJ., (2003), "The study of infectious intestinal disease in England: socio-economic impact", Epidemiol. Infect. (2003), 130, 1–11.
11. Schlundt, J., Tozofuku, H., Jansen, J., and Hebrst, S.A., (2004): Emerging food-borne zoonoses.
12. Swanenburg, M., Berends, B.R., Urlings, H.A., Snijders, J.M., van Knapen, F., 2001a. Epidemiological investigations into the sources of *Salmonella* contamination of pork. Berl Münch Tierärztl Wochenschr 114: 356-359 Rev. sci. tech. Off. int. Epiz. 23, 513-533.
13. Wegener, H.C., Tine, H., Danilo Lo Fo Wong, Mogens Madsen, Helle Korsgaard, Flemming Bager, Peter Gerner-Smidt, Kåre Mølbak (2003): *Salmonella* Control Programs in Denmark. Emerging Infectious Diseases 9, 774-780.
14. *** Community summary report on trends and sources of zoonoses and zoonotic agents and food-borne outbreaks in the European Union in 2008. *The EFSA Journal (2010)* 8(1), 1496.
15. *** EFSA 2009, The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2009 EFSA Journal 2011;9(3):2090.
16. *** ERS-USDA., April 21, 2003. "*Economics of Foodborne Disease: Salmonella*" from http://www.ers.usda.gov/Briefing/FoodborneDisease/*Salmonella*.htm.
17. ***ISO 6579 Microbiologia produselor alimentare şi furajere. Metoda orizontală pentru detectarea bacteriilor din genul Salmonella, Iunie 2003.
18. ***WHO (2005). "*Drug-resistant Salmonella*" http://www.who.int/mediacentre/factsheets/fs139/en/.

SEROEPIDEMIOLOGICAL STUDY OF BOVINE PESTIVIRUS (BVDV) INFECTION IN VASLUI AND VRANCEA COUNTY

Dragoș ANIȚĂ
Faculty of Veterinary Medicine Iași, 8, Alley Mihail Sadoveanu, Iași, Romania
danita@uaiasi.ro

Abstract
Bovine viral diarrhoea, caused by the bovine viral diarrhoea virus (BVDV) in the Pestivirus genus of the Flaviviridae, is one of the most important diseases of cattle world wide causing poor reproductive performance in adult cattle and mucosal disease in calves. BVDV are ubiquitous viral pathogens of cattle with a high degree of sequence diversity amongst strains circulating in livestock herds. BVDV infect a range of domestic and wild ruminants. Clinical presentation varies depending on strain of virus, species of host, immune status of host, reproductive status of host, age of host, and concurrent infections. Blood samples were collected from 212 cattle and tested for BVDV infection, by detection of specific antibodies. This paper describes the serological study for antibodies to BVDV and in a cattle population in Vaslui and Vrancea county. Control of BVDV infections rests on reducing exposure by removing BVDV infected animals, increasing herd resistance by vaccination, and instituting biocontrol methods that limit the opportunity for introduction of BVDV into herds and management units.
Keywords: cattle, BVDV, ELISA

Bovine viral diarrhea virus (BVDV), a major cattle pathogen, has a global distribution, and is responsible for a wide spectrum of clinical manifestations, including reproductive failure, and respiratory and gastrointestinal tract diseases (Baker JC., 1995). In the pregnant animal the virus can be transmitted vertically to the foetus. The timing of transmission is critical to the epidemiology of the disease; infection prior to 100–125 days of gestation may result in foetal death or in birth of a live calf persistently infected with BVDV, due to the infection occurring prior to the development of immune competence (Potgeiter L, 2004). The most damaging effect of BVDV infection is the establishment of a persistently infected animal; these cattle may appear to be asymptomatic while shedding the virus and infecting other animals (Grooms DL, 2004). Such cattle are likely the most important reservoir of virus for susceptible cattle, as was illustrated when 70% to 100% of susceptible nonvaccinated penmates became infected after exposure to PI calves (Fulton RW, 2005). Despite the widespread use of modified live and killed vaccines, BVDV persists as a pathogen that causes a wide variety of subclinical and clinical infections and immunosuppression (Brock KV, 2004).

Because of the clinical and economical importance of BVDV, many European countries (including Denmark, Sweden, Norway, Finland, Austria, Germany, and Switzerland) have implemented BVDV eradication programs (Houe H, 2006).

Materials and methods

An indirect ELISA has been used for detection of antibodies to BVDV in individual serum samples. Detection of BVDV antibodies was performed with the use of a commercially available kit IDEXX BVDV Total Ab (IDEXX Laboratories), according to the manufacturer's recommendations. The IDEXX BVDV Ab Test identifies animals that have been recently infected or re-infected with BVDV: a strong indicator that PI cattle are present in the herd and require identification.

Two hundred and twelve bovine serum samples were collected from two counties: Vaslui and Vrancea. Ninety samples were collected from household system cattle in County Vrancea; 122 serum samples were collected from farm diary cows in Vaslui County (three farms). All serum samples were stored at -20^{0}C and were tested individually. Non-reactive and reactive controls were included for each plate.

Results and discussions

Ninety serum samples were collected from healthy cattle from eight villages in Vrancea counties. Out 90 samples tested were identified 69 sera positives for anti-BVDV antibody. The overall prevalence of anti-BVDV positive antibodies in cow sera was 76.66% (69/90) across the Vrancea County.

Table 1. The results of serological detection of anti-BVDV antibodies in serum samples collected from cattle in Vrancea County

Village	No. of tested samples	No. of positive samples	No. of negative samples
Tifeşti	12	12	0
Ruginești	10	7	3
Cimpuri	10	2	8
Boghesti	10	10	0
Tătăranu	10	6	4
Vulturu	12	11	1
Panciu	14	9	5
Reghiu	12	12	0
Total	***90***	***69***	***21***

The detection of serum antibodies in the 69 seropositive cows may have been a result of virus exposure and seroconversion to circulating BVDV.

Anti-HEV antibody village prevalence ranged from 20% to 100%. The highest prevalence of anti-HEV antibodies was observed in three villages, showing a 100% seroprevalence, followed by one village showing a 91.60% seroprevalence.

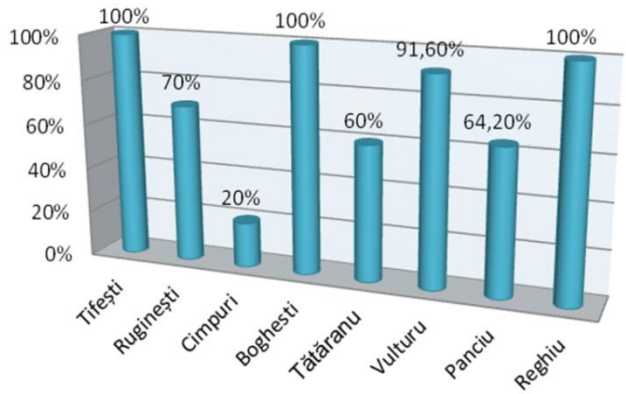


Fig. 1.The percentage of seropositive samples collected in Vrancea County

The prevalence rates of seroconversion in diary cattle sera from Vaslui County were examined with ELISAs specific for total anti-HEV antibodies. The overall prevalence of anti-HEV positive antibodies in cattle sera was 100% across the all three farms from Vaslui County.

Table 2. The results of serological detection of anti-BVDV antibodies in serum samples collected from cattle in Vaslui County

No.	Farm	No. of tested samples	No. of positives samples	No. of negative samples
1	Farm A	14	14	0
2	Farm B	31	31	0
3	Farm C	77	77	0
Total		***122***	***122***	***0***

Detection of 100% seropositive cattle for antibodies to BVDV in Vaslui County is due to a sustained campaign of vaccination as a means of control and eradication of bovine virus diarrhea-mucosal disease in diary cattle farms.

Accurate detection of BVDV infection in cattle is important in all herds, but less than perfect sensitivity and the potential of false negative test result is amplified in large herds with BVDV PI cattle (Goial SM, 2005).

Conclusions

1. The household system (Vrancea County) BVDV seropositivity can be justified by the existence of animals exposed to the virus, in those cases there is no implemented program to actively fight against BVD-MD.
2. The results of this study demonstrate that cattle are exposed to BVDV and become infected, as evidenced by seroconversion. These data have important implications for the management of bovine virus diarrhea-mucosal disease.
3. Simple, easily interpretable strategies to detect BVDV infections in cattle herds with a wide variety of management practices are necessary to provide herd-level information essential for a BVDV control program.

Aknowledgements

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References

1. Baker JC., 1995, The clinical manifestations of bovine viral diarrhea infection. Vet Clin North Am Food Anim Pract.; 11:425–445.
2. Brock KV, 2004, Strategies for the control and prevention of bovine viral diarrhea virus. Vet Clin North Am Food Anim Pract, 20:171–180.

3. Fulton RW, Briggs RE, Ridpath JF, et al., 2005, Transmission of bovine viral diarrhea virus 1b to susceptible and vaccinated calves by exposure to persistently infected calves. Can J Vet Res.; 69:161–169.
4. Goyal SM, 2005, Diagnosis In: Bovine viral diarrhea virus: diagnosis, management, and control, Goyal SM, Ridpath JF, 1st ed.: 197–208. Blackwell, IA.
5. Grooms DL, 2004, Reproductive consequences of infection with bovine viral diarrhea virus. Vet Clin North Am Food Anim Clin; 20: 5–19.
6. Houe H, Lindberg A, Moennig V, 2006, Test strategies in bovine viral diarrhea virus control and eradication campaigns in Europe. J Vet Diagn Invest, 18:427–436.
7. Potgeiter L, 2004, Infectious Diseases of Livestock. Bovine viral diarrhea and mucosal disease. Southern Africa: Oxford University Press. 946–969.
8. Rush DM, Thurmond MC, Munoz-Zanzi CA, Hietala SK, 2001, Descriptive epidemiology of postnatal bovine viral diarrhea virus infection in intensively managed dairy heifers. J Am Vet Med Assoc, 219:1426–1431.

IMMUNOSUPPRESSIVE ACTION OF DEOXYNIVALENOL (DON) ON BURSA FABRICII IN CHICKENS

Carmen SOLCAN, C. COTEA, Cristina CIORNEI, C. TODIREANU, Lavinia NICHITA
USAMV Iasi, Faculty of Veterinary Medicine

Abstract
The most common effects of prolonged dietary exposure of experimental animals to DON are decreased weight gain, anorexia, decreased nutritional efficiency and altered immune function with species differences (Marrs et al., 1986). The study aimed to prove the immunosuppressant action of deoxynivalenol (DON) in chickens experimentally treated each day, from the 7th day of life, using 5,4mg/kg b.w in E group for 28 days (since 35 day old). Histopathological studies of bursa of Fabricius were made on 7th , 14^{th,} 21st and 28th days of experiment. Lymphoid depletion of Fabricii bursa appeared from the begining and persisted during the recovery phase of experimental poisoning. After 21 and 28 days of exposure bursa revealed a lack of cortico-medullar differentiation, lymphoid depletion and necrosis.

Key words: Deoxynivalenol, immunosuppressant, chickens, bursa Fabricius

Deoxynivalenol (DON, vomitoxin) is a type B trichothecene, an epoxy-sesquiterpenoid, produced by fungi of the *Fusarium* genus, i.e. *Fusarium culmorum* and *Fusarium graminearum (*Eriksen and Alexander, 1998). It is also know as vomitoxin due to his strong emetic effect after consumption, because it is transported into the brain, where it runs dopaminergic receptors. The International Agency for Research on Cancer (IARC) concluded in 1993 that "There is inadequate evidence in experimental animals for the carcinogenicity of deoxynivalenol." Overall, DON was placed in Group 3, "not classifiable as to its carcinogenicity to humans". All animal species evaluated to date are susceptible to DON according to the rank order of pigs > mice > rats > poultry ≈ ruminants (Prelusky et al., 1994).

Deoxynivalenol has negative influences on the health and performance of chicks. However, there is little information available regarding the effect of DON on DNA fragmentation in blood lymphocytes, on lipid peroxidation and lymphocyte DNA fragmentation in broilers (Awad and col. 2012). Most trichothecenes inhibit protein synthesis, their potency depending on structural substituents and requiring an unsaturated bond at the C9–C10 position and integrity of the 12,13-epoxy ring. The 12,13-epoxid group of the DON molecule is responsible for its toxicity (Feinberg B and col 1989). Trichothecenes bind to the 60S subunit of eukaryotic ribosomes and interfere with the activity of peptidyltransferase. Deoxynivalenol, which lacks a substituent at C-4, inhibits chain elongation (Ehrlich & Daigle, 1987). Inhibition of protein synthesis is considered to be the primary toxic effect of trichothecenes, including deoxynivalenol.

Materials and methods

Experiment were used 40 chickens, which after a period of one week of accommodation to living conditions provided, were randomly divided in 2 groups: experimental (E) and control (C). Chickens were reared on sawdust litter, were provided specific microclimate conditions for age, room temperature gradually decreasing from 28°C, to 18°C. Commercial-type food, free of DON was administered ad libitum. E group received

daily by gavage deoxynivalenol (DON-Sigma Chemicals Co.) eluted in sterilized sunflower oil at a dosed 5,4 mg/ kg b.w. The control group received only eluent (sterilized sunflower oil). At the end of each week during the experiment, chicks were individually weighed. Five chickens were selected by random from each group and were killed at 7^{th}, 14^{th} , 21^{st} and 28^{th} day of the experiment.

Histopathology was performed on bursa Fabricius fragments fixed in 10% formalin solution, embedded in paraffin, sectioned at 5μm and stained by HEA and PAS.

Results and discussions

In our study cellular depletion in the folicle medulla of the bursa Fabricii appeared from the begining and persisted during the recovery phase in experimental poisoning with DON. Bursa Fabricius after 14 and 21 days of exposure to DON showed mild to severe atrophy of the lymphoid follicles with small necrotic foci, infolded epithelium, hydropic epithelial degeneration, and proliferation of reticular cells (fig.1a). Fibrous tissue gradually increased, and large mucoid cysts were evident (fig. 1b).

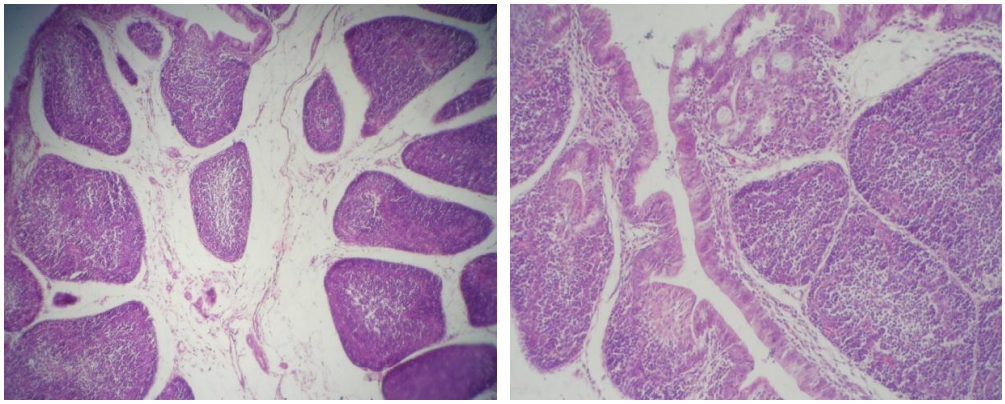

Fig.1. Bursa Fabricius after 14 days of exposure to DON (a), nonlymphoid cells from interfollicular and epithelium associated to proliferated follicle and cysts formed by epithelial cells and (b). PAS stain x100 (a); HE stain x200 (b)

After 21 days of exposure bursa Fabricius in DON treated group revealed a lack of cortico-medullar differentiation and generalized lymphoid depletion and necrosis (fig.2a). Bursal follicles have a reduced cortical zone in which necrotic foci may be seen. Folicle associated epithelium is folded and consists from 2 layers of cells, showing numerous necrotic foci (fig.2b)

After 28 days of exposure bursa Fabricius in DON treated group revealed a lack of cortico-medullar differentiation and generalized lymphoid depletion and necrosis (fig.3a). Bursal follicles have a reduced cortical zone in which necrotic foci. Follicle associated epithelium is folded and consists from 2-3 layers of cells, showing numerous necrotic foci (fig.3b).

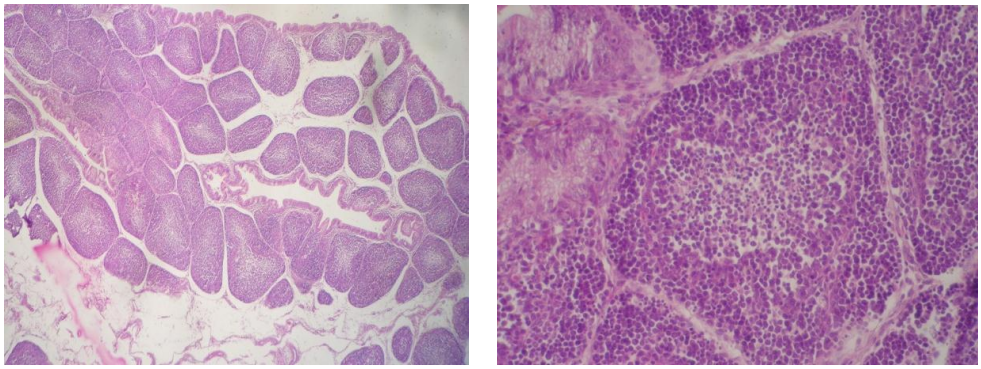

Fig.2. Bursa Fabricius after 21 days of exposure to DON.
Depletion of lymphocytes from the medullary region of bursal follicles.
Necrosis area in cortical and medullar zones. Epithelium associated to folicle proliferated (b).
Hematoxilin-eosin stain; a x60, b x400

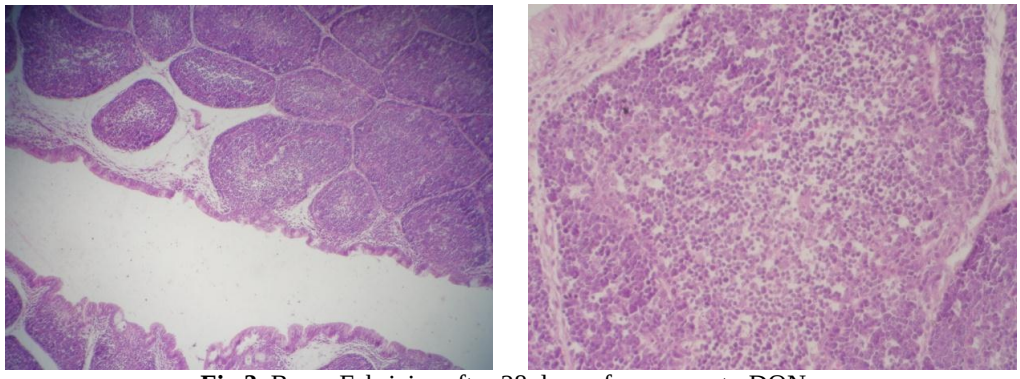

Fig.3. Bursa Fabricius after 28 days of exposure to DON.
Depletion of lymphocytes from the medullar region of bursal follicles.
Necrosis area in cortical and medullar zones. Epithelium associated to follicle proliferated (b). PAS stain; x100 (a); HE stain x400 (b)

The germinal center of the lymphoid follicles appears to provide a microenvironment for the generation of memory B and plasma cells. Following stimulation with antigen, B-cell blasts seed the primary follicle wich matures to a secondary germinal center with well defined anatomic zones.

Discussions

Many studies of host resistance, mitogen-induced lymphocyte proliferation, and humoral immune response have yielded a common theme that trichothecenes are both immunostimulator and immunosuppressive depending on dose, exposure frequency and timing relative to functional immune assay (Pestka *et al*., 2004). Trichotecene poisoning in poultry can cause oral lesions, haemorrhages, depletion and necrosis in the lymphopoetic organs and death.

DON-induced immunosuppression in poultry may be manifested as decreased antibody production to antigens (e.g. sheep red blood cells) and impaired delayed hypersensitivity response (e.g. dinitrochlorobenzene), reduction in systemic bacterial clearance (e.g. *Salmonella, Brucella, Listeria* and *Escherichia*), lymphocyte proliferation (response to mitogens), macrophage phagocytotic ability, and alterations in CD4+/CD8+ ratio, reduced immune organ weights (spleen, thymus and bursa of Fabricius), and histological changes (lymphocyte depletion, degeneration and necrosis). Mycotoxins, have been shown to down regulate proinflammatory cytokine levels including those of interferon (IFN)-γ, IFN-α, interleukin (IL)-1β, and IL-2 in broiler chickens. *Fusarium* mycotoxins exert part of their toxic effects by altering cytokine production in poultry. Impact of feed-borne mycotoxins on avian cell-mediated and humoral immune responses on thymus and bursa Fabricius is associated with lymphoid necrosis and premature atrophy of these tissues (Girish, T. K. Smith 2008).

The capacity of DON to expand polyclonally IgA-secreting cells in mice is mediated by increased cytokine production by macrophages (Mφ) and T cells (Bondy and Pestka, 1991, Pestka et al., 1990, Warner et al., 1994).

High doses of trichothecenes promote rapid onset of leukocyte apoptosis and this is manifested as immunosuppression (Pestka et al., 2004). DON induces apoptosis in T-cells, B-cells and IgA+ cells in vitro (Pestka et al., 1994). These in vitro data are relevant to the whole animal since in vivo administration of trichothecenes to rodents results in apoptosis in Peyer's patches, thymus, and bone marrow (Ihara et al., 1997, Ihara et al., 1998, Islam et al., 1998, Miura et al., 1998, Shinozuka et al., 1997).

In contrast to the above, exposure to high concentrations of trichothecene can induce apoptosis in macrophages (Yang et al., 2000, Zhou and Pestka, 2003) thereby suppressing innate immune function (Pestka et al., 2004). DON induces apoptosis in RAW 264.7 murine macrophages, however, murine peritoneal macrophages might be less susceptible (Zhou et al., 2005).

Mononuclear phagocytes appear to be exquisitely sensitive to trichothecenes. Stimulation of macrophages by low doses or concentrations of trichothecenes upregulate expression of inflammation-related genes in vivo and in vitro including COX-2 (Moon and Pestka, 2002), proinflammatory cytokines (Zhou et al.,2005), and numerous chemokines (Chung et al., 2003, and Kinser et al., 2004).

Conclusions

In bursa Fabricius intense histological lesions appeared at 14, 21, 28 days after experimentaly intoxication with DON. Stromal cells hyperplasia is severe following lymphocyte necrosis. Bursal follicles have a reduced cortical zone, whith necrotic foci. Follicle associated epithelium is folded and consists from 2-3 layers of cells, showing numerous necrotic foci. Many lymphocytes shown histological changes: depletion, degeneration and necrosis.

Bibliography

1. Awad W. A., Ghareeb K, Dadak A. , Gille L, Staniek K, Hess M., Böhm J. , 2012 - Genotoxic effects of deoxynivalenol in broiler chickens fed low-protein feeds Poult. Sci. March 2012 vol. 91 no. 3 550-555

2. Bondy G.S., Pestka J.J. , 1991 - Dietary exposure to the trichothecene vomitoxin (Deoxynivalenol) stimulates terminal differentiation of Peyer's patch B cells to IgA secreting plasma cells Toxicol. Appl. Pharmacol., 108, pp. 520–530
3. Chung Y.J., Yang G.H., Islam Z., Pestka J.J., 2004- Up-regulation of macrophage inflammatory protein-2 and complement 3A receptor by the trichothecenes deoxynivalenol and satratoxin G Toxicology, 186 (2003), pp. 51–65,
4. Ehrlich, K.C. & Daigle, K.W. 1987- Protein synthesis inhibition by 8-oxo-12,13- epoxytrichothecenes. *Biochem. Biophys. Acta,* 923, 206–213.
5. Eriksen, G.S. & Alexander, J., 1998- Fusarium Toxins in Cereals —A Risk Assessment (TemaNord 1998:502). Copenhagen: Nordic Council of Ministers, 115 pp.
6. Girish CK, Smith TK ., 2008 - Effects of feeding blends of grains naturally contaminated with Fusarium mycotoxins on small intestinal morphology of turkeys. Poult Sci. Jun;87(6):1075-82.
7. Ihara T., Sugamata , M, Sekijima M., Okumura H., Yoshino N., Ueno Y., 1997 -Apoptotic cellular damage in mice after T-2 toxin-induced acute toxicosis Nat. Toxins, 5, pp. 141–145
8. IharaT., Yamamoto T, Sugamata M., Okumura H., Ueno Y., 1998 -The process of ultrastructural changes from nuclei to apoptotic body, Virchows Arch., 433 (1998), pp. 443–447
9. Islam Z., Nagase M., Yoshizawa T.,. Yamauchi K, Sakato N., 1998 - T-2 toxin induces thymic apoptosis in vivo in mice Toxicol. Appl. Pharmacol., 148, pp. 205–214
10. Kinser S,. Jia Q, Li, M. Laughter A., Maizewell P., Corton J.C.,. Pestka J, 2004-Gene expression profiling in spleens of deoxynivalenol-exposed mice: immediate early genes as primary targets J. Toxicol. Environ. Health A, 67 (2004), pp. 1423–1441
11. Marrs T.C., Edginton J.A., Price P.N., Upshall D.G., 1986 - Acute toxicity of T2 mycotoxin to the guinea-pig by inhalation and subcutaneous routes Br. J. Exp. Pathol., 67, pp. 259–268
12. Miura K., Nakajima Y., Yamanaka N., Terao K., Shibato T., Ishino S., 1998 Induction of apoptosis with fusarenon-X in mouse thymocytes Toxicology, 127, pp. 195–206
13. Moon Y., Pestka J.J., 2002 - Vomitoxin-induced cyclooxygenase-2 gene expression in macrophages mediated by activation of ERK and p38 but not JNK mitogen-activated protein kinases Toxicol. Sci., 69, pp. 373–382
14. Pestka J.J., Yan D., King L.E., 1994 - Flow cytometric analysis of the effects of in vitro exposure to vomitoxin (deoxynivalenol) on apoptosis in murine T B and IgA+ cells Food Chem. Toxicol., 32, pp. 1125–1136
15. Pestka J.J., Dong W., Warner R.L., Rasooly L., Bondy G.S., Brooks K.H., 1990 - Elevated membrane IgA+ and CD4+ (T helper) populations in murine Peyer's patch and splenic lymphocytes during dietary administration of the trichothecene vomitoxin (deoxynivalenol) Food Chem. Toxicol., 28, pp. 409–420
16. Pestka J.J., Zhou H.R., Moon Y., Chung Y.J., 2004 - Cellular and molecular mechanisms for immune modulation by deoxynivalenol and other trichothecenes: unraveling a paradox Toxicol. Lett., 153, pp. 61–73
17. Prelusky D.B., Gerdes R.G., Underhill K.L., Rotter B.A., Jui P.Y., Trenholm H.L., 1994- Effects of low level dietary deoxynivalenol on the haematological and clinical parameters of the pig. *Nat. Toxins.*;2:97–104.
18. Shinozuka J., Guanmin L., Uetsuka K., Nakayama H., Doi K., 1997 - Process of the development of T-2 toxin-induced apoptosis in the lymphoid organs of mice Exp. Anim., 46, pp. 117–126
19. Warner R.L., Brooks K., Pestka J.J., 1994 - In vitro effects of vomitoxin (Deoxynivalenol) on lymphocyte function: enhanced interleukin production and help for IgA secretion Food Chem. Toxicol., 32, pp. 617–
20. Yang G., Jarvis B.B., Chung Y., Pestka J.J., 2000 - Apoptosis induction by the satratoxins and other trichothecene mycotoxins: relationship to ERK, p38 MAPK and SAPK/JNK activation Toxicol. Appl. Pharmacol., 164, pp. 149–160
21. Zhou H.R., Islam Z., Pestka J.J., 2003 - Rapid, sequential activation of mitogen-activated protein kinases and transcription factors precedes proinflammatory cytokine mRNA expression in spleens of mice exposed to the trichothecene vomitoxin Toxicol. Sci., 72, pp. 130–142
22. Zhou H.R., Islam Z., Pestka J.J., 2005 - Induction of competing apoptotic and survival signaling pathways in the macrophage by the ribotoxic trichothecene deoxynivalenol, Toxicol. Sci., 87 (2005), pp. 113–122

DYNAMICS OF DIGESTIVE AND PULMONARY PARASITIC ELEMENTS IN CARPATHIAN GOATS, AT THE END OF STABULATION

Olimpia C. IACOB
Faculty of Veterinary Medicine Iași

Abstract
The research has been made in April 2011 on a number of 1450 Carpathian goats, private property (Costești – Botoșani), purchased from all areas and grown in semi intensive system. Their body condition was medium to weak, goats being exploited for milk production. In the grazing season the goats had special summer shelters and wide grazing areas with rich flora and young trees. In the winter, the modern shelters offered all the necessary conditions to assure biological comfort. The diet consisted from natural hay, corn silage, supplemented with concentrate feed and mineral salts, given concerning the age and the physiological state. Watering has been done automatically, in grooves. To increase the health state of the herd, at the end of the stabulation period a digestive and pulmonary parasitogram has been made to 10% of the goat popullation, concerning the age and the physiological state. Feces samples were individually collected by trans rectal prelevation and were analyzed by qualitative (Willis, Vajda) and quantitative (McMaster, Euzeby) ovoscopic and larvoscopic methods, estimating the intensity (OPG) and extensivity (E%) of the parasitic infestations. The obtained coproscopic results revealed parasitic elements belonging to protozoa, Eimeria genus (OPG: 0-2500, E%: 0-100%), cestodes, Moniezia genus ((OPG: 0-300, E%: 20-30%), nematodes, Trichostrongylidae family (OPG: 0-5100, E%: 0-100%), pulmonary nematodes, Protostrongylus genus (Lpg: 0-180 E% 0-100%), suggesting a variable level of infestation with a variable extensivity which represents a perpetual source of parasitic pollution of the grazing areas.
Key words: goats, stabulation, digestive and pulmonary parasitogram

During the last years, in our country, there has been a special interest in the intensive farming of goats. The breeds that are raised are Carpathian (70%) and White of Banat, (30%). The high quality of milk and dairy products, low colesterol meat, the production of hair and down, and last but not least, the simple conditions of growth and exploitation, recommend this species as economically advantageous. It is appreciated for being a species resistant to some diseases (brucellosis, tuberculosis), but it is sensitive to cold and humidity (5). Since the flocks of sheep usually include goats, the parasites infecting the two species are studied as being similar, which is not correct, given the fact that the goat has many features that set it apart from sheep. The fact that goats and sheep are reared together, favor the passage of some parasitic and/or infectious elements from one species to the other, which maintains them in flocks and on common grazing lands. Establishing the parasitic profile of a herd of Carpathian goats, purchased from most of the areas of the country, has established itself as a necessity for understanding the species that make up the parasitic fauna of the digestive tube, the liver and the lungs, in view of selecting the most appropriate antiparasitic substances for the medical therapy and prophylaxis of the herd.

Materials and methods

The herd of 1450 goats in the study is structured as follows: adult pregnant does, barren does, Saanen breed bucks, youth from previous year, youth from current year. The fecal samples were collected individually by rectal prelevation from 5% of the group, at the end of the stabulation period. The samples were analyzed in the Parasitic Diseases Clinic of the Faculty of Veterinary Medicine in Iasi, through copro-parasitological, ovoscopical and

larvoscopical methods, both qualitative (Willis, Vajda) and quantitative (Mc.Master, Euzeby), as well as repeated washing and free sedimentation. The anthelmintics used two weeks before going out to pasture were parenteral Ivermectin administered subcutaneously at a dose of 0.2 mg / kg body weight and Vermitan (Albendazole) at the dose of 0.75 ml/10kg body weight, administered orally by automatic gun.

Results and discussions

The results of the coproscopical ovoscopical and larvoscopical examinations made on samples from goats, according to age and physiological state, are included in the tables, and the dynamics of invasional elements is graphically represented. The coproscopical results obtained for one year old does, during their first gestation period, are shown in table 1. The goat youth studied represent the first generation of mixed breed between the Carpathian does and Saanen bucks for the improvement of milk production.

Table 1. Intensivity (OPG, Lpg) and extensivity (E%) of digestive and pulmonary infestation in one year old does, (pregnant) at the end of the stabulation period. Costești-Botoșani (May 3rd 2011)

		Methods: Willis, Mc. Master						Methods: Vajda, Euzeby			
Nr. Crt.	Nr. matr	*Eimeria*		Cestodes		*Trichostrongylidae*		*Dictyocaulus filaria*		*Protostrongylus*	
		OPG	E%	OPG	E%	OPG	E%	Lpg	E%	LpG	E%
1	183	150		0		0		0		4	
2	036	350		0		0		0		4	
3	297	0		0		0		0		0	
4	239	250		0		0		0		0	
5	030	300	90	0	20	0	10	0	0	20	40
6	032	650		0		50		0		0	
7	186	200		0		0		0		0	
8	090	150		0		0		0		0	
9	081	100		250		0		0		0	
10	123	250		300		0		0		28	

The coproscopical results obtained from goat youth on their first gestation reflect the dominant presence of the genus *Eimeria*, with a low level of OPG (0-650) but with an extensivity (E%) of 90%. Also, digestive and pulmonary helminths have a reduced extensivity, between 0-40%, suggesting a very diminished population. The cestodes of the *Moniezia* genus, are present in 20 % of the samples with an OPG of 250-300, the species of the *Trichostrongylidae* family have an extensivity of 10%, and the OPG: 50; the species *Dictyocaulus filaria* has not been identified, while the genus *Protostrongylus* was present in

40 % of the samples. This was due to the fact that the young pregnant does were treated with Vermitan (Albendazole)10%, 0,75 ml/10kg body weight, administered orally, two weeks before the coproscopical examination (April 15th, 2011). Removing the helminth fauna in the digestive sistem through preventive therapy, created favorable conditions for the development of the *Eimeria* population, and the pollution of the resting and feeding space with oocysts, thus favoring the occurrence of clinical episodes in newborn kids.

Examining the samples through the method of repeated washings and free sedimentation showed eggs belonging to the *Dicrocoelium lanceatum* species.

The dynamics of digestive and pulmonary invasional elements in young pregnant does, one year of age, is shown in figure 1. a, b.

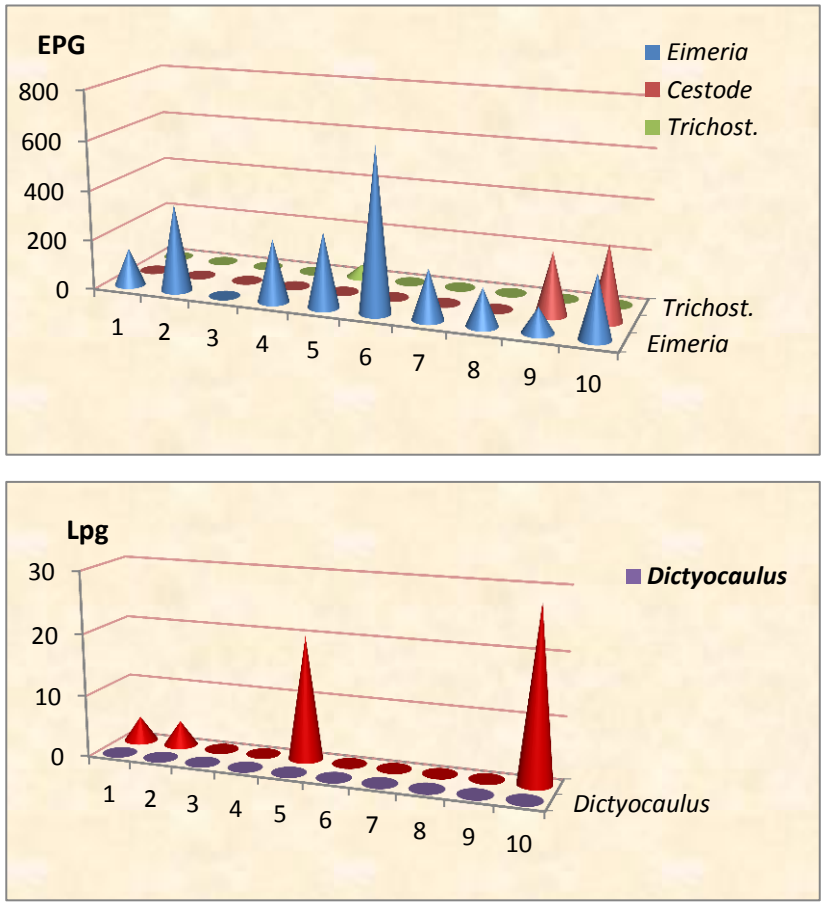

Fig. 1. a, b. Dynamics of intensivity (OPG) of digestive and pulmonary invasional elements in pregnant, one year old does, at the end of the stabulation period, Costești -Botoșani (May 3rd 2011)

The coproscopical results of the herd of multiparous lactating adult does, aged between 3-7 years, purchased from most of the areas of the country, which had not been previously treated with anthelmintics, is shown in table 2.

Table 2. Intensivity (OPG, Lpg) and extensivity (E%) of digestive and pulmonary infestation in multiparous lactating does of the Carpathian breed, aged between 3-7 years (May 3rd 2011)

Nr. crt.	Nr. matr.	Methods: Willis, Mc.Master						Methods: Vajda, Euzeby			
		Eimeria		Cestodes		*Trichostrongylidae*		*Dictyocaulus filaria*		*Protostrongylus*	
		OPG	E%	OPG	E%	OPG	E%	Lpg	E%	LpG	E%
1	081	2000		50T		2000		0		16	
2	660	2500		0		2500		0		20	
3	355	2000		0		4200		0		12	
4	823	2400		0		3800		0		20	
5	213	1500	100	0	30	4900	100	0	0	20	100
6	204	2100		0		5100		0		36	
7	298	2200		50		3000		0		100	
8	924	1500		0		3500		0		180	
9	598	1800		50		3900		0		60	
10	033	2500		0		4600		0		100	

The coproscopical digestive and pulmonary examination of adult goats, at the end of the stabulation period, showed different aspects. The population of *Eimeria* is best represented, with an OPG of 1500-2500 and an extensivity of 100%, constituting a main source of pollution of shelters with oocysts, but it is dominated by nematodes of the Trichostrongyilidae family, which have high values of OPG: 2000-51000 and an extensivity of 100%. This increase in the OPG of Trichostrongillids observed in adult goats, can be attributed to the phenomenon known as „spring rise". This phenomenon starts in spring with the improvement of the climate, contributing to the pollution of pastures with new invasional elements which will ensure a new population of infesting larvae, since the residual larvae that

survived temperatures in winter are in small number (2). A new population of larvae will find a new population of kids, generating severe clinical episodes, on their first infestation.

Pulmonary nematodes are also represented in this case, with the genus *Protostrongylus* showing a gradual infestation from a low level to a massive and strong level (Lpg: 12-180) and an extensivity of 100%. In this herd, the pulmonary infestation was clinically manifest.

Examining the samples through repeated washings and free sedimentation led to the rare identification of eggs belonging to the species *Dicrocoelium lanceatum*, which suggests the presence of adult parasites in the liver.

The dynamics of digestive and pulmonary infestive elements in multiparous lactating adult goats, aged between 3-7 years is shown in fig. 2 a, b.

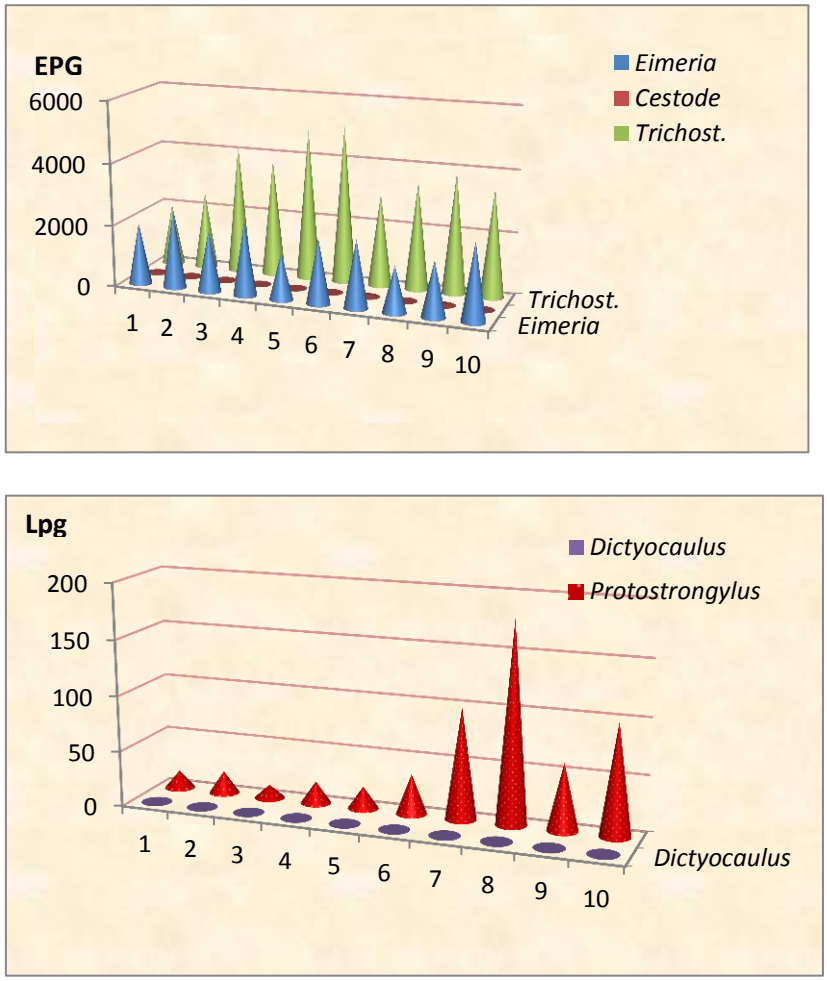

Fig. 2. a, b. Dynamics of intensivity (OPG) of digestive and pulmonary invasional elements in adult goats between 3-7 years, at the end of the stabulation period, Costești -Botoșani (May 3rd 2011)

The coproscopical results obtained from imported Saanen bucks, regarding the digestive and pulmonary infestation at the end of the stabulation period are included in table 3.

Table 3. Intensivity (OPG, Lpg) and extensivity (E%) of digestive and pulmonary infestation in imported Saanen male goats Costeşti-Botoşani (May 3rd 2011)

Nr. crt.	Nr. matr.	Methods: Willis, Mc.Master						Methods: Vajda, Euzeby			
		Eimeria		Cestodes		*Trichostrongylidae*		*Dictyocaulus filaria*		*Protostrongylus*	
		OPG	E%	OPG	E%	OPG	E%	Lpg	E%	LpG	E%
1	171	100		0		0		0		0	
2	271	150		0		0		0		0	
3	272	50	100	0	0	0	0	0	0	0	0
4	317	100		0		0		0		0	
5	318	150		0		0		0		0	

The coproscopical analysis of the bucks shows the presence of the genus *Eimeria* in all samples, with a very low OPG, between 50-150, being the only type of parasite present. It should be noted that male goats received preferential feeding and husbandry. The bucks had been treated with Ivermectin at a dose of 0,2mg/kg, administered subcutaneously two weeks before samples were taken. Examining the samples through repeated washings and free sedimentation did not reveal eggs belonging to trematode species. The dynamics of digestive and pulmonary invasional elements in bucks is shown in fig. 3.

The coproscopical digestive and pulmonary analysis of the herd of goats taken into study shows different aspects of the parasitogram according to age, phisiological state, sex and economical interest. Thus, we observed a differentiated approach of age categories, considering the fact that the group of adult goats will be progressively reduced or replaced with improved young ind. In the digestive and pulmonary parasitic biocenosis we notice the value oscillation of OPG and Lpg, according to internal and external factors.

In all age categories the genus Eimeria is present both in treated animals and in those which had not been treated before going out to pasture, which suggests a perpetuation of the genus Eimeria both in the goat herd and in the shelters belonging to the husbandry unit. The dominance of Trichostrongillids in adult untreated goats suggests that the other age categories were also affected by these species, but the results changed after anthelmintic treatment.

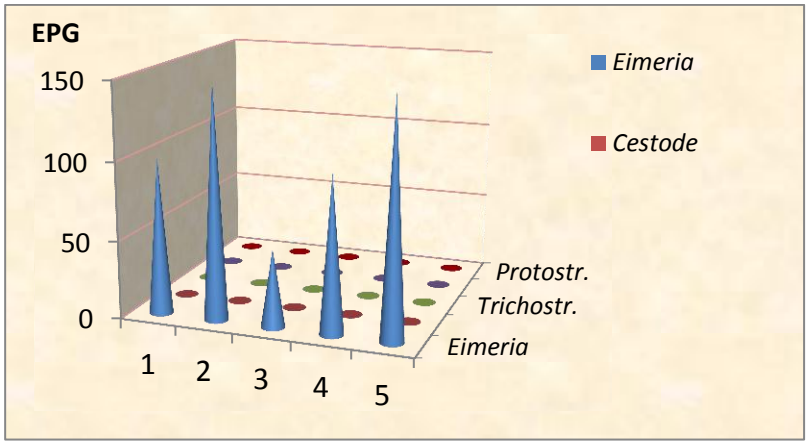

Fig. 3. Dynamics of intensivity (OPG) of digestive and pulmonary invazional elements in adult bucks , Costeşti -Botoşani (May 3rd 2011)

Pulmonary nematodes represented by the genus *Protostrongylus*, species *Protostrongylus rufescens*, appeared as a unique infestation in all the herd; larvae of the species *Dictyocaulus filaria* have not been identified. These results show that the areas atributed for grazing are located in a hill area, with slopes and brushes, favourable to the development of intermediary hosts (terrestrial gasteropodes) for Protostrongylidae and unfavourable to the development of the species *Dictyocaulus filaria.* The results obtained are consistent with scientific literature (1, 3). The coproscopical evalution of the herd contributes to the identification of species of parasites, selecting antiparasitical substances according to the type of parasites, obtaining superior terapeutical results and preventing the development of resistence of parasites to the medicines used (4, 6). Medical prophylaxis correctly applied in the groups of animals leads to the observation and control of parasitic infestations, reducing economical losses and the pollution of the environment with invasional elements.

Conclusions

1. During the period April-May 2011, we made a coproscopical evaluation of a herd of 1450 goats of Carpathian breed, private property, in the area Costeşti-Botoşani, revealing different aspects of their digestive and pulmonary parasitogram.
2. The genus *Eimeria* was dominant in all age categories, with an extensivity of 90-100% and a value of OPG between 0-2500.
3. Nematodes of the family Trichostrongylidae were dominant in adult goats, with an extensivity of 100% and an OPG value between 2000-5100, which defines a strong infestation; in young goats and bucks (treated against parasites two weeks previous to going out to pasture), trichostrongillids had an extensivity of 0-10% and a value of OPG of 0-50.

4. Pulmonary nematodes of the genus *Protostrongylus*, species *Protostrongylus rufescens*, were dominant through unique infestation in adult goats and pregnant youth.
5. Cestodes were represented by the family *Anoplocephallidae*, genus *Moniezia* and rarely *Thysaniezia*, with a dominance of *Moniezia* genus, species *Moniezia expansa*. The extensivity was of 0-30% and the OPG between 0-300.
6. Trematodes of the genus *Dicrocoelium*, species *Dicrocoelium lanceatum* were represented by rare invasional elements identified in young individuals and adult does.
7. Medical prophylaxis applied selectively in the herd, according to age and short-term economical interest, contributes to the infestation and disease of animals at their first grazing, to the parasitical pollution of grazing areas, and to the recording of much higher economical losses.

References

1. Altas, M. G., Sevgili, M., Gokcen, A., Aksin, N., Bay-Burs, H.C. The prevalence of gastro-intestinal nematodes in hair goats of the Snliurfa region. Turkiye Parazitoloji Dergisi (2009)33 (1)20-24 Bornovo, Turkey.
2. Duk, J.Van, Morgan, E.R. The influence of temperature on the development hatching and survival of Nematodirus battus larvae. Parasitology (2008) 135 (2) 269-283 Cambridge, UK.
3. Meshram, M.D., Shirale, S.Y., Khillare, K.P. Prevalence of helminthic infection in goats. Indian Veterinary Journal (2007) 84 (9)992 Chennai, India.
4. Ved Prakash, Siddiqua Bano. Comparative study on treatment of gastro-intestinal nematodes of goats in Kanpur. Asian Journal of Animal Science (2008) 3 (1) 77-78 Muzaffarnagar, India
5. Pascal C. Creșterea ovinelor și caprinelor. (2007) Ed. Pim, Iași, pp. 441-448.
6. Paraud, C. Kulo, A., Pors, I., Chatier, C. Resistance of goat nematodes to multiple anthelmintics on a farm in France. Veterinary Record (2009) 164 (18) 563-564 London, UK.

ASSESSMENT OF THE PHARMACODYNAMIC EFFECT OF ROBENACOXIB IN CATS WITH MUSCULOSKELETAL PAIN AND INFLAMMATION

Mariana GRECU, Valentin NĂSTASĂ, Mihai MAREŞ, Ramona MORARU
University of Agricultural Sciences and Veterinary Medicine of Iasi, Romania;
Faculty of Veterinary Medicine; marygrecu@gmail.com

Abstract
In this study, 8 cats with musculoskeletal pain and inflammation were included. They have received robenocoxib orally once a day, 1 mg / kg body weight, for 14 to 21 days. During this period, the analgesic and anti-inflammatory effects were evaluated and the side reactions were also noticed. For all patients, blood tests (i.e. urea, alanine phosphatase, alanine transaminase, gamma-glutamyltransferase, creatinine, total protein, lactat dehidogenase) and clinical surveillance for mucosal hemorrhages at 0, 14 and 21 days were assessed. Data were statistically analyzed. Analgesic and anti-inflammatory effects of robenocoxib was registered for 89% of treated cases. No clinical adverse reactions and no changes in serum or blood parameters were noted, indicating that the drug was well tolerated by cats.

Key words: robenacoxib, pain and inflammation musculoskeletal, analgesic and anti-inflammatory effect

Introduction

Robenacoxib is a new drug substance category nonsteroidal anti-inflammatory (NSAIDs) (King et al. 2009), used in veterinary medicine to treat acute pain and chronic inflammation in dogs and cats. Substance belongs to the coxib class of inhibitors of cyclooxygenase 2 (COX 2) preferential and selective therapeutic effect indicate in their analgesic and anti-inflammatory, but especially for digestive safety they offer, compared with a COX 1 inhibiting substances (Budsberg 2009b, EMA EPAR - Onsior 2008a, Giraudel et al., 2009, Innes et al.2010, Whittle 2000).

COX-2 is an inducible isozyme, its expression is induced by proinflammatory cytokines and endotoxins at inflammation (macrophages, monocytes, endothelial cells, fibroblasts) (King et al. 2009, Lascelles et al. 2007, Papich 2008). Cyclooxygenase 2 is involved in the synthesis of prostaglandins, which are in turn involved in inflammation and pain. Prostaglandins have been identified both in inflamed tissues, contributing to the spread of nociceptive (pain) and gastrointestinal tract, with gastroprotective effects. Some researches (David 2006, Whittle 2000) have shown that COX-2 is expressed and constitutive in some tissues: kidney epithelium, particularly in the macula dense, vascular endothelium, central nervous system, ovaries and uterus. This isoenzyme is inhibited secretion of steroids (King et al. 2009, Whittle 2000).

Robenacoxib our country is less known and used, which motivates this study. Thus, we evaluated the efficacy and tolerability of the substance also in cats with acute pain and inflammation associated with musculoskeletal disorders.

Material and methods

Research case studies were conducted on a private veterinarian for a period of several months (September 2011 - February 2012), taking the study a total of eight cats (from a total

of 21 cases), three females and five males, selected following predetermined criteria that excluded pregnant cats or those diagnosed with digestive disorders, kidney, liver and heart.

Cats studied came from different races, were aged from 3.2 to 9.4 years and weighing 2.5 to 4.7 kg and had various musculoskeletal disorders osteoarticular acute, confirmed after clinical and laboratory examinations (radiography). They have undergone therapy robenacoxib product is commercial tablets 6 mg gram specifically for cats, the dose was 1 mg / kg bodyweight per day managing, observing the same time.

Treatment was performed over a period ranging from 14 to 21 days for complete resolution of disease, and by agreement with the owners of cats, robenacoxib they administered oral doses as recommended by the manufacturer, the label veterinarian. Before treatment (t0) and at its end (TF) blood was collected from each case, 5 ml / patient and biochemical analyzes were performed: alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, amylase, gamma glutamyl transferase (γ GT), creatinine and urea.

To assess gastrointestinal tolerability, both at baseline and at its end, and samples were collected in the feces (the rectal ampul) using cotton swabs. Samples were analyzed using a standard test (Hemoccult test) using filter paper impregnated with guaiac, a reagent is in contact with fecal sample and reagent for positive samples induces a blue coloration of the filter paper by forming a halo. The analysis detected the presence of blood (microhemoragies), which comes normally from the gastrointestinal tract.

Pain was evaluated on a scale from 0-3, based on patient behavior, painful sensitivity of the response on (to palpation of the inflamed area) and mobility.

Cats were evaluated globally throughout the treatment, so the owner, the daily marking of all clinical signs appear, but particularly by veterinary clinicians at established time intervals (3, 7, 14, 18 and 21 days) evaluating the clinical or the patient's home or by presenting it to the office, the changes following therapy. Thus, we studied the phenomenon of pain intensity reduction, inflammation, mobility and improve motor function by resuming the general condition of cats.

Results

Efficacy and tolerability robenacoxib were analyzed based on clinical matters recorded throughout the treatment. It was considered that the patients responded favorably to treatment with robenacoxib if there is pain and inflammation, with remission track parameters and locomotor function and resume if there were no adverse effects or other digestive (vomiting, diarrhea, anorexia, loss of appetite, abdominal pain sensitivity, etc.).

Each evaluation of recorded data showed significant improvement with each examination carried out at 3, 7, 14, 18 and 21 days (Figure 1). Complete resolution of pain and inflammation phenomena associated with osteoarticular disease, was recorded at the end of treatment for more than 92%.

Cats have completed treatment without serious reaction. The only evident clinical changes were transient loose stools or diarrhea, after 11 to 14 days after the robenacoxib, 3 of cats (2 females and 1 male) and should be taken the antidiarrhea. Weight of the cats was not altered in any stage of treatment. Hemoccult test results conducted on fecal samples (performed before and at the end of treatment - t_0 - t_f) were negative for all cats. Also, no significant changes were observed in the study of biochemical parameters at the end of

treatment compared with results t_0, serum enzyme activities (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, amylase) were all within reference (table 1).

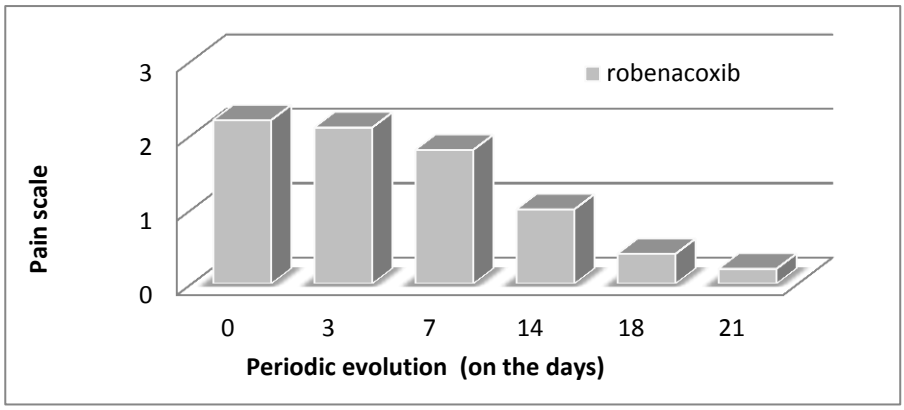

Fig. 1. Efficacy robenacoxib (score / days)

Table 2. Median values (minimum to maximum) for serum biochemistry data in cats

Variable	Before treatment	14 days	21 days	Reference range
Total protein (g/dL)	7.9 (6.8–8.1)	7.4 (6.2–8.3)	7.6 (6.6–8.5)	6.5–8.9
Albumin (g/dL)	3.3 (2.7–3.6)	3.2 (2.6–3.9)	3.4 (2.6–4.0)	3.2–4.7
Creatinine (mg/dL)	1.2 (0.8–1.6)	1.1 (0.7–1.4)	1.1 (0.8–1.3)	0.6–2.3
Serum urea nitrogen (mg/dL)	23 (18–35)	27 (22–31)	27 (20–32)	17–35
Alanine aminotransferase (U/L)	68 (40–250)	60 (37–424)	60 (36–316)	35–134
Aspartate aminotransferase (U/L)	27 (17–112)	21 (13–68)*	23 (15–47)	13–46
Alkaline phosphatase (U/L)	27 (15–44)	37 (19–62)*	39 (22–50)	15–96
g-Glutamyltransferase	2 (2–2)	2 (2–2)	2 (2–2)	0–2
Amylase	963(782–712)	941 (631–272)	866 (651–547)	515–1500
Lactat dehidrogenase	9.2 (7.9 – 8.8)	7.5 (6.4 – 7.2)	6.9 (6.9 – 7.0)	1 - 10
Creatine kinase	341(120–123)	320 (83–722)*	237 (133–649)*	71–502

The mean (± SD) between sampling periods with significant differences ($p < 0,05$) between the values of indices*

Robenacoxib showed a good efficiency by improving locomotor function markedly, with a rapid action and hence regression pain of the inflammatory process.

Discussion

Robenacoxib is licensed for dogs and cats. The flavoured tables have been shown to be very palatable in dogs and especially so in cats. Robenacoxib has an elimination half life of only two hours, but persists in inflamed tissue. This has been termed tissue selective and makes once-daily dosing sufficient despite rapidly falling plasma levels. This has been discussed as one of the reasons for a low incidence of side effects with maintained treatment effect (EMA EPAR Onsior - 2008a). It has been licensed for acute pain in cats and dogs and chronic use in dogs. The tablets were studied over six days in cats with acute musculoskeletal disorders, for six weeks in toxicity studies in cats and for up to one year in aged dogs with osteoarthritis. It is licensed for up six days in cats and infinitive use in dogs. In dogs the tablets should be administered without food to improve bioavailability; in cats they can be given with a small amount of food (Giraudel et al., 2009, King et al. 2009).

Although demonstrated efficacy and safety robenacoxib use this product on cats, some studies (Clarke et al. 2006, Giraudel et al. 2009, King et al. 2009) have reported different nature of gastrointestinal side effects, liver or kidney following the administration of this substances. In our research, product management led to improved disability cats without major side effects, even if the treatment period exceeded the recommendations specified by the manufacturer. Thus, the present study demonstrated that medical treatment is relevant because robenacoxib reduced pain and inflammation, improving mobility and quality of life of patients treated also. Side effects manifested by loose stools or diarrhea, were observed in 3 cases, but these were transient and were overlooked both the veterinary and by owner, because these clinical signs resolved in specific treatment of diarrhea. Similar findings were reported in other medical studies robenacoxib administered to cats (Innes et al. 2010).

Conclusions

Robenacoxib use in musculoskeletal disorders in cats studied osteoarticular caused a good analgesic and anti-inflammatory efficacy without side effects and biochemical parameters without changing the entire treatment period, indicating that the drug was well tolerated by cats.

Bibliography

1. Budsberg S.C. (2009b) - Nonsteroidal Antiinflammatory Drugs. In: *Handbook of Veterinary Pain Management (2nd edn).* MO, USA. pp. 183–209.
2. Clarke S., Bennett D. (2006) - Feline osteoarthritis: a prospective study of 28 cases. *J Small Anim Pract*; 47: 439–45.
3. David J., Graham M.D. (2006) **-** COX-2 Inhibitors, Other NSAIDs, and Cardiovascular Risk. *JAMA*; 296*.*
4. EMA EPAR (2008a) Onsior - European Public Assessment Reports. London, UK.
5. Giraudel JM, Toutain PL, King JN et al. (2009) - Differential inhibition of cyclooxygenase isoenzymes in the cat by the NSAID robenacoxib. *J Vet Pharmacol Ther* 32, 31–40.
6. Giraudel J.M., King J.N., Jeunesse E.C., Lees P., Toutain P.L. (2009) - Use of a pharmacokinetic/pharmacodynamic approach in the cat to determine a dosage regimen for the COX-2 selective drug robenacoxib. *J Vet Pharmacol Ther*, 32, 18–30.
7. Innes J., O'Neill T., Lascelles D. (2010) - Use of non-steroidal anti-inflammatory drugs for the treatment of canine osteoarthritis. *In Practice*; 32: 126-137.

8. King J.N., Dawson J., Esser R.E., Fujimoto R., Kimble E.F., Maniara W., Marshall P.J., O'Byrne L.O., Quadros E., Toutain P.L., Lees P. (2009) - Preclinical pharmacology of robenacoxib: a novel selective inhibitor of cyclooxygenase-2. *Journal of Veterinary Pharmacology and Therapeutics,* 32, 1–17.
9. Lascelles B., Court M., Hardie E., et al. (2007) - Nonsteroidal anti-inflammatory drugs in cats: a review. *Vet Anaesth Analg*; 34: 228–50.
10. Papich M.G. (2008) - An update on nonsteroidal anti-inflammatory drugs (NSAIDs) in small animals. *Vet Clin North Am Small Anim Pract*; 38(6): 1243-66.
11. Whittle B.J.R. (2000) - COX-1 and COX-2 products in the gut: therapeutic impact of COX-2 inhibitors*. J. Am. Vet. Med. Assoc*; 47: 320—3.

ZOOTHERAPY AS NON DRUG THERAPY IN ALZHEIMER'S DISEASE: THE ROLE OF THE VETERINARIAN

L.F. MENNA, M. TRAVAGLINO, M. FONTANELLA, A. SANTANIELLO, F. GIRARDI, E. AMMENDOLA
Universita' Degli Studi di Napoli Federico II, Facolta' di Medicina Veterinaria
Dipartimento di Patologia e Sanita' Animale
menna@unina.it

Abstract
When we speak of therapies with the help of the animals prefer to use the specific term zootherapy instead of pet therapy because we want to define those therapies in which intervention protocols are encoded with a specific method different from that of normal pet therapy. The zootherapy is a health service and as such is of clinical attributable to of both human and veterinary. The therapeutic setting, so it's made by the veterinarian with the animal cooterapist, the patient and the clinician human, be it a doctor or a psychotherapist all with interdisciplinary training. The zootherapy in Alzheimer's disease is a valid summary of all non-drug therapies currently in use, because it allows during a session to stimulate through rehabilitative multi-strategy the area of cognitive, affective and motor improvement of the patient with detectable by the tests in use after the session. The results obtained on five patients will be reported.
Keywords : zootherapy, Alzheimer, non-drog therapy

When we speak of therapies with the help of the animals we prefer to use the specific term zootherapy instead of pet therapy because we want to define those therapies which encoded protocols with a specific method that we are studying at the Faculty of Veterinary Medicine, University of Naples Federico II. Usually Assisted Activities by Animals and Therapy Assisted by Animals are distinguished between their the first like an activity for recreation and the second like therapy, instead we refer to the meaning of the term health, given by WHO says the health is a state of physical, mental and social well-being so we considered the zootherapy an health service without difference between activity and therapy assisted by animals.

For this purpose it is necessary that the whiting the therapeutic setting there is the zootherapeutic equipe formed by human doctor or psichotherapyst, a veterinarian doctor, animal cootherapist and patient; for all members of the team professional Interdisciplinary training is required.

The zooantropology is a new discipline that studies the meaning of the relationship between man and animal. Actually, we know that Animal is able to influence the ontological development of man and so the relationship can become therapy. In our universities we are training veterinary students with the interdisciplinary vision with skills of science and humanities. In this historical period we are seeing many changes in several fields including the cultural disciplines. There is a need to the broader cultural vision, i.e. a cultural interdisciplinary education as manifested by the integration for example of bioethics and Zooantropology (Tugnoli C., 2003). The zooanthropology is the theoretical framework upon which is the pet therapy.

People affected by Alzheimer disease and other dementias are about 36 million worldwide of which 1 million in Italy. These data are expected to increase dramatically in coming years.

The etiology of this disease is not known but it is knows that is an progressive, irreversible and primary dementia is due to progressive atrophy of the cerebral cortex with loss of cholinergic neurons.

There is not a homogeneous pathogenesis in all patients and also the symptoms are variable but the memory disorders (Backman L., 1992) are commons, but it may be disordes of language, echolalia, aphasia, dyscalculia, Defective orientation space-time, idiomotor apraxia, impaired social behavior, alteration affect, paranoid state – hallucinations. Cours average: 8-10 years (Bergamini L., 1986). The pharmacological therapy of the Alzheimer disease is symptomatic and is based on the administration of acetylcholinesterase inhibitors, instead not pharmacology treatment is based on use of techniques of cognitive stimulation, aimed to stimulate the neuroanatomical not compromised (redundancy) (Rosenzweig M.R., 1996).

Materials and methods

Our studies about Alzheimer disease suggest that the zootherapy as valid summary of all non-drug therapies currently in use. This insight comes from the observation that the zootherapy allows to stimulate the area of cognitive, affective and motor during a session through rehabilitative multi-strategy (Viola L.F., 2011), the improvement of the patient is detectable by in use specific tests after the session.

The techniques of cognitive stimulation are the following:

- **Rot** (Reality orientation therapy), repetition of the multimodal verbal, visual, written stimulation to reorient the patient to himself and space (Zanetti O., 1995);
- **Reminiscence therapy**: is a psychotherapeutic rehabilitation to reinforce the identity.
- **Contextual therapy**: cognitive behavioral intervention to improve affectivity and social life of the patient.
- **Validation Therapy**: (Day C.R, 1997) build an empathic relationship with the patient to improve the feelings and emotions(Gagnon D.L., 1996).

The presence of an animal makes easier to propose and get physical activity for disabled patient. The pet therapy and particularly the contact with animal promote the neurorehabilitation through repeated stimulation of proprioceptors that come the stimulus from the periphery to the cerebral cortex, favoring the reactivation of the compromised areas (Rosenzweig M.R.,1996). Through the sensory and emotional stimulation you can see an improvement in attention and social and dialogic skills, and a reduction in behavioral disturbances. The patients are subjected to diagnostic tests like Vineland adaptive behavior scales, Cargiver burden inventory (CB) (Thompson S.G., 2002) CIBIC-Plus and this specificity allows to draw up individual protocols.

The intervention of Zootherapy was carried out at the Center Alzheimer, Asl Napoli 1 – Frullone Hospital, with Professional staff composed by Psycologist, reference Geriatrics (Zisselman M.H., 1996), health workers, Veterinarian Zootherapist and dog co-therapist. Preliminary step: Patient selection taking into account the following criteria: no denial,

fear, dislike of the dog, no total compromised memory; acceptance of the interaction by the dog (recognition of the therapeutic value of the dog as an expert on nonverbal language), not total impairment of memory, not participation in other social activities, the need for individual treatment setting.

Description of patients and goals of therapy.

1. **Patient A** (man with moderately severe Alzheimer), minimize continuous and persistent decrease, ambulation and reduce deficit of attention.
2. **Patiente B** (woman 49 years old mild Alzheimer), improve mood.
3. **Patient C** (man 70 years old moderately severe Alzheimer), reduce attention deficit.
4. **Patiente D** (man 80 years old moderately severe Alzheimer), stimulation of memory.
5. **Patient E** (woman 80 years old moderately severe Alzheimer), stimulation of memory.
6. **Patiente F** (man 80 years old mild Alzheimer), improve mood.

The method of operation was like Frequency: once a week for six months.

Duration: 60 minutes, 20 minutes with the dog: the dog co-therapist Nina was a Labrador Retriever female with good tempered, accustomed to relationships with humans Educated according to the specific protocol for zootherapy.

Setting was structured with phases of the sessions:

1) Psychomotor exercises
2) Coordination exercises
3) Exercises of space-time orientation

Results and discussions

1) **Patient A**. Initially shows above all a difficulties Relationship and Minimum level of attention/ concentration for activities we used a chronometer but at the last of treatment he was.. Attention/concentration: improved seating up to 20 min.
2) **Patient B**. Initially shows continuous involvement and cooperation at various stages of the treatment cycle, she has increased socialization, improved memory.
3) **Patient C**. Initially: Difficulties throughout the therapeutic steps at the end his Attention/concentration improved.
4) **Patient D**. Shows Defici of memory that improved at the end of treatment
5) **Patient E**. Initially: Difficulties throughout the therapeutic steps and she improved memory.
6) P**atient F**. Initially shows the deficit of the mood improved it by relationship with the dog.

Conclusions

It should be emphasized that the pet therapy is always in support of drug therapies (Zanetti O., 2004).

For this reason, it can be a valuable aid to identify the efficacy of drug therapies:

1. We can considered the Zootherapy as the synthesis of non-drug therapies because involves biological mechanisms, emotional mechanisms, physical mechanisms and psychological mechanisms.
2. This non conventional therapy is "tailored" to suit the style of personality of patient. Other very important element is to consider the Dog like value added to therapy because is a reader of non-verbal language, diagnostic aid, a strong social and emotional catalyst.
3. In conclusion, the zootherapy is a new therapeutic treatment and should be included in the non-pharmacological treatment protocol for patients with Alzheimer disease.
4. Veterinarians should be the primary responsible of the Zootherapy. Furthermore, as it is impossible to think of a farm without the advice of a zoothecnical veterinarian, it is equally impossible to think of zootherapy as a health service not provided by the veterinarian zooterapeuta. Veterinarian Zootherapist with the Interdisciplinary trainig is a doctor who has skills on animal health control, visit behavioral, archetypes and ethology of species of animals involved. He has same language and health training with human doctor and medical *forma mentis*.

Reference

1. Backman L. *Memory training and memory improvement in Alzheimer's disease: rules and exceptions*. Acta Neurol Scand 1992;39(Suppl. 1):84-9.
2. Bergamini L., Bergamasco B., Mutani R., - *Manuale di neurologia clinica* Libreria editrice scientifica Cortina, 1986, Torino
3. Day CR. *Validation therapy. A review of the literature*. J Gerontol Nurs 1997;23:29-34.
4. Gagnon DL. *A review of reality orientation, validation therapy, and reminiscence therapy with the Alzheimer's client*. *Physical and occupational therapy.* Geriatrics 1996;14:61-77.
5. Rosenzweig MR *Aspects of the search for neural mechanisms of memory*..Annu Rev Psychol. 1996;47:1-32. Review.
6. Rosenzweig MR, Bennett EL *Psychobiology of plasticity: effects of training and experience on brain and behavior..*Behav Brain Res. 1996 Jun;78(1):57-65. Review.
7. Thompson SG, Galbraight M, Thomas C, Swan JJ, Vrungos S. *Caregivers of stroke patient family members:behavioral and attitudinal indicators of overprotective care*. Psycol Health 2002;17:297-312.
8. Tugnoli Claudio, *Zooantropologia. Storia, etica e pedagogia dell'interazione uomo animale*. Franco Angeli 2003, Milano
9. Viola, Nunes, Yassuda et al. (2011). *Effects of a multidisciplinar cognitive rehabilitation program for patients with Alzheimer's disease,* Clinical Science , 66 (8).
10. Zanetti O, Frisoni G, De Leo D, Dello Buono M, Bianchetti A, Trabucchi M. *Reality orientation therapy in Alzheimer's disease: useful or not?. A controlled study*. Alzheimer' Dis Assoc Disord 1995;93:132-8.
11. Zanetti O, Calabria M, Cotelli M. *L'efficacia dell'associazione tra farmaci e Reality Orientation Therapy*. G Gerontol 2004;52:408-11.
12. Zisselman M.H, Rovner B.W., Shmuely Y., Ferrie P., 1996 *A pet therapy intervention with geriatric psychiatry inpatients*, American Journal of Occupational Therapy, Jan;50(1), pp. 47-51.

RESEARCHES REGARDING *YERSINIA ENTEROCOLITICA* INCIDENCE IN POULTRY CARCASSES DESTINED TO HUMAN CONSUME

*** Carmen CREŢU, V. FLORIŞTEAN, I. BONDOC, M. CARP CĂRARE**
*****Faculty of Veterinary Medicine Iași
carmenccretu@yahoo.es

Abstract
Yersinia enterocolitica is a bacterium that can be found in the digestive tract of healthy poultry, the presence of faecal caontamination on carcasses during evisceration showing. Action taken to reduce food poisoning caused by Yersinia enterocolitica are the same used for other pathogens. From the 140 cases investigated microbiologically poultry carcasses were isolated 9 strains of Yersinia enterocolitica on fresh carcasses, the positivity rate of 6,4% and 4 strains from carcasses chilled, the positivity index of 2,8% of samples examines. Yersinia enterocolitica fundamental property of synthesis of cilia in the temperature of incubation is also presnt in the 13 strains studies. It is recognized that the optimum temperature to highlight the mobility is between 22 – 30 ^{0}C. In our research at the 13 strains we studied the presence of cilia indirectly following environmental MIU mobility (mobility – indole - urea) at 29 ^{0}C and 37 ^{0}C. All strains were mobile and immobile at 37 ^{0}C to 29 ^{0}C.
Keywords: carcasses poultry, Yersinia enterocolitica, neck skin

The main way of transmission of *Y. enterocolitica* is by fecal-oral, and may involve contaminated water sources. Most common modes of transmission were food (especially animal) and water (1,3)

The presence of *Y. enterocolitica* in food is not always associated with illness, in this case, it is recommended that all food isolates to be tested for pathogenicity (coagulase evidence synthesis, cell invasiveness, presence of virulence plasmids). *Y. enterocolitica* is widespread in the environment and was isolated from the intestinal tract of many domestic and wild mammals, but also in rodents, birds, fish, frogs, molluscs, crustaceans and humans (3)

Yersinia enterocolitica is a bacterium that can be found in the digestive tract of healthy birds, the presence of faecal contamination on carcasses during evisceration showing. Action taken to reduce food poisoning caused by *Yersinia enterocolitica* and *Salmonella* are the same used (1,3).

Poultry may be the source of contamination with *Y. enterocolitica* vegetables and prepared foods in kitchens unhygienic products are handled, or stored in the chilled.

Home contamination of meat, with most significance for them conservabilitatea and sanitation, contamination occurs during the primary: bleeding, grooming (washing), cooling plants. Original flora is mainly determined by surface contamination of carcasses, acquired during these operations. Surface contamination of carcasses is always present, its level can vary considerably depending on the time of slaughter hygiene (2).

Material and method

For species *Yersinia enterocolitica* were harvested a total of 140 samples, respectively broiller fresh chicken carcasses chilled at 25g which were harvested from the neck skin.

Determination of *Yersinia enterocolitica* species was made according to ISO 10273/2003. Under this standard enrichment broth made PSB and CIN agar isolation (6).

Yersinia enterocolitica is a bacteria that form colonies typical psihotrof on solid selective media, with biochemical properties that define pathogenicity. The method is basically the presence or absence of *Yersinia enterocolitica* in a predetermined amount of product.

For detection of putative pathogenic bacteria of the species *Yersinia enterocolitica* is required proceeding sequentially through the following three stages:

Enrichment in selective media. The suspensions were inoculated samples obtained from 2-enriched environments as follows: PSB broth (peptone, sorbitol and bile salts), incubated at 25 ° C for 3-5 days; ITC broth (irgasan, ticarcillin and potassium chlorate), incubated at 25 ° C for 48 hours.

Strierea and identification. After incubation of enriched environments, it continued: PSB broth culture obtained was dispersed on the surface of a plate with CIN agar (cefsulodin, and novobiocin irgasan) with a circular loop, in order to obtain isolated colonies.

With a sterile pipette were transferred to 0.5 ml of culture broth PSB in 4.5 ml potassium hydroxide solution (0.5%) and mix. After 20 ± 5 seconds, dispersed on the surface of CIN agar plates with a circular loop, in order to obtain isolated colonies.

ITC broth culture was dispersed on the surface of agar plates with CDSS (Agar Salmonella / Sigella with sodium deoxycholate and calcium chloride) using a circular loop, in order to obtain isolated colonies.

CIN agar plates and CDSS were incubated upside down at 30 ° C, 24 hours, then checked the presence of characteristic colonies of *Yersinia enterocolitica*. This was done with magnifier equipped with oblique transmitted light.

a) The appearance of typical colonies of *Yersinia enterocolitica* on CIN agar: small colonies (≤ 1 mm) and the edges of translucent and red center, and on examination with oblique transmitted light, colonies neirizant look slightly grainy;

b) The appearance of typical colonies of *Yersinia enterocolitica* on agar CDSS: lactose-negative colonies, small (≤ 1 mm) and gray, non-distinctive edge, and slightly granular neirizante the examination with transmitted light obliquely.

Confirmation. Typical colonies present on the solid selective media were subjected to confirmatory tests consisted of biochemical tests, biotype, blood tests and API 20E.

From each plate with selective medium, colonies were considered characteristic transplanters 5 or suspicious. Selected colonies were dispersed in molten nutrient agar Petri plates to obtain pure cultures and isolated colonies. Plates were incubated at 30 ° C, 24 hours, then were examined to verify the purity of cultures.

The enzymatic activity of strains of *Yersinia enterocolitica* from various metabolisable substrates was monitored by techniques used to identify *Enterobacteriaceae*. These substrates were grouped and studied in two stages.

In the first stage (presumptive biochemistry) to investigate the behavior of *Yersinia enterocolitica* strains isolated from substrates that may allow differentiation from other *Enterobacteriaceae*:

The presence of oxidase was determined by the method Kovacs, the cultures studied were put in contact with a drop of oxidase reagent on a piece of filter paper previously moistened with distilled water. Dark blue color appeared after more than 10 seconds, so the reaction is negative.

The presence of catalase was investigated on the blade that is deposited drops of 0.3% aqueous solution of H2O2 as it examined suspension culture. Appearance of gas bubbles (O2) shows the presence of catalase.

The presence of urease was achieved by making insemination of cultures investigated in broth wishes / indole, then were incubated 24 hours at 30 ° C. Appearance of pink-purple or pink-red in 1-5 minutes signified positive urease reaction.

In parallel we used M.I.U. environment (Mobility-indole-urea). Bacteria hydrolyze urea, determines that environmental staining in red (original is yellow), resulting from the presence of urea ammonia.

Production of indole was followed by the reference model Kovacs, the existing cultures in broth wishes / indole were added a few drops of Kovacs reagent. The appearance of a red ring on the surface of cultures in 15 minutes meant the positive indole reaction.

Behavior towards glucose: insemination were performed on Kligler agar (poured into tubes, 3 cm column and gradient 5 cm) column prick and dispersion slope, incubation was performed at 30 ° C, 24 hours.

Interpretation: Column yellow - positive (glucose fermentation); slope red or unchanged - unfermented lactose.

In the second stage (confirmre biochemistry) and *Yersinia* species objective was to confirm compliance with the biotypes.

For serological identification: using rapid slide agglutination technique (RAR) and slow tube agglutination. All strains were agglutinated with serum agglutinative antiyersinia enterocolitica O3. Strains not agglutinated by this serum were classified serologically by sera preparation antiyersinia enterocolitica (O1-O10).

Results and discussion

Yersinia enterocolitica is a species widespread in the environment and has been isolated from the intestinal tract of many wild and domestic mammals but also poultry (5).

Yersinia enterocolitica is a bacterium that can be found in the digestive tract of healthy birds, the presence of faecal contamination on carcasses during evisceration showing. Involvement of bacteria in the production of food poisoning in humans is relatively recent, which has spurred research in this field in our country (1,4).

From the 140 cases investigated microbiologically broiller chicken were isolated nine strains of Yersinia enterocolitica on fresh carcasses and 4 strains from carcasses chilled, the positivity rate of 9.2% of the cases examined (Table 1).

Table 1. *Yersinia enterocolitica*/25g values. neck skin from fresh and chilled poultry carcasses

CARCASSES POULTRY	NR. SAMPLES	POSITIVE SAMPLES		NEGATIVE SAMPLES	
		Nr.	%	Nr.	%
Fresh	140	9	6,4%	131	93,5%
Chilled	140	4	2,8%	136	97,1%

The 13 isolates were subjected to identification which: morphology, mobility, cultural characteristics, metabolic characteristics and antigenicity.

Morphology- In all the literature is presented as a *Yersinia enterocolitica* Gram-negative bacteria, nesporulată, enveloped virus. Classic form is a tendency to pleiomorfism cocobacil as temperature of incubation, culture age, composition of media, biotype (4).

Mobility *Yersinia enterocolitica* fundamental property of synthesis of cilia in the temperature of incubation is also present in the 13 strains studied. It is recognized that the optimum temperature to highlight the mobility is between 22-30 ° C.

In our research at the 13 strains we studied the presence of cilia indirectly following environmental MIU mobility (mobility-indole-urea) at 29° C and 37° C. All strains were mobile and immobile at 37° C to 29° C.

The cultural aspect - He pursued the cultural aspect of Yersinia enterocolitica strains on solid media CIN, CDSS and Api 20E test.

Yersinia enterocolitica colonies are translucent edges and red heart on CIN agar (fig.1.).

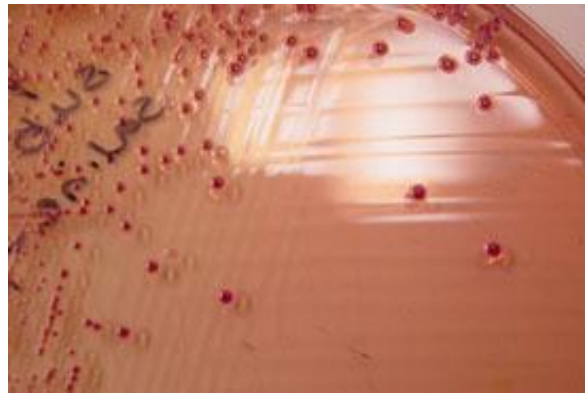

Fig. 1. CIN Environment - Culture of *Yersinia enterocolitica*

Research enzymatic activity of these strains allowed on one side of the taxon *Yersiniei enterocolitica*, and on the other metabolic changes have led to some substrates to subdivide this species in chimiotipuri and their correlation with other properties.

Conclusions

1. Were investigated bacteriologically and serologically 13 strains of *Yersinia enterocolitica*. Analysis of studies allowed the selection of a test set taxon correct and expeditious, adapted to the veterinary laboratories.
2. Strains studied were from fresh and frozen carcasses from chicken carcasses broiller: isolation rate was 6.4% in strains isolated from fresh carcasses and 2.8% in strains of *Yersinia enterocoltica* of chilled carcasses .
3. Mobility strains of Yersinia enterocolitica from 37oC to 29oC and immobility causes its introduction the scheme to distinguish them from other *Enterobacteriaceae,* which are immobile at 29oC.
4. You can get less contaminated carcasses from microbiological point of view, provided that at all stages of obtaining their respect and hygiene technology for each stage of processing.

Acknowledgments

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References

1. Condrea M., 1996 – Cercetări bacteriologice şi serologice asupra Yersiniei enterocolitica izolată de la animale. Teză de doctorat, Iaşi.
2. Floccari ME, Carranza MM, Parada JL., 2000 - Yersinia enterocolitica biogroup 1A, serotype O:5 in chicken carcasses, 63(11):1591-3.
3. Fukushima H., 1985 – Direct insolation of Yersinia enterocolitica from meat, Appl. Env. Microbiology, vol. 50, nr.3, p. 710–712.
4. Yashoda K.P., Sachindra N.M., Sakhare P.Z., Rao D.N., 2001 - Microbiological quality of broiler chicken carcass processed hygienically in a small scale poultry processing unit. Journal of Food Quality vol. 24, p. 249 – 259.
5. Yoshinori T., Gou I., şi col., 1999 - Survival of bacteria at a subfreezing temperature (-1^0C), Yonago Acta medica, vol. 42, p. 147–152.
6. SR EN ISO 10273/2003. Microbiologia produselor alimentare şi furajelor. Metoda orizontală pentru detectarea bacteriilor din genul Yersinia enterocolitica ASRO Standard Român

DETERMINING PATHOGENICITY STRAINS OF *CAMPYLOBACTER SPP.* ISOLATED FROM THE CARCASSES OF POULTRY

*** Carmen CREŢU, M. OBADĂ, V. FLORIŞTEAN, I. BONDOC, M. CARP CĂRARE**
*****Faculty of Veterinary Medicine Iași
carmenccretu@yahoo.es

Abstract
Pathogenic mechanisms of Campylobacter spp. are not fully known, but in the last decade have made significant progress for the correct understanding of virulence factors, as discussed above. Consumption of contaminated poultry meat is considered to be one of the key causes of disease in humans. The frequency with which bacteria isolated from chicken carcasses is usually low but may reach alarming levels in certain circumstances. Incidence is less on the frozen carcassses compared with the chilled carcasses.The research was conducted in a slaughterhouse in Iasi. 120 samples from poultry carcasses were collected and analyzed microbiologically. Samples were obtained from the surface of poultry carcasses.The study results allowed the identification of Campylobacter species in a number of 23 samples. The identification of isolation species of Campylobacter from the taken samples using the biochemical test for confirmation, those samples show us that 17 samples have been positive to Campylobacter jejuni, 3 samples was positive to Campylobacter coli and 3 samples to Campylobacter lari, those are the most common species which are impicated in human pathology.
Keywords*:* carcasses poultry, Campylobacter spp., neck skin, pathogeniticity

Today it is known that species *Campylobacter jejuni* and *Campylobacter coli* cause acute enterocolitis in humans, often with epidemiological data of food poisoning, which is why Expert Committee FAO / WHO on food safety include them on the list of agents that cause diseases of origin food.

Campylobacter jejuni is the dominant species of the genus by its significance for human health, accounting for 85-95% of cases of acute gastroenteritis in humans campilobacteriosis with epidemiological data of food poisoning, and sometimes is involved in urinary tract infections. Is widespread in birds and mammals (pigs, cattle, sheep, dogs, cats and rarely in rodents)(1,3).

Campylobacter coli long been considered a biochemical version *Campylobacter jejuni*, was separated as distinct species based on DNA hybridization. This species is involved in 5 -15% of cases of enteritis in humans campilobacteriosis. It is hosted in the intestine of different animals, but its usual host is the pig (1).

Campylobacter lari is found mainly on carcasses, and poultry livers. It produces enteritis in humans and poultry and *Campylobacter coli* resembles that develop at 35 º C and does not hydrolyze hippurate.

Campylobacter jejuni is sensitive to acid naldixic and cephalothin resistant, sensitive to amoxicillin and clavulanic acid, gentamicin, chloramphenicol and sulphonamides.

The isolation medium there may be two kinds of colonies: colony type flat, gray, fine grained, translucent and tend to stretch and type of colonies convex, shiny, smooth, translucent edges and darker center, brown slightly opaque (2)

Material and method

Researches were initiated in period 2012, in a slaughtering unit from Iassy 120 samples from poultry carcasses were gathered and analyzed microbiologically.

The parameters investigated were represented by microorganisms *Campylobacter spp*.The samples were obtained using carcasses washing fresh.

The samples poultry carcasses were analyzed, taking neck skin, in sterile conditions and then was made a quantity check (25g).

When the bacteria of the genus *Campylobacter* surveillance order for the approval of State for the application of the program of supervision, prevention and control of animal diseases, those transmitted from animals to humans, animals and environmental protection for 2012 respectively and surveillance and control program on food safety for 2012, prepared by ANSVSA.

Isolation and confirmation of microorganisms of the genus *Campylobacter* will be in accordance with the method described in ISO 10272-1: 2006, and *Campylobacter* species isolated setting will be made using phenotypic methods described in ISO 10272-1: 2006 (6).

To isolate and identify bacteria of the genus *Campylobacter* is envisaged that there are germs and chemoorganotrofi microaerophilic and requires special nutritional substrates for cultivation, growing only on average higher peptones.

According to ISO 10272-1: 2006 for detection of *Campylobacter* spp used as enrichment media: Bolton, Brucella broth as a selective isolation medium: Agar - Coal - cefoperazone - deoxycholate, Columbia Blood Agar, the medium for identifying and confirming : oxidase reagent for research, research reagents hipuratului hydrolysis of sodium nihidrină solution 3.5% Mueller-Hinton blood agar.

In developing the practical part method was used to detect horizontal thermotolerant *Campylobacter* species according to EN ISO 10272-1: 2006.

This method comprises the following steps:- Preparing basic suspension - Analyzed fraction is obtained by removing the skin of the poultry neck, if present, together with the skin on one side of the casing, avoiding fatty parts with a sterile instrument and aseptic technique. Fraction obtained is introduced into a stomacher bag. The addition of 9 volumes agar Bolton, Enrichment in liquid medium - was achieved with Bolton broth. Along with enrichment is achieved and direct strierea (for products believed to be heavily contaminated with thermotolerant *Campylobacter)* surface jealousy Bolton Karma and the environment.

Incubation initial suspension of enrichment media, is performed in an atmosphere of microaerophilic (5% oxygen, 10% carbon dioxide and 85% nitrogen) at 42 ° C for 18 hours broth Preston, and if puree Park and Sanders incubated at 32 ° C for 4 h, after which antibiotic is added to solution B in a concentaţie a concentration of 5% (v / v) and then reincubează at 37 ° C for 2h.

For isolation and identification, a part of the culture obtained from each enrichment broth, are seeded in parallel on two selective media Karmali and another agar selective medium of choice: Skirrow agar, ACCDE, Butzler modified agar or agar Preston.

For confirmation, presumptive colonies transplanters thermotolerant *Campylobacter*, (isolated on selective media), by inoculation in biochemical environments. Skirrow and Butzler agar the colonies appear two types occurring differences in their size, color is gray or brown gray brown. On ACCD, typical colonies are gray, often with metallic luster, flat wet and tends to spread.

Confirmatory tests are selected a total of five typical colonies and / or suspected on plates with selective culture media: - you test morphology and mobility, preparation of pure cultures, growth test at 25 ° C, oxidase test, test using the absence of sugars, catalase test, research susceptibility to nalidixic acid and resistance to cephalothin, study hippurate hydrolysis of sodium.

Test interpretation and expression of results is carried out after 10 minutes to maintain crop water bath at 37 ° C. The results are in accordance with table 1.

Table 1. Expression of results

Morphology	small curved bacilli
Motility	characteristic
Microaerobic growth at 25 ° C	-
Aerobic growth at 41.5 ° C	-
Oxidase	+

Results and discussion

Genus *Campylobacter* bacteria produce a variety of pathogenic infections, most common are acute diarrheal diseases, in essence these organisms can cause infection at any location, especially in compromised hosts and cause sequelae nesupurative.

The main pathogen that causes diarrhea is *Campylobacter jejuni*, responsible for 80% to 90% of all diseases recognized as campylobacterii products (4,5).

Other microorganisms that cause diarrheal disease include *Campylobacter coli, Campylobacter upsaliensis, Campylobacter lari* şi *Campylobacter fetus*. As no aerobic or strictly anaerobic, microaerophilic these organisms are adapted to the survival in gastrointestinal mucosal layer. These germs are bacteria curved, spiral, mobile, nesporulate, microaerophilic and Gram-negative, can be found in ancient cultures and as cocoide (fig.1.).

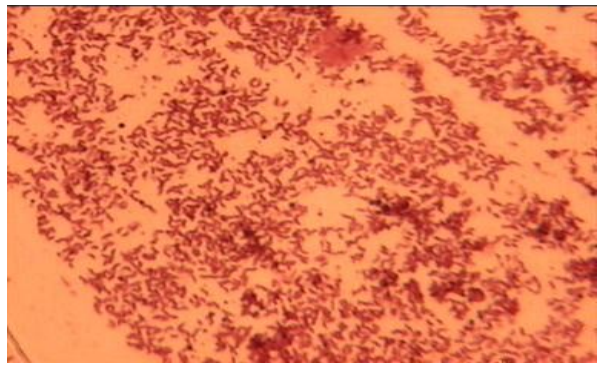

Fig. 1. Cultural aspect characteristic of *Campylobacter jejuni* isolated from poultry carcasses

In most cases, campylobacteriile be transmitted to people through raw or uncooked food, or by direct contact with infected animals. In the United States and other developed

countries, the most common way of acquiring infection (50% to 70% of cases) is ingestion of contaminated chicken that was not cooked enough (2).

Of the 120 samples collected from poultry carcasses and subject to investigation, 23 samples were presented and *Campylobacter spp.* 97 samples were free of *Campylobacter spp.*(fig.2.).

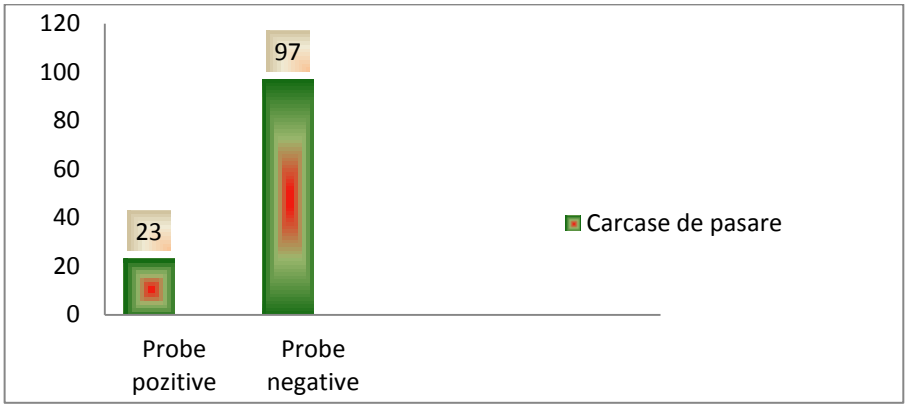

Fig. 2. The incidence of *Campylobacter spp.* positive samples isolated from poultry carcasses

Of the 23 samples positive for *Campylobacter spp*, 17 samples showed *Campylobacter jejuni* and *Campylobacter coli* 3 samples and 3 samples *Campylobacter lari*. Note that the largest share is occupied by *Campylobacter jejuni* from samples analyzed with a rate of 14.16% (table 2).

Table 2. The incidence of *Campylobacter strains* isolated from poultry carcasses

Nr.	***Campylobacter jejuni***	***Campylobacter coli***	***Campylobacter lari***
1.	**4/20**	**0/20**	**0/20**
2.	**3/20**	**1/20**	**1/20**
3.	**4/20**	**1/20**	**0/20**
4.	**2/20**	**0/20**	**1/20**
5.	**3/20**	**0/20**	**1/20**
6.	**1/20**	**1/20**	**0/20**
TOTAL	**17/120 (14,16%)**	**3/120 (2,5%)**	**3/120 (2,5%)**

I expected more cases of human infection by eating food from diseased animals or carriers. *C. jejuni* frequently contaminates poultry meat, the carcasses and organs (the percentage of 20-100% of poultry marketed). This is not entirely surprising given that many healthy birds carry this bacteria in the intestinal tract (3).

Conclusions

1. Although all poultry slaughterhouses are equipped with modern processing lines poultry, throughout the technological cutting poultry carcass contamination is possible even if the conditions of hygiene.
2. He oversaw the presence of bacteria of the genus *Campylobacter* in poultry during 2012, a total of 120 carcasses poultry.
3. The basis of this study state ISO 10272-1: 2006 - Microbiology of food and forage for detection and enumeration of *Campylobacter spp* Part 1: Method of detection.
4. Of the 120 samples collected in 2012 and subject to investigation, 23 samples showed *Campylobacter spp* and 97 samples were free of *Campylobacter spp.*
5. The largest share is occupied by *Campylobacter jejuni*, with a rate of 14.16% of samples analyzed.

Acknowledgments

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References

1. Georgssona F., Asmundur E. and col., 2007 - The influence of freezing and duration of storage on Campylobacter and indicator bacteria in broiler carcasses, Food Microbiology, vol. 23, p. 677–683.
2. Hutchison M.L., Walters L.D. and col., 2006 - Measurement of Campylobacter numbers on carcasses in British poultry slaughterhouses, J Food Prot. Feb, vol. 69, nr. 2, p. 421-425
3. Reich Felix, Viktoria Atanassova, Eberhard Haunhorst and Günter Klein, 2008 - The effects of Campylobacter numbers in caeca on the contamination of broiler carcasses with Campylobacter, International Journal of Food Microbiology, Volume 127, Issues 1-2, Pages 116-120
4. Rosenquist Hanne, Helle M. Sommer, Niels L. Nielsen and Bjarke B. Christensen, 2006 - The effect of slaughter operations on the contamination of chicken carcasses with thermotolerant Campylobacter, International Journal of Food Microbiology, Volume 108, Issue 2, Pages 226-232
5. Wagenaar J.A., Mevius D.J., 2006 - Campylobacter in primary animal production and control strategies to reduce the burden of human campylobacteriosis, Rev. Sci. Tech. Off. int. Epiz., vol. 25, nr. 2, p. 581-594.
6. *** 2006 - SR EN ISO 10272 – 1 : 2006 - „Microbiologia alimentelor şi a nutreţurilor pentru detectarea şi enumerarea Campylobacter spp. Partea 1: Metodă de detectare.

ELISA DETECTION OF EQUINE VIRAL ARTERITIS INFECTION IN EASTERN ROMANIA

Oana TANASE[1], C. PAVLI[1]
[1] FMV Iași, tanase_oana@yahoo.com

Abstract
Equine viral arteritis (EVA) is a contagious disease of horses caused by equine arteritis virus (EAV). The virus is present in horse populations throughout the world, aided by international movement of horses and infective cryopreserved semen. The primary mode of transmission of the virus is by the aerosol or respiratory route when horses are in close contact with one another. The virus is present in nasal secretions in acutely infected horses for up to 16 days. It may also be transmitted by the use of infective semen from carrier stallions during live cover, or insemination with fresh, cooled or frozen semen. Virus transmission rate from a carrier stallion to a naïve mare is 85 to 95 percent. The epidemiology of EVA involves virus, host, and environment-related factors, including variability in pathogenicity among naturally occurring strains of the virus, routes of transmission, existence of the carrier state in the stallion, and the nature of acquired immunity to infection. Viral transmission can be widespread at racetracks or on breeding farms; such occurrences are not always associated with the appearance of clinical illness characteristic of EVA. In fact, the vast majority of cases of natural infection with the virus are asymptomatic.

Keywords: equine arteritis virus, ELISA, Romania

EVA can vary in clinical severity both between and within outbreaks. EVA cannot be diagnosed based on clinical signs alone, as case presentation is similar to various other infectious and non-infectious equine diseases. Laboratory confirmation is required for diagnosis. Serological diagnosis of the disease may be performed by virus neutralization or by ELISA. The ELISA technique, unlike virus neutralization, may be used for the analysis of cytotoxic sera, and it is rapid and easy-to-use.

Materials and methods

The research was conducted on the equine that are equine infectious anemia free (EIA), the infected animals being removed from the flock gradually as required by ANSVSA plan. Investigations were undertaken on a number of 164 equine spread in households from Iasi county, Bacau county, Vaslui county and Botosani county.

It was used indirect-ELISA immunoassay test for detection and titration of anti-virus specific antibodies of equine viral arteritis serum samples, as a confirmatory test produced by ID-VET.

Immunoassay test is based on specific reaction between antigen fixed on polystyrene plate and antibodies for equine viral arteritis from equine test sera.

Results and discussion

The investigated animals were located in different regions (county Bacau, Iasi, Vaslui, Botosani) with very diverse climate and vegetation. The results showed that from tested animals, 29.53% were positive for EVA antibodies and 70.47% were negative, (table 1).

The main conclusion was that the EAV antibodies were produced consequently the infection of horses with the wild virus, since in Romania horses were never vaccinated against this disease.

Horses that were the subject of research in the present experiment are coming from households and there were no swine at the moment when the samples were collected, to avoid in this way any possible cross-reactions with PRRS virus.

To limit losses caused by abortions, the positive stallions detected by EVA ELISA assay will be castrated, thus interrupting their role of the disease dissemination in the flocks. Also it has been collected semen from which will be performed a virological examination to reveal the presence of equine viral arteritis virus (EVA).

Table 1. The results of tested animals by county

Total of tested animals	**Distribution results by county**							
	Bacău		**Iaşi**		**Vaslui**		**Botoşani**	
164	**Results pozitives**	**Results negatives**	**Results pozitives**	**Results negatives**	**Results pozitives**	**Results negatives**	**Results pozitives**	**Results negatives**
	12	23	9	18	21	38	12	31

Using indirect ELISA assay were examined a total of 164 sera from horses, of which 54 positively reacted, with a seroprevalence average of 29.53% and 110 samples were negative which is 70.47%.

Distribution of results by administrative region was as follows: Bacau county were 12 positive samples which represents 52.17% of the samples analyzed in this county. From the samples analyzed were positive Iasi 9 of 18, representing a 50%, in Vaslui positive were 21 of 38 samples, representing a rate of 55.26% and in Botosani positive samples were detected in 12 of 31, coresponding to 38.7%.

Notice that the degree of infection of equine viral arteritis virus is close to one county to another, especially in neighboring regions.

Conclusions

1. Given that in Romania the horses are not vaccinated against equine viral arteritis, the conclusion is that specific antibodies have appeared consecutively natural infection of the animals.
2. Out of 164 sera samples coming from a group of equine serological tested using EVA ELISA diagnostic test from ID^{VET}, there were identified as positive a number of 54 samples, representing 29.53% of analyzed samples;
3. Because there were no swine in the households when the samples were collected, it can be concluded that there are not present possible cross-reactions with PRRS virus.

Acknowledgements

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Bibliografy

1. Anthony E., Herschelle P., (*2004)* – *Veterinary Diagnostic Virology.* A practitioners gquide.
2. Fukunaga Y., Wada R., Imagawa H., Kanemaru T., (2003) – Veneral infection of mares by equine arteritis virus and use of killed vaccine against the infection. Journal Comparative, Pathology, no 117 (3).
3. Ionescu Aurelia*, (2006)*- Cercetari privind arterita virala ecvina, Teză de doctorat, Iași.
4. Mânzat, R.M., (2005) – *Boli virotice şi prionice ale animalelor*, Editura Brumar, Timişoara.
5. Perianu, Tudor,(2005) – *Bolile infecţioase ale animalelor*, Viroze, vol. II, Ed. Universitas XXI, Iassy.
6. Timony P., (1984) – Epidemiological features of the outbreak of equine viral arteritis in the troughbred population in Kentucky, USA. Am. Vet. J., vol 28, no 11.

SEROLOGICAL EVIDENCE OF HEV INFECTION AMONG FARM PIGS IN EAST OF ROMANIA

Adriana ANIȚĂ, Gheorghe SAVUȚA
Faculty of Veterinary Medicine Iași 8, Aleea Mihail Sadoveanu, 700489, Iași, Romania
aeanita@uaiasi.ro

Abstract
Hepatitis E virus (HEV) is a fecal-orally transmitted member of the family Hepeviridae that causes acute hepatitis in humans and is widely distributed throughout the world. Swine hepatitis E virus is a ubiquitous agent and majority of swine ≥ 3 month of age in herds from the United States and Europe were seropositive. There exist four major genotypes of HEV, and HEV isolates identified in samples from pigs belong to either genotype 3 or 4. Pigs have been reported as the main source of genotypes 3 infection to humans in non-endemic areas. To investigate HEV infection in farm pigs from different counties of east region of Romania we performed serological analyses of serum samples from 176 adult pigs. The commercial ELISA kit we used was PrioCHECK® HEV Ab porcine (produced by Prionics). Among the animals tested, 15.9% (28/176) were positive for specific anti-HEV antibodies. The HEV-specific positive antibody rate ranged from 0% to 56.25%. These results constitute the serologic evidence of swine HEV circulation in the east region of Romania.
Keywords: swine, HEV, antibodies.

Hepatitis E virus (HEV) is a small non-enveloped RNA virus classified in the genus *Hepevirus*, the only member of the *Hepeviridae* family (Emerson et al, 2003). The HEV RNA is about 7.2 kb long and contains three open reading frames, ORF1–ORF3. ORF1 encodes for non-structural proteins, ORF2 encodes the capsid protein and ORF3 encodes the cytoskeleton-associated phosphoprotein (Jameel et al, 1996). There are two species of HEV, mammalian and avian HEV. At least 4 major genotypes have been identified but only 1 serotype of HEV is recognized (Schlauder et al, 2001). The transmission of HEV is by the faecal-oral route through contaminated water or food. The virus is responsible for large epidemics of acute hepatitis in the human population in many developing countries. In developed countries, HEV infection is sporadic in humans but has become increasingly important (Dalton et al, 2008).

HEV was first isolated from swine in 1997 at commercial farms in the USA (Meng et al. 1997). Today, it is widely known that strains of genotype 3 HEV circulate extensively in swine across all continents and anti-HEV antibodies have also been found in other species of domestic and wild mammals (Lu et al, 2006; Bouwknegt et al. 2007; Caprioli et al., 2007). Hepatitis E virus is considered enzootic worldwide. Infected swine appear clinically normal but show microscopic evidence of hepatitis (Halbur et al., 2001). In addition, there have been reports of high genetic relatedness between HEV isolates obtained from humans and those from swine in the same geographical region (Goens et al, 2004).

Materials and Methods

A total of 176 samples were collected from apparently healthy adult swine. The study was performed during September 2011 and March 2012. The sampled pigs were derived from 12 different swine farrowing to finishing farms located in eastern region of Romania. All blood samples included in this study were centrifuged (3 000 g per min) and the serum was harvested and stored at −20°C until testing for HEV antibodies.

All the serum samples were evaluated for anti-HEV antibodies using a commercial ELISA diagnostic kit - PrioCHECK® HEV Ab porcine (produced by Prionics). The PrioCHECK® HEV Ab porcine is an enzyme immunoassay that is based on HEV genotype 1 and 3 antigens. All assay procedures were carried out following the manufacturer's instructions. The samples with optical density (OD) less than the cutoff value (mean OD for the two cut-off controls on each plate multiplied by 1.2) were considered negative. Samples with OD greater than or equal to the cutoff value were considered positive. The absorbance was determined at 450/620 nm.

Results and discussions

The prevalence rates of seroconversion in swine sera were examined with ELISAs specific for total anti-HEV antibodies. The overall prevalence of anti-HEV positive antibodies in swine sera was 15.90% (28/176) across the eastern regions of Romania. Anti-HEV antibody prevalence ranged from 0% to 56.25%.

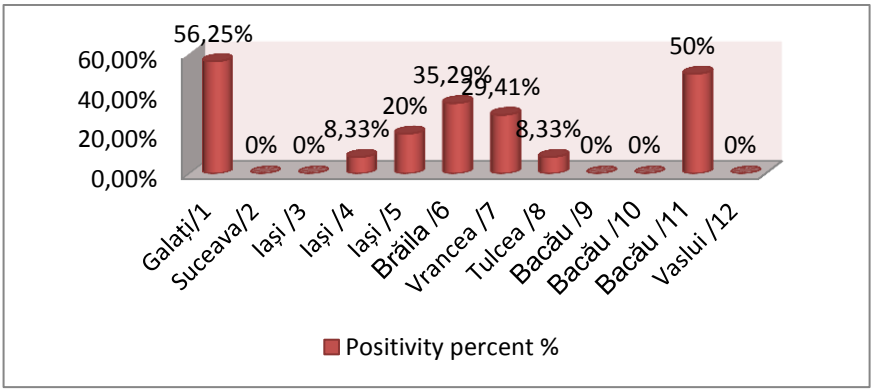

Fig.1. Distribution of anti-HEV antibody prevalence

The highest prevalence of anti-HEV antibodies was observed in one farm from Galați County, showing a 56.25% seroprevalence, followed by one farm from Brăila County showing a 35.29% seroprevalence. With respect to herd prevalence of anti-HEV antibodies, 7 out of 12 pig farms had HEV-positive pigs, demonstrating a 58.33% prevalence in the pig herds.

Our results support the finding that the farm swine populations in eastern region of Romania have been subclinically infected by the HEV. Epidemiological studies conducted so far shows that infection with hepatitis E virus in pigs is worldwide (Meng XJ, 2009): most pigs older than 3 months are seropositive, and most of those aged than 2 months are negative (F. Huang et al., 2002).

The force of infection depends on many factors influencing spread of infection (biological, environmental, demographic factors). As the disease is transmitted through virus contamination, breeding methods and other determinants affecting exposure are likely to influence the HEV infection prevalence.

Anti-HEV antibodies have been detected in almost 85% of pigs older than 5 months of age. Antibodies against HEV have been found in pigs which were originally negative in hepatitis E infection, after their close contact with seropositive pigs (Kasorndorbua et al, 2002).

Conclusions

Of the 176 serum samples from 8 counties and 12 farms, 28 samples have been identified as seropositive for hepatitis E virus infection. Our study indicates the presence of seropositive animals and hepatitis E virus circulation in pig farms, pointing to the risk of zoonotic transmission.

The impact of pigs as a reservoir of hepatitis E virus infection in humans requires study to assess whether and how zoonotic transmission should be controlled. Particular attention should be given to genetic variability and recombinant hepatitis E virus from animals to prevent the potential occurrence of several HEV strains pathogenic to humans.

Aknowledgements

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References

1. Bouwknegt M, Lodder-Verschoor F, van der Poel WH, Rutjes SA, de Roda Husman AM, 2007, Hepatitis E virus RNA in commercial porcine livers in The Netherlands. Journal of Food Protection, 70: 2889–2895.
2. Caprioli A, Martelli F, Ostanello F, Di Bartolo I, Ruggeri FM, 2007, Detection of hepatitis E virus in Italian pig herds. Veterinary Record, 161: 422–423.
3. Dalton HR, Bendall R, Ijaz S, Banks M, 2008, Hepatitis E: an emerging infection in developed countries. Lancet Infectious Diseases, 8: 698–709.
4. Emerson SU, Purcell RH, 2003, Hepatitis E virus. Review of Medical Virology, 13:145-154.
5. Goens SD, Perdue ML, 2004, Hepatitis E viruses in humans and animals. Animal Health Research Reviews, 5:145-156.
6. Halbur PG, Kasorndorkbua C, Gilbert C, Guenette D, Potters MB, Purcell RH, Emerson SU, Toth TE, Meng XJ, 2001, Comparative pathogenesis of infection of pigs with hepatitis E viruses recovered from a pig and a human. Journal of Clinical Microbiology, 39:918–923.
7. Huang FF, Haqshenas G, Guenette DK, Halbur PG, Schommer SK, Pierson FW et al., 2002 - Detection by Reverse Transcriptase-PCR and genetic characterization of field isolates of swine hepatitis E virus from pigs in different geographic regions of the United States. Journal of clinical microbiology, 40: 1326-1332.
8. Jameel S, Zafrullah M, Ozdener MH, Panda SK, 1996, Expression in animal cells and characterization of the hepatitis E virus structural proteins. Journal of Virology, 70: 207–216.
9. Kasorndorkbua C, Halbur PG, Thomas PJ, Guenette DK, Toth TE, Meng XJ, 2002, Use of a swine bioassay and a RT-PCR assay to assess the risk of transmission of swine hepatitis E virus in pigs, Journal of Virology Methods, 101, 1-2, 71-8.
10. Lu L, Li C, Hagedorn CH, 2006, Phylogenetic analysis of global hepatitis E virus sequences: genetic diversity, subtypes and zoonosis. Review of Medical Virology 16: 5–36.
11. Meng X.J., 2009, Hepatitis E virus: animal reservoirs and zoonotic risk, Veterinary microbiology, 140: 256–265
12. Schlauder GG, Mushahwar IK, 2001, Genetic heterogeneity of hepatitis E virus. Journal of Medical Virology 65:282–292.

SEQUELAE RECOVERY BY PHYSIOTHERAPY AFTER SPINAL CORD INJURIE

Adina ZBÂNGU, Mihaela ARMAȘU, Cristina BARBAZAN, M. MUSTEAȚĂ, E.V. ȘINDILAR, Gh. SOLCAN
Faculty of Veterinary Medicine, Aleea Mihail Sadoveanu nr.8, Iasi, Romania
zbangu.adina@yahoo.com

Abstract
A 6 mounths old, half breed female dog, weighting(with the weight of) 19,5 kg, has entred in our attention, aproximatelly 1.5 mounths ago, when it was brought to the Medical Clinic of the Faculty of Veterinary Medicine with acute non-progressive paraplegia and urinary incontinence and fecal incontinence. The result of neurological and imaging examination (radiology, mielography and MRI) was the diagnosis of vertebral subluxation at T13-L1 intervertebral space level. In addition to the surgery that has aimed the decopressing of the spinal cord and drug therapy, the physiotherapy had a major role in the patient recovery process. In this respect(in acest sens) a physical rehabilitation program has been applied that used as manual recovery methods the massage and passive range of motion, completed by special recovery methods as the Low Level Laser Therapy and ultrasound therapy. At the end of the physical therapy program has been regained (redobandita) the superficial and deep sensivity, partial recovery of patellar and withdrawal reflexes, sensitivity and motility of the tail and slight increase of atrophied muscle mass from hindlimbs.
Keywords: *physical rehabilitation, spinal cord injury, dog.*

Introduction

The spinal injuries are a frequent cause of disability small animals and they can be internal or external nature. The diseases associated spinal injuries include contusion, fracture and vertebral dislocation. The most common cause of spinal injuries is road accidents that reach up to 60% of causes. (11)

Regarding to localization, the trauma is frequent located at the T10-T12 for approximately 50% of causes due to the positioning of rigid thoracic vertebrae and muscular lumbar vertebrae (11). The spinal trauma is common in young dogs of the large breeds. Regarding to clinical evolution, the traumatic condition onset acute non-progressive causing to nervous system commotions, compression, tearing and ischemia. (11,16)

In terms pathophysiological, the traumatic condition include 2 principal stages: primary lesion and secondary lesion. The primary lesion can include the fracture or luxation, traumatic hernia of the intervertebral disc and bleeding followed by to destruction of blood vessels. The secondary lesion includes all the biochemical mechanisms from the traumatic spinal cord impact side that lead to further spinal damage over 24-48 hours after primary injury.

The degree of spinal cord disease due to physical impact is assessed by reference to four factors: duration and degree of compression speed and power cord concussion (11). Primary damage can be of functional or structural nature. In the first case the damage is reversible, while in the second case there are opportunities to recover by supporting nerve structures that have survived trauma.

Generally the diagnosis of spinal cord trauma is easily established by correlating the history information and neurological examination, but the information regarding the exact

location, degree impact and damage of nerve tissue are obtained after imaging exam such as radiography, computer tomography (CT) and magnetic resonance nuclear (MRI).

Rarely, the native radiography and with contrast agent can be considered sufficient to diagnose injuries and / or spinal cord contusion. Taking into consideration multiple trauma occur about 5-10% of causes (Bali M.S., Lang J., Jaggy A., 2009) implying a more complex imaging. In cases where a trauma is still suspected and radiographs plans are not conclusive, the best subsequent choice is CT or MRI. (11)

CT and MRI are extremely valuable techniques imaging that offer significant advantages over conventional radiographs and myelography. A study comparated the diagnostic ssensitivity of survey radiographs and CT in dogs with confirmed spinal cord fractures and luxation. Radiographs missed approximately 25% of the lesions detected on CT (Kinns J., Mai W., Seiler G., 2006). The general diagnostic sensitivity of spinal cord disease belongs MRI that proved superior CT even if images of bone are relatively poor. (14)

The overriding objective of treatment of spinal cord trauma is to provide an environment in which damaged neural tissues can recover optimal function. The primary aims are to limit further damage (which could occur through persistent instability) by maintaining adequate oxygenation infusions of medullar tissue associated with administration of neuroprotective agents and release spinal compression by surgery. (10)

After spinal cord injury, the recovery program by physiotherapy can take several forms depending on the stage after injury. Initial aim is reduction of pain that must be associated with permissive methods of recovery.

The intensity of care required for animals that have suffered medullar trauma is variable depending on the location and nature of lesion, severity of the initial deficit, age and evolution of damage and the type of intervention that has been used (1)

In general, care for animals that have undergone surgery is more straightforward, because stability at the injury site allows for more vigorous handling without causing pain. (10). All these are necessary to be done as clear distinction between types of neural tissues which were affected and to estimate recovery and development a rehabilitation program, as appropriate. (1)

Recovery of the neural tissue from central nervous system is not produced by regeneration of nervous tissue but rather by supporting its survival function axons harmed. (10) This phenomenon is evident for "complete" physical medullar lesions that cause permanent paralysis, compared to situations where there is a continuity tissue from lesional side that would allow the possibility of a potential recovery. (6)

Thus, through various adequate and applied physiotherapy methods can stimulate this functional plasticity of nervous tissue. (10) The first category is the manual methods such as the massage, stretching, pasive range of motion completed bt the special methods such as electric stimulation (ES), ultrasound (US) and low level laser therapy (LLLT). Each of these physiotherapeutic means must be well defined in the recovery program. (3)

Objective

Neurological disorders of spinal origin is a special circumstance where physiotherapy plays a critical role in the maintenance and recovery of nerve function. In clinical practice the diagnosis is limited to clinical and radiological native exam, which most of the times are not enough and often fails. In its absence, therapeutic conduct it is simplier leaving the need of

surgery as the last chance. The prognosis is evaluated based on the presence or absence of deep pain and physiotherapy program is often overlooked.

In neurology, physiotherapy idea is confused with the regeneration of nervous tissue, which is totally wrong. The role of physiotherapy in spinal nervous system is to support the survival of injured nerve tissue. This article is a case report that describes the clinical evolution of a patient with chronic spinal cord injury, to emphasize the degree of recovery of nerve function lost, after physiotherapy program.

Materials and methods

The study was made on a 6 mounths old, half breed female dog, brought at the Medical Clinic of the Faculty of Veterinary Medicine Iasi. The dog underwent neurological, hematological, biochemical and imagistic examination in order to establish the diagnosis. The blood sample for the hematological and biochemical examination was taken from the jugular vein. The hematological examination was conducted on an ABC Vet machine for blood collected on EDTA; the biochemical examination was performed on a Scan 200 machine.

Native and with contrast agent (Iohexol 300 in dose of 0.5 ml / kg) X-ray and MRI exam was performed under general anesthesia using a combination of metedomidine (Domitor® Phizer) in dose of 0,03 mg/kg i.v. and ketamine in dose of 0,3 ml/kg i.v. MRI examination was performed with Toshiba de 1,5 T on the vertebral dorsal - lumbar area. MRI image was captured before and after the administration of iodinated contrast substance gadobenat dimeglumine at a dose of 1 mg / kg, i.v. (Multihance®).

After the diagnosis of T13-L1 vertebral subluxation and herniated disc L2-L3, L3-L4, L6-L7, the dog was underwent laminectomy surgery on the T13-L1 to decompression of marrow.

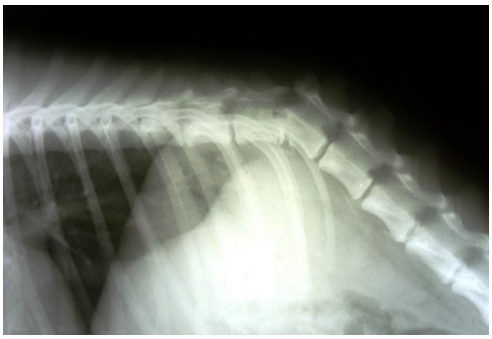

Fig.1 Thoraco-lumbar lateral radiographic image. T13-L1 intervertebral joint subluxation. Spinous process of T13 vertebra fracture
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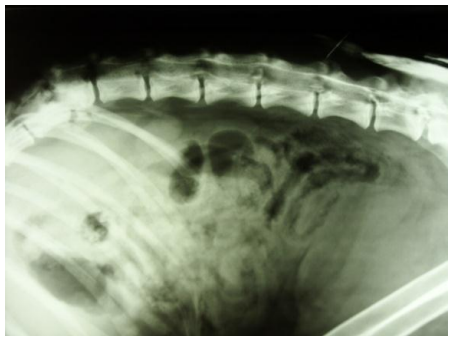

Fig.2 Thoraco-lumbar myelography examination after injection of contrast at L7-S1. Side view - deviation line contrast agent into spinal canal next to the intervertebral space L2-L3, L3-L4, L6-L7.

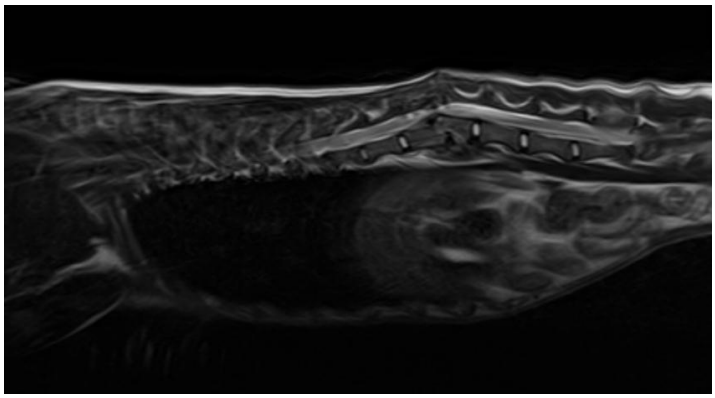

Fig. 3 Image thoraco-lumbar sagittal T2 MRI.
T13-L1 intervertebral joint subluxation associated with dorsal-ventral spinal cord compression severe

Before surgery, the dog was given antibiotics - amoxicillin and acid clavulamic 5 days (Synulox ® 8.75 mg / kg / day), anti-inflammatory drug - meloxicam 0.4 ml/10 kg (Metacam® Boehringer Ingelheim), vitamin B complex for 10 days (Multiject® Bomac) at a dose of 0,25-1 ml/9 kg and as an adjunct neurotropic - hydrolyzate of porcine brain protein (Cerebrolysin ®) at a dose of 2 ml / day (430.4 mg / animal). Treatment continued for 9 days postoperatively Synulox ®, Multiject ®, Cerebrolysin ® and 3 days for Metacam ®.

After 3 weeks postoperative, the patient underwent rehabilitation program making the manual methods such as massage and passive range of motion and special methods as laser therapy and ultrasound.

Massage was applied to the hind limbs atrophied muscles to strengthen the muscles, boost circulation and to support the patient psyche. The massage technique was easy friction for 5 minutes followed by a passive range of motion with a frequency of 10-15 repetitions every 2 times daily for 7 days. (Fig. 4, 5)

Laser treatment was applied using IR laser device 27 series Rolland following features: soft-infrared laser emission, well equipped with a diode 27 W, wavelength 905 nm, frequency adjustable from 5 to 6500 Hz.

Application of laser therapy was performed on the intervertebral spaces T12-T13, T13-L1, L1, L2, one side of the spine. Initially the area was clipped and disinfected.

After marking the contact points, ultrasound gel was applied to facilitate the penetration of laser radiation with tissue. (12) (Fig. 7) Gradually, during meetings energy density was reduced to 72 J / cm ², on the step at 64.8 J / cm ², 57.6 J / cm ², 50.4 J / cm ², 43.2 J / cm ², 36.1 J / cm ² and 28.8 J / cm ².

Treatment with therapeutic ultrasound was taken with Vet Advanced Intellect, Chattanooga Group, equipped with 2 channels of ultrasonic transducers (US) and 2 channels with 4 electrodes for electrostimulation.

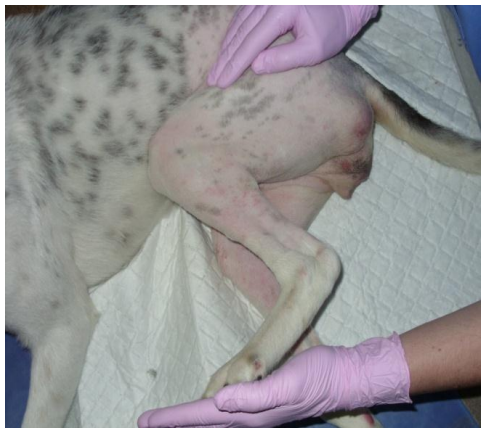

Fig.4 Passive range of motion (flexion-tibio-patellar femoral joint)

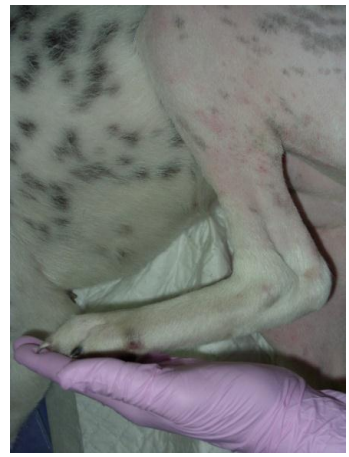

Fig.5. Passive range of motion (flexion of the tarsus)

Therapeutic ultrasound was applied in a continuously transmitting a frequency of 3.3 MHz and intensity 0.8 W / cm ² for 5 minutes for each member. (Fig. 6)

US have a beneficial tissue effects by stimulating local circulation and formation of collagen.

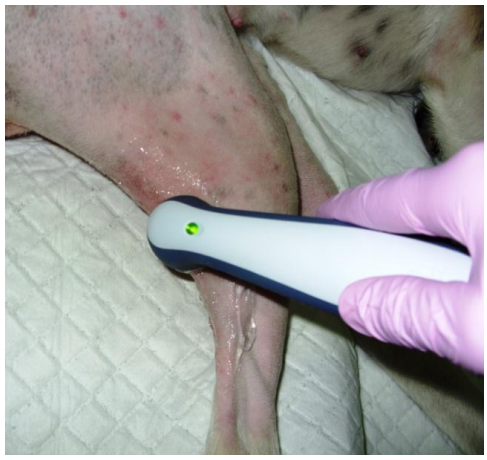

Fig.6 Application of therapeutic ultrasound

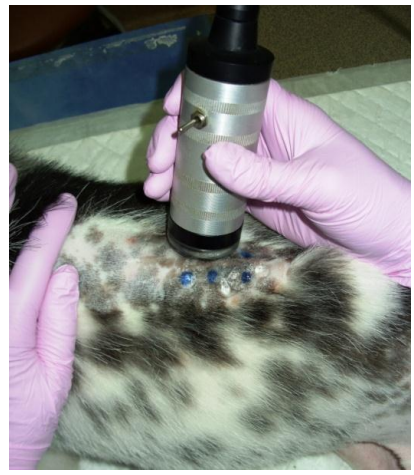

Fig.7. Application of laser therapy

US application was made with trasductorul 5 cm ² on the flexors tendons of the hind limbs in an area of 10 cm². Initially the area was clipped, disinfected and covered with ultrasound gel state in an amount so that the to ensure intimate contact between the head of U.S. trasductorului and animal skin. (3,5)

Results and discussion

The dog was brought to the clinic as it had paraplegia and incontinence of faeces and urine. General physical examination was normal. In the neurological examination have found no postural reactions, reflexes, sensation and motility of hindlimbs. Perianal reflex was absent and panicular reflex was present to the T11 vertebra. Tail lacked motility and sensitivity. On palpation of spine was found pain and kyphosis to the T13-L2. Defecation and urination were made incontinent. Cranial nerve examination was unchanged.

The results of blood counts and blood biochemical examination for liver profile, kidney, Ck and total protein showed normal values.

The result of the native X-ray exam was conclusive for the T13-L1 vertebral subluxation (Fig. 1) and myelography exam was possible evidence of extramedullary masses carbide, specific aspect of intervertebral disc protrusion of the spaces L2-L3, L3-L4, L6-L7. (Fig. 2) Injurious degree of medullary tissue was performed by MRI. (Fig. 3), (8.14)

The result of MRI examination was conclusive for dorsal-ventral spinal cord compression due to severe joint subluxations at the T13-L1, with zero chance of recovering lost nerve functions.

The laminectomy treatment of vertebral subluxation (T13-L1) was motivated by the fact that the loss of axonal and myelin damage was not significant, decompressing of medullar tissue would produce a major reversal of signs.

Damaged myelin requires more time to be recovered, but remyelinization of axons in central nervous system can occur leading to the recovery of function. If the axons were damaged, when is frequent in chronic compressive lesions, the potential for recovery of function is reduced. (7)

Preoperative administration of meloxicam's aimed at improving moderately severe acute pain by preventing production of prostaglandins from medullary tissue, the analgesic effect starting after 30-60 minutes from administration.

As a neurotropic adjuvant were administered Cerebrolysin ® which is a hydrolyzate of porcine brain protein involved in reducing local edema formation following trauma and vitamins B (Multiject ®) effect on pain and hyperalgesia, but also nutrition, axonal transport and neurotransmitter synthesis. (Zimmermann, 1988)

Postoperatively the patient was isolated in a cage to restrict movements of rest during which was monitored continuously.

Cerebrolysin ® was administered daily dose of 2 ml (430.4 mg / animal) in the course of 10 days with 14 days rest after human regimen. B vitamins and antibiotic were administered for 10 consecutive days. The program was held during 14 daily sessions and each session began with massage and ended with passive range of motion.

Massage therapy was initially performed slightly rubbing of the muscles of the hind limb and then gradually more energetic to stimulate blood circulation, as advanced muscle atrophy was accompanied by a reduction in advanced mobility with shorter hind limb flexors tendons back straight. Deep pressure through hand contact activates tactile receptors, proprioceptive neuromuscular and sensory conscious ways. Proper hand placement provides safety and support for the unstable segment body and can stimulate muscle contraction. As well, massage and training serves the patient following his methods inducing relaxation.

Passive range of motion is the mobility of joints, produced without using a muscle contraction but with the help of external forces that contribute to its mobilization.

The exercises were applied to prevent joint contracture of joints and adaptive shortening of soft tissues, maintaining mobility between layers of soft tissue, lymph and blood flow increase and improve production and diffusion of synovial fluid. (1,3)

The initial objective of application of LLLT on the uncompressed medullar tissue by laminectomy to the T12-T13 and L1-L2 intervertebral space was reducing local inflammation and then biostimulation. Based on this reason the treatment was started with an energy density that gradually decreased to 72 J / cm ² to step up to 28.8 J / cm ² to step.

US have been applied to prevent and treat contracture hind flexors tendons. (5)

After completing the program of physiotherapy, the patient was neurologically reassessed. Evolution of clinical signs of neurological patient from hospitalization and after physiotherapy program can be seen in Table number 1.

In assessing the degree of recovery of spinal cord trauma, perception of deep sensitivity is one of the major objectives. Its absence occurs only in severe bilateral sectioning and indicates a functional spinal cord sectioning because the nerve cords are deeply located and designed on both sides of the spinal white matter.

Most of the times, in clinical practice it is not known precisely the time of trauma and can not be appreciated the evolutionary stage of spinal cord injury and the prognosis is initially assessed on the basis of the neurologic examination. When have pasted more than 4 hours after the spinal trauma accompanied by the absence of deep sensitivity, the prognosis is considered grave. (9.15)

Table 1. Showing the evolution of clinical neurological signs

Postural reaction	**Examination1 (preoperative)**		**Examination 2 (3 weeks after surgery)**		**Examination 3 (after physiotherapy)**		**Evolution**	
	left	rightt	left	rightt	left	right	left	right
Conscious proprioception	+2	+2	+2	+2	+2	+2	-	-
MT	0	0	0	0	0	0	-	-
HL								
Biped anterior	+2	+2	+2	+2	+2	+2	-	-
Hopping	+2	+2	+2	+2	+2	+2	-	-
reaction	0	0	0	0	0	0	-	-
FL								
HL								
Cervical tonus	+2	+2	+2	+2	+2	+2	-	-
Spinal reflexes								
Patellar reflexs (L4-L6)	0	0	0	0	+1	+1	↑	↑
Cranial tibial reflex (L6-L7)	0	0	0	0	+1	+1	↑	↑
Extensor carpi radialis reflex (C7, C8, T1)	+2	+2	+2	+2	+2	+2	-	-
Triceps reflex (C7, C8, T1)	+2	+2	+2	+2	+2	+2	-	-

Flexor reflex	+2	+2	+2	+2	+2	+2	-	-
FL HL	0	0	+1	0	+1	+1	↑	↑
Perianal (S1-S3)	0		+1		+2		↑	
Panniculus (C8, T1)	T11	T11	L2-L3	L2-L3	L5	L5	↑	
Control of sphincters								
Urinary	Incontinent		Incontinent		Incontinent		-	
Defecation	Incontinent		Incontinent		Voluntary		↑	
Sensitivity								
Superficial	+2		+2		+2		-	
FL HL	0		0		+2		↑	
Deep	+2		+2		+2		-	
FL HL	0		0		+2		↑	
Tail								
Sensitivity	0		+1		+2		↑	
Motility	0		+1		+2		↑	

FL - Forelimb (0) – no response (reaction /reflex) (-) – no change
HL -Hindlimb (+1) – diminished response, low (↑) – improved response
(+2) –normal response

Based on main effects of laser radiation I hhave tried reducing the local inflammation and therefore pain, followed by biostimulation of nervous tissue. The reduction of inflammatory reaction occurs due to the increased number of leukocytes and activation of phagocytic process locally. Also, the decrease of inflamatory effect is based, also, on the increased production of PGE2 and PGF2 alpha, growth lisosomale membranes and reducing of swelling.

Laser radiation has a major control of local microcirculation. By stimulating lymphatic drainage occurs vasodilatation and an increase in concentration of nutrients. This leads to cell oxygenation that stimulates local energy chemical reactions by the increase of protein synthesis, enzymatic functions and energetic metabolism through a higher ATP production at the cellular level. By stimulating the increase of the mitochondrial activity, it is stimulated the increase of the nucleic acids (DNA and RNA) production. In general, all cell functions are stimulated. (4,12)

This type of laser radiation penetration with 2 cm direct effect and 5 cm with indirect effect stimulated and reorganized local microcirculation ensuring the survival of affected tissue function by supporting injured axons. Thus, the recovery of deep sensitivity shows restored functionality of the fascicles of deep sensitivity.

The leucomedulary deep location of them confirms that laser therapy protocol chosen have allowed the delivery of energy required.

Perianal sphincter neurogenic incontinence is often considered as a cause of disease cauda equina (Jones B.2000), as well as paralysis of the tail, but has been reported in dogs with signs of central motoneurons. (Skeen T.M., Olby N.J., Munana K.R., 2003)

Thus, recovery of perianal reflex associated with improved of voluntary defecation confirms the effectiveness of surgical therapy (Lynch AC, Anthony A, Dobbs BR, 2000) associated with the delivery of adequate doses of neurotropic substances and laser energy. In this way it is explained, as well, the recovery of motility and sensitivity of the tail.

Persistent contraction of the posterior limb flexors tendons as a cause of the absence of active muscle contraction is a consequence of chronic disease (5), but, also, the current inability to accurately measure the temperature distribution through the US. (Gail ter Haar, 1999)

Non-recovery of flexion and patellar reflexes of the hind limbs only in a minimum manner, indicates that axons of lumbar spinal cord were irreversibly affected by demyelination of neurons and axons injured because of the persistent spinal cord compression. (11)

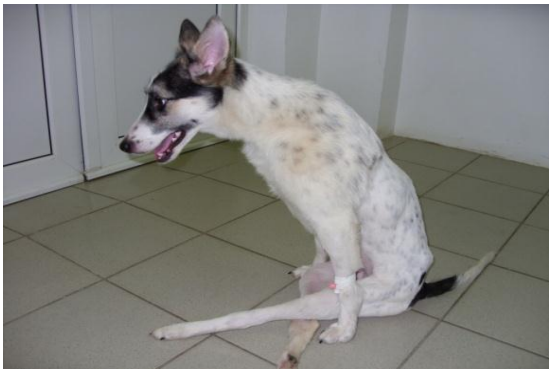

Fig.8 Paraplegia with amyotrophy (preoperative)

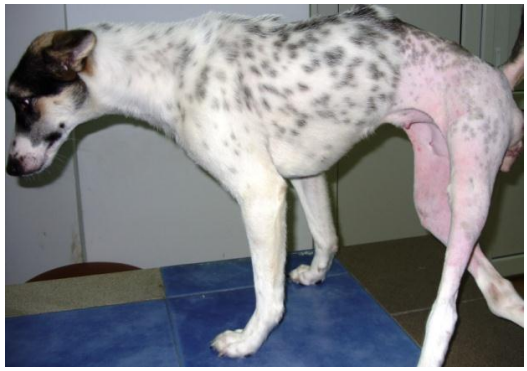

Fig.9 Paraparesis with right posterior limb flexors tendon shortening (after physiotherapy)

Conclusion

1. The absence of deep pain as a factor in prognosis, it is not a decisive factor for all clinical cases.
2. Surgical decompression of the spinal cord can produce a dramatic reversal of clinical signs if axonal loss and myelin damage are not significant.
3. The dose of laser energy applied for obtaining an photochemical cell effect by direct and effective penetration, must be accordingly with the objectives of the recovery program.

Bibliography

1. Catherine M. McGowan, Lesley Goff, Narelle Stubbs – *Animal Physiotherapy Assessment, Treatment and Rehabilitation of Animals*, 2007 by Blackwell Publishing;
2. Darryl L. Millis, David Francis, Caroline Adamson – *Emerging Modalities in Veterinary* Rehabilitation - Veterinary Clinics of North America, 35 (2005) 1335-1355, Elsevier 2005;
3. Darry L. Millis, David Levine, Robert A. Taylor - *Canine rehabilitation and physical therapy*, Elsevier 2004;
4. Ion Dan Aurelian Nemes - *Ghid de electroterapie si fototerapie*, Ed. Orizonturi Universitare, Timisoara 2000;
5. Jaqueline R. Davidson, Sharon C. Kerwin, Darry L. Millis – *Rehabilitation for the orthopedic patient* - Veterinary Clinics of North America, vol. 35 (2005), Elsevier;
6. Jeffery N.D., Blakemore W.F. – *Spinal cord injury in small animals*; 1. *Mecanisms of spontaneous recovery*, The Veterinary Record, 1999;
7. Jeffery N.D., Blakemore W.F. – *Spinal cord injury in small animals*; 2. *Current and future options for therapy,* The Veterinary Record, 1999;
8. Liptak J.M., Watt P.R., Thomson M.J., Copeland S.E., Galoway A.M. – *Hansen type I disk disease at T1-T2 in a Dachshund*, Australian Veterinary Journal, vol. 77, Nr.3, 1999;
9. Michael D. Lorenz ,Joan R. Coates, Mark Kent – *Handbook of Veterinary Neurology,* Fifth edition, Elsevier 2011;
10. Natasha Olby, Krista B. Halling, Teresa R. Glick – *Rehabilitation for the Neurologic Patient* - Veterinary Clinics of North America, vol. 35 (2005), Elsevier;
11. Nick D. Jeffery - *Vertebral fracture and luxation in Small Animals* – Veterinary Clinics of North America, vol. 40 (2010), Elsevier;
12. *Operating manual IR 27 laser therapy unit*, Elettronica Pagani S.R.L.;
13. Paul Ionescu, Ed. Tehnică - *Laserul - Aspecte biomedicale în medicina veterinară*, Bucureşti 1999;
14. Ronaldo C. da Costa – *Advanced Imaging of the Spine in Small Animals* – Veterinary Clinics of North America, vol. 40 (2010), Elsevier;
15. Simon R. Platt, Natasha J. Olby – *Manual of Canine and Feline Neurology*, Third edition, Bsava2004 .

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RESEARCH REGARDING THE HEAVY METALS (LEAD AND CADMIUM) RESIDUES IN THE DRY PET FOODS

G. AXINTE[1], GH. SOLCAN[2], R.N. MĂLĂNCUȘ[2]
[1]DSVSA Bacău, [2]UȘAMV Iași

Abstract
Heavy metals represent very dangerous compounds mainly because of their cumulative character, the detection of these metals in pet food being of utmost importance in the appreciation of the sanitation degree of this products. Numerous implications in diseases such as dental, bone or nerve disorders make of great importance the investigation of different types of pet food in order to determine the amount of lead and cadmium. Following our research it has been found the exceedance of the maximum allowed limit for cadmium and also for lead in one type of food out of six (16,6%) (a sort of cat food). This can happen because of the use of some ingredients with high degree of contamination, possibly prohibited for human consumption. It is required to identify the contamination sources and to act in order to address this cause.
Key words: heavy metals, pet food, cadmium, lead

Introduction

Heavy metals and their compounds found in pet food in amounts that exceed the allowed limits produce disorders of the vital functions. Many substances begin to exercise their harmful action only when they cumulate in organism in a sufficient amount.

Lead act on nervous system and on blood producing paralysis, tooth loss, gastric ulcer and important hematological disorders (1, 2, 5).

Cadmium has a toxic action against renal tissue, fact confirmed in different experiments on lab animals, but also has a toxic action on testicular tissue. Damage of this tissue in chronic evolution, not only by chemical pollution of water, soil, air, plants (forage, vegetables, etc.) but also by intoxication of animals that consists raw material in pet food industry is very dangerous for pets. The negative effects of cadmium and lead on spermatogenesis can be observed from an inappropriate quality of sperm to its absence.

Not less significant are the destructive actions against dentine (cavities), bone system (bone pain, decalcification, cracks), taste buds (loss of taste), against intestinal fluid balance (persistant diarrhea) and against central nervous system (4).

Because the data regarding the presence of heavy metals like cadmium and lead in pet food are limited, the research observed the presence of these contaminants in pet food industry and their possibly source of contamination.

Materials and methods

In industrial pet food can be determined important amounts of heavy metals that can reach there in several ways: as raw material;with auxiliary ingredients;with water;following corrosive processes of equipment and containers.

In this study have been used 6 types of pet dry food, three types of dry food for dogs and three types of food for cats.

The most common types of pet dry food from pet-shops and pharmacies have been chosen to be tested.

These determinations haven`t been made on granules, these requiring to be

preliminary prepared. This preparation consists in fine grinding and chopping of the granules and their passage for at least two times through a meat grinder and keeping them until the moment of analysis in a covered crystallizer and under an adequate temperature.

Physical and chemical analyzes used for determination of heavy metals in pet dry food have been made throughout a period of two years in the Laboratory of Food Safety and Veterinary Health Department Bacau in four sets of analyzes, one at each 6 months.

These analyzes tried to show if over this period of two years the pet food is healthy and if the chemical contaminants represented by heavy metals are present and within normal limits. In Residues Control Laboratory within DSVSA Bacau, for the determination of heavy metal content (lead and cadmium) from forage and forage raw material have been used the spectrophotometry of atomic absorption. This method is based on the determination of a chemical element from the analyzing sample concentration, by measuring the absorption of an electromagnetic radiation with a specific wave length, when passing through a medium where the free atoms of the element are freely distributed.

Results and discussions

According to the Waltham Nutritional Research Center the maximum allowed limits from pet dry food are 0,3 mg/kg for cadmium and 0,4 mg/kg for lead. We observed the constant exceeding of the maximum allowed limit not only for cadmium but also for lead for type 3 of dry food for cats. This fact can be caused by the use in the industry of pet food of some ingredients with a raised degree of contamination, possibly prohibited for human consumption.

Table 1. Concentration of cadmium residues from pet dry food

Cadmiu Mg/ kg	Determinatio n 1	Determinatio n 2	Determinatio n 3	Determinatio n 4	Determinatio n 5
Type 1 Dog	0.261	0.260	0.269	0.262	0.267
Type 2 Dog	0.164	0.165	0.166	0.168	0.167
Type 3 Dog	0.168	0.167	0.162	0.164	0.165
Type 1 Cat	0.112	0.113	0.111	0.115	0.112
Type 2Cat	0.118	0.118	0.117	0.116	0.118
Type 3Cat	0,321	0.319	0.318	0.319	0.319

Table 2. The evolution of lead amount in pet dry food throughout two years of study

Plumb Mg/ kg	Determinatio n 1	Determinatio n 2	Determinatio n 3	Determinatio n 4	Determinatio n 5
Type 1 Dog	0.331	0.321	0.391	0.321	0.131
Type 2 Dog	0.166	0.165	0.164	0.167	0.165
Type 3 Dog	0.388	0.389	0.387	0.388	0.389
Type 1 Cat	0.169	0.163	0.169	0.167	0.171
Type 2Cat	0.159	0.160	0.159	0.159	0.160
Type 3Cat	0.471	0.472	0.471	0.472	0.471

Considering that the acute toxic dose for rat is 141 mg/kg of cadmium and for rabbit of 3 g/kg of cadmium it is unlikely the installation of acute poisoning. Both metals have a powerful cumulative character thus being possible the installationof the undesired effects following chronic exposure. In blood, cadmium is tied of red blood cells and serum proteins (especially albumin), afterwards being transported to liver. In cadmium poisoning the liver is one of the target organs because it plays a major role in the anabolism and catabolism of plasmatic proteins and the changes of their characteristics showan hepatic lesion. But the most affected organ is the kidney, cadmium inducing micro- and macro renal lesions. In samples with raised cadmium and lead concentration have been seen significant changes of hematological parameters. Erythropenia, decreased hemoglobin and hematocrit, indicates oligocythemic anemia. The anemic effect produced by decreased hemoglobin and hematocrit is determined by the disturbance of iron absorption which, along with cadmium, is significantly reduced. In organism with iron deficiencies the toxic effect is much more pronounced.

In the present context it is clear that pet food pollution with heavy metals has been identified and remains to act in order to address this problem.

Most pet food producing countries claim zero tolerance for cadmium and lead presence, so in Romania where there is a pioneer in terms of industrial production of pet food it is also recommended zero tolerance.

Conclusions

Following our research it has been found the exceed of the maximum allowed limit for cadmium and also for lead in one type of food out of six (16,6%) (type 3 - cat food). This can happen because of the use of some ingredients with high degree of contamination, possibly prohibited for human consumption. It is required to identify the contamination sources and to act in order to address this cause.

Bibliography

1. Alfven T., Jarup L., Elinder C G., 2002, *Cadmium and lead in blood in relation to low bone mineral density and tubular proteinuria,* Environmental Health Perspectives, 110 (7): 699-702;
2. Bianu Elisabeta, 2005 - *Monitorizarea efectului toxic al cadmiului asupra stării de sănătate a unor specii de animale în zona Copşa Mică*, Teză de doctorat, FMV Bucureşti;
3. Bondoc I., Șindilar V. E., 2002 - *Controlul sanitar veterinar al calitatii si salubritatii alimentelor*, *vol. I si II*, Editura „ Ion Ionescu de la Brad" Iasi;
4. Hura Carmen, 1997 - *Poluarea chimică a alimentelor şi sănătatea*, Ed. "Hermes", Bucureşti;
5. Solcan Gh., Beşchea Chiriac I. S. 2005**-** *Toxicologie veterinară – manual practic.* Ed. Tehnopress, Iaşi

INVESTIGATIONS REGARDING THE RADIOACTIVITY LEVEL IN PET DRY FOOD

G. AXINTE [1], R.N. MĂLĂNCUȘ [2]
[1]DSVSA Bacău, [2]UȘAMV Iași

Abstract
Pet food radioactivity is a major reason of concern for dogs and cats owners but also for pet food manufacturers because of their multiple and long lasting harmful effects.The research has targeted the concordance with radioprotection international regulations. It has sought to show if over a period of two years pet food remains healthy and if the radioactivity level stays within normal limits.Contamination recorded in our study falls within permissible limits for farm animals. It can be considered that even a low level of radiation can be a possible risk for health.
Key words: radioactivity, Cs-134, Cs-137, pet food

Introduction

Recent research has led to the development of some food products that adopt the concept of positive nutrition. In the same time big pet food manufacturers invest millions of euros in research field that aims to improve the health and longevity of pets but also in the industrialization of production lines in order to maintain a high standard of hygiene.

Pet food radioactivity is a major reason of concern for dogs and cats owners but also for pet food manufacturers because of their multiple and long lasting harmful effects.

The research has targeted the concordance with radioprotection international regulations. It has sought to show if over a period of two years pet food remains healthy and if the radioactivity level stays within normal limits.

Material and methods

In this study have been used 6 types of pet dry food, three types of dry food for dogs and three types of food for cats.

The most common types of pet dry food from pet-shops and pharmacies have been chosen to be tested.

Investigations have been made in Animal Forage Radioactivity Control Laboratory from DSVSA Bacau where has been used the simultaneous or distinct determination method for Cs-134 and Cs-137 gamma nuclides that are present in forage.

The method used for the determination of radioactive contamination degree has been the gamma spectrometry of low resolution with NaI (sodium iodide) detector (crystal). The result is represented by the sum of Cs-137 and Cs-134 in relation with Kg/pb ratio.

The method principle is based on concomitant recordings of gamma radiations produced by the gamma nuclides mixture from the analyzed sample. Every component is identified based on the correspondent energies and is calculated the content (volume activity) of every gamma nuclide in the mixture. The result expresses the sum of the two nuclides.

The method allows the detection o gamma nuclides with energies greater than 100 KeV if using the NaI scintillation detector.

Preparation of all six samples of pet dry food (three for dogs and three for cats)

consisted in preliminary grinding in the meat grinder. Afterwards have been weighted in the electronic scale 150 g of every sample and have been introduced in Sarpagan plastic boxes. These boxes are used in order to respect the measuring geometry in which is calibrated the system and are placed directly on the end of the detector from the shielding enclosure.

Results and discussions

Determination of radioactive contamination degree of pet dry food has been made over a period of two years, by four sets of analyzes every six months (Table 1).

Table 1. Radioactive contamination degree of pet dry food

Radioactivity Bq / kg	Determinatio n 1	Determinatio n 2	Determinatio n 3	Determinatio n 4	Determinatio n 5
Type 1 Dog	123	126	123	128	132
Type 2 Dog	179	178	183	185	187
Type 3 Dog	232	238	234	231	233
Type 1 Cat	154	152	152	155	151
Type 2 Cat	119	115	116	116	119
Type 3 Cat	112	111	112	113	116

In 1989 the European Parliament recommended the limits for forage radioactivity in order to protect animal health (Table 2).

Table 2. Limits regarding forage radioactivity (Cs -134, Cs-137)

Animal	Bq /Kg
Pig	1250
Poultry,lamb,cow	2500
Cat,dog	5000

In 2008 the European Council Regulation 733/2008 regarding the import requirements for original agricultural products from third party countries following Chernobyl nuclear central accident, with subsequent changes and completions states that the maximum cumulative radioactivity of animal forage for Cs-134 and Cs-137 must be of 1250 Bq/kg.

This statement of the EC has been taken by the Health Ministry and has been published in the Official Gazette no. 297/2002 and states that the maximum cumulative radioactivity for animals must not exceed 1250 Bq/kg. Compared, for children food is permitted a limit of 370 Bq/kg and for other human consumption products the limit is of 600 Bq/kg.

It is sometimes a serious confusion between pet food radioactivity (their radioactive content) and the irradiation of some sort of pet food (which is a technological technique used for extra preservation).

The presence of radionuclides in pet food is caused by both natural causes (the existence in geosphere of natural radionuclides) and by industry, accidents, nuclear experiments, all of these being human activities. The harmful action of radiations against

living tissues is mainly due the presence of abnormal ions that produce disorders of cellular metabolism until the death of the cell. Great importance is represented by the alteration of the genetic message, this leading to neoplasic disease. The most affected are the tissues with a quick renewal ratio: mucosa and skin epithelium, lymphoid tissue, bone marrow, testicles and ovaries. In all these tissues the radiation causes cell division stagnation (4).

It is known that at international level the theoretical and experimental basis to define the Maximum Allowed Concentration (CMA) for animal food chemical pollution is not fully clarified, this making very difficult the interpretation of results. Much more difficult is taking a decision over the animal forage radioactivity control.

In conclusion, when controlling the pet food radioactive content there are not stated any maximum allowed concentrations, as for chemical and biological contamination. Depending on specific conditions, the control accredited laboratory and animal health accredited institutions can state intervention limits for pet dry food radioactivity, their exceed causing the use of radioprotection measures. Contamination recorded in our study falls within permissible limits for farm animals. It can be considered that even a low level of radiation can be a possible risk for health.

Bibliography

1. Bondoc I., Sindilar V. E., 2002 - *Controlul sanitar veterinar al calitatii si salubritatii alimentelor, Vol I si II*, Editura „ Ion Ionescu de la Brad" Iasi;
2. Burows I., 1988 - *Nutritia cainelui si pisicii,* Waltham;
3. Hura Carmen, 1997 – *Poluarea chimică a alimentelor şi sănătatea,* Ed. "Hermes", Bucureşti;
4. Solcan Gh., Beşchea Chiriac I. S. 2005**-** *Toxicologie veterinară – manual practic.* Ed. Tehnopress, Iaşi;
5. ***Regulamentul (CE) nr. 854/2004 al Parlamentului European din 29 aprilie 2004 de stabilire a normelor specifice de organizare a controalelor oficiale privind produsele de origine animală destinate consumului uman;
6. ***Regulamentul (CE) nr. 1774/2002 al Parlamentului European și al Consiliului din 3 octombrie 2002 de stabilire a normelor sanitare privind subprodusele de origine animală care nu sunt destinate consumului uman;
7. *** Ordin al Ministerului Sănătăţii şi al Ministerului Agriculturii, Pădurilor, Apelor şi Mediului privind completarea Ordinului Ministerului Sănătăţii şi Familiei şi al Ministerului Agriculturii, Alimentaţiei şi Pădurilor nr. 84/91/2002 pentru aprobarea normelor privind contaminanţii în alimente., Monitorul Oficial al României, anul 172, nr. 142, 17 februarie, 2004.

ULTRASONOGRAPHIC ASPECTS OF LIVER DISEASE IN COWS

Elena LOPATNICU
Faculty of Veterinary Medicine, Ion Ionescu de la Brad University of Agricultural Sciences and Veterinary Medicine, Aleea Mihail Sadoveanu nr. 3, Iaşi, 700490, România
elena.lopatnicu@yahoo.com

Abstract
The clinical aspects of liver diseases may be nonspecific. The studies included 29 cows various breed with exclusive digestive disorders. The ultrasonographic examination was performed on the right side of the standing animal using a 3,5 MHz transducer. Out of 29 animals the most common diagnosis was fatty infiltration of the liver (n=6) and hepatic abscesses(n=2).
Keywords: ultrasound, cow, liver

Introduction

Diagnosis of hepatic disease is difficult because clinical signs may be nonspecific. Ultrasonographic examination is of considerable value in the diagnosis of several hepatic disorders, including hepatic lipidosis, liver abscess, fascioliasis and caudal vena caval thrombosis, allowing visualization of changes in localized or diffuse parenchymal liver. A complete examination can offer information about position, size, ultrasonographic parenchymal pattern of the liver and the gallbladder.

Materials and methods

The study was carried out in the period April 2010- June 2011 on 29 cows aged from 1 to 8 years old (mean age ±5 years, mean body weight ± 545,9 kg) admitted to a large animal veterinary clinic, referred after a previous consultation of a veterinarian. Liver examination is part of the routine first exam consult.

Ultrasonograhpy was performed on the right side of the abdomen in standing position. The site for ultrasonography was prepared by shaving the skin between the Xth intercostal space and the last rib. After the application of a transmission gel (Aquasonic Gel, Parkers Laboratories, USA) the B-mode ultrasonography was performed using a grey-scale equipment (Aloka, SSD 500) with a 3.5 MHz transducer. The examination starts caudally, from the last intercostal space moving toward to the six intercostal space. Each area is examined in the dorsal-ventral direction keeping the probe parallel to the coast.

In some cases additional paraclinical exams, like serum biochemistry, were made.

Results and disscutions

The most common complaint was decreasing of milk production (n=8) followed by other reasons like anorexia (n=6), diarrhea (n=2) and persistent hyperthermia(n=2).

In the studied period, 6 cows (20,68%) where diagnosed with fatty liver degeneration and increased liver area, 2 cows (6,9%) received a diagnosis of liver abscesses and 1 cow (2,9%) received the diagnosis of liver congestion. The most common concurrent disease was

left displacement of the abomasum observed in 4 cows (13,8%) and primary acetonemia in 5 (14,5%) cows. There rest of 11cows (31,9%) where diagnosed with other digestive disorders.

The normal liver has a homogenous hypoechoic texture. The hepatic vasculature is imaged as anechoic circular to tubular structures with anechoic content. (M. Mahdi Komeilian,et al, 2011). The portal veins have an echogenic wall and diverge from thmain portal vein, while the hepatic veins do not have walls and echogenic converge towards the caudal vena cava (Braun et al., 1996). The bile ducts are not visible. Regarding some of the pathologies with reference to diffuse hepatic disease: hepatic lipidosis or fatty liver degeneration is one of the most common (figure 1 and 2). This results in augmentation of hepatic area, rounding of liver margins, increased echogenicity due to relatively low acoustic impedance of adipose tissue comparing to other soft tissues and placement of the parenchyma near the abdominal wall.(Robert N et al 2007). It is likely that only large vessels can be seen. The poor visualization of small hepatic vessels is due to compression of the swollen hepatic tissue (Braun, 2009). In advanced stages of the disease, the liver appears white on ultrasonograms and is difficult to differentiate from surrounding tissue. From the same class, hepatic congestion is less often seen (figure 3).

The ultrasonographic appearance of hepatic abscesses in cattle is variable. The content of the abscess appears anechogenic to hyperechogenic. Abscesses may be homogeneous or heterogeneous. In terms of its evolution and maturation stage, the capsule of the abscess is not always visible. When is present like in figure 4, it appears as an echogenic line delimitating the contents of the abscess.

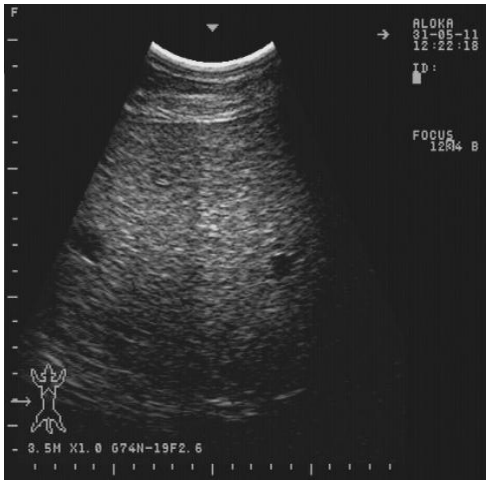


Fig. 1. Hepatic lipidosis- diffuse increased echogenicity of the parenchyma, and reduced visibility of small hepatic vessels

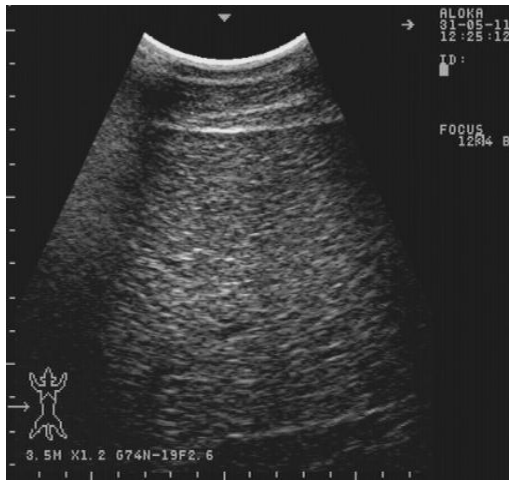


Fig. 2. Hepatic lipidosis, demonstrating diffuse increased echogenicity, reduced visibility of small hepatic vessels, and deep attenuation of the image

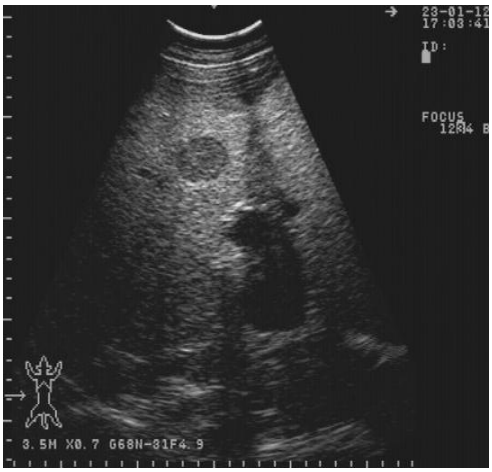


Fig. 3. Ultrasonogram of bovine liver-hepatic congestion and focal hypoecogencity of the tissue

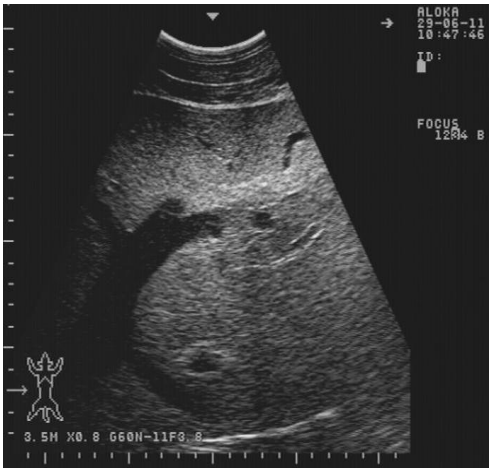
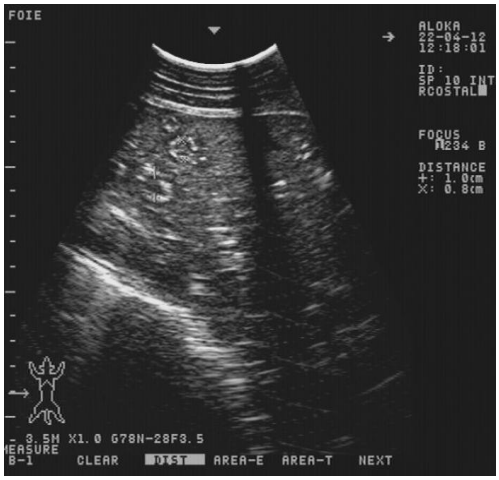


Fig. 4. Ultrasonogram from a cow with a: developing absces



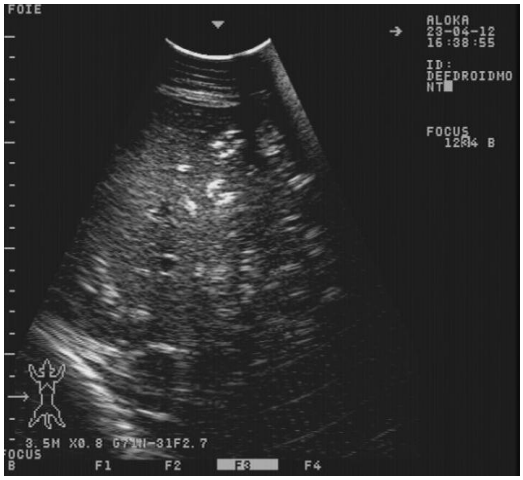


Fig. 5 and 6 Ultrasonograms from a cow with multiple hepatic abscesses. Imagine from the tenth and eighth intercostal space

In the figures 5 the liver abscesses in terms of ultrasound reveals are characterized by multiple sizes, for example 0.8 cm to 1 cm, they are widely disseminated in the liver parenchyma and have a hypercogenic aspect. A heterogeneous ultrasonogram with single or multiple foci and no evidence of a capsule indicates an early stage in the development of the abscess like in the figure 7. The heterogeneous is completed with a deterioration of the contrast between the hepatic parenchyma and hepatic vessels specific for fatty degeneration.

The gallbladder is situated on the visceral surface of the liver and ultrasonographically has an image typical of a fluid-filled vesicle surrounded by a thin white rim. Dilatation of the gallbladder alone with a normal content, like in the figure 8, is common observed in anorexic cows; due to loss of appetite there is no reflex stimulus for emptying.

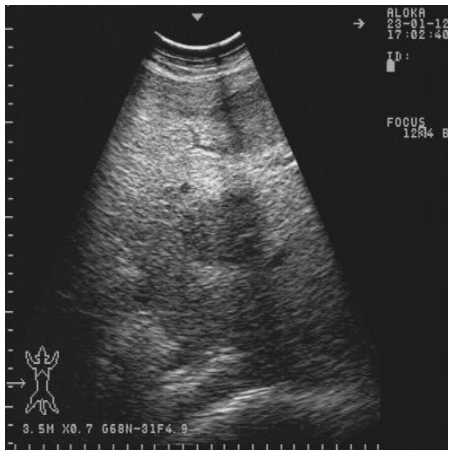


Fig. 7. Ultrasonogram of the liver parenchima heterogenous aspect with alternation of hypoechoic and hyperechoic areas

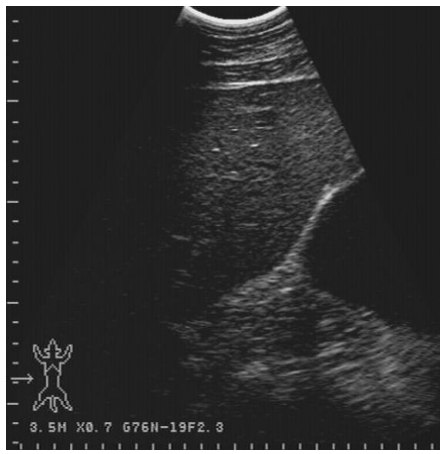


Fig. 8. Ultrasonogram of dilatated gallbladder

Conclusions

1. Ultrasound has the disadvantage of not being able to explore the entire liver parenchyma particularly the left side and parts of the lobes that are covered by the lungs.
2. Fatty liver infiltration is characterized by the decreased of the contrast between the liver and vessels and it was observed in 6 cows.
3. Given the importance of this pathology in the dairy farming sonography is appropriate to confirm the diagnosis of fatty infiltration beside the paraclinical determinations.
4. A liver abscess in 2 cases was confirmed by circumscribed structural changes in the hepatic parenchyma.

References

1. Braun, U., Ultrasonography of the liver in cattle. Veterinary Clinics of North America: Food Animal Practice, 2009; 25:591-609.
2. M. Mahdi Komeilian, Mehdi Sakha,. Gholi Nadalian, Abbas Veshkini, Hepatic ultrasonography of dairy cattle in postpartum period: Finding the Sonographic Features of Fatty Liver Syndrome, Australian Journal of Basic and Applied Sciences, 2011; 5: 701-706.
3. Robert N. Streeter, D.L. Step, Diagnostic ultrasonography in ruminants, Vet Clin Food Anim, 2007:541–574.

ASSISTED REPRODUCTION IN QUEEN USING VAGINAL INSEMINATION WITH EXTENDED SEMEN

Constntin PAVLI, Oana TANASE, Georghe SAVUTA
FMV Iași; pavli_constantin@yahoo.com

Abstract
Although AI has been widely used in domestic livestock its use in queen is limited. This study purpose was to present this artificial insemination technique, in all cases we used similar induction of estrus and extended semen. Fresh semen with 5-18 x 10^6 spermatozoa was extended prior use with a noncommercial extender. Pregnancy rate was established using ultrasonography after measuring progesterone levels (ovulation indicator-needed in conception rate establish).
Keywords: Artificial insemination, Electroejaculation, Progesterone

Materials and methods

Cats

We used six queens and two tom cats; all mixed breeds aged 2-5years, housed in individual cages (1.5x1x2 meters) and feed with commercial cat food (cat chow-Purina), the light cycle was 10-12h/24h. The animals were provided with ambient light and artificial light in order to complete the light cycle of 12 hours (artificial light depending season), the project begun in February 2012.

Semen collection

Semen was collected using electroejaculateing procedure described by C.C.Platz, procedure conducted under sedation with medetomidine hydrochlorid (Domitor orion pharma)- ketamine (ketaminol 10 – intervet), this procedure could by routinely repeated twice weekly.

The semen was evaluated as row semen or extended (we used an egg yolk based extender). The ejaculate was evaluated for sperm motility, counted with haemocytometer. A complete spermogram was difficult to obtain on a single ejaculate considering the volume, a number of 2-4 ejaculates being needed to asses a mail. An increased interest was shown to sperm morphology; we used stained slides (staining for cytological material - cytocolor-merck) and formol-saline wet smears – phase contrast microscopy.

For artificial insemination we used a tirss–egg yolk based extender, all females were grouped in order to assure a safe use of the three males during electroejaculateing procedure.

Hormonal estrus induction

In the experimental protocol ovarian cycle was manipulated using hormonal treatments with eCG and hCG. Ovarian activity was monitored using ultrasonography (figure 1-2), vaginal cytology and hormonal assessments (progesterone and estrogens dosage).

One administration of 100 IU eCG (Folligon, Intervet International Inc., The Netherlands) intramuscularly to anoestrous cats, followed 80 hours later by an injection of 100 IU human chorionic gonadotrophin (hCG), produces ovulation and pregnancy results comparable to those of natural matings.

Blood was collected via cephalic or jugular venipuncture at the time of artificial insemination and ten days later. The serum was separated by centrifugation and stored in

plastic tubes at minus 20^0C until assayed. The hormonal analysis was performed in a private laboratory renar certified. The serum was analyzed for progesterone using Elisa assay.

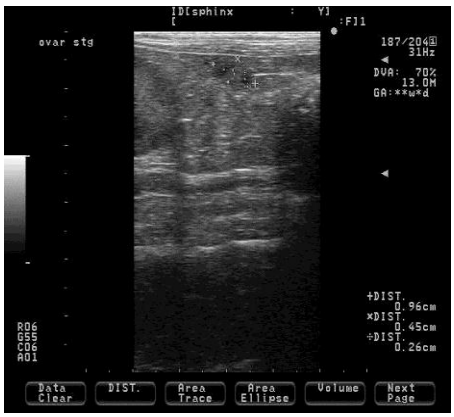


Fig. 1. Ovarian activity

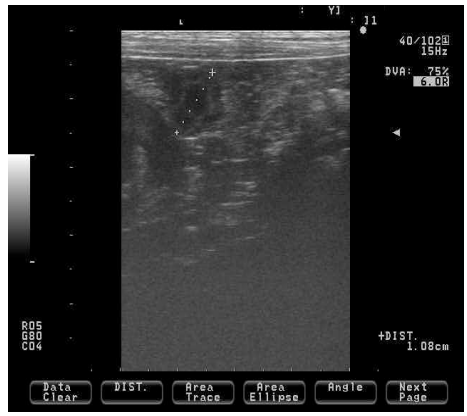


Fig. 2. Ovarian activity

Intravaginal insemination (IVI)

General anesthesia was induced in the queens by i.m. treatment with Medetomidine (Orion pharma) and 10 mg/kg ketamine hidrocloride (Ketaminol Intervet Holland).

An open-ended tomcat catheter (1.2-mm diameter) was used for IVI; it was inserted toward the cervix until no further cranial movement could be achieved. Tris buffer was added to the fresh semen (total insemination volume, 250 mL).

The queen's hindquarters were elevated at an angle of 45 degree for 10 min to promote the passage of semen from the vagina into the uterus. Ultrasonography (for pregnancy diagnosis) and blood collection (for serum concentrations of progesterone) were performed 30–40 days after AI (Figure 3).

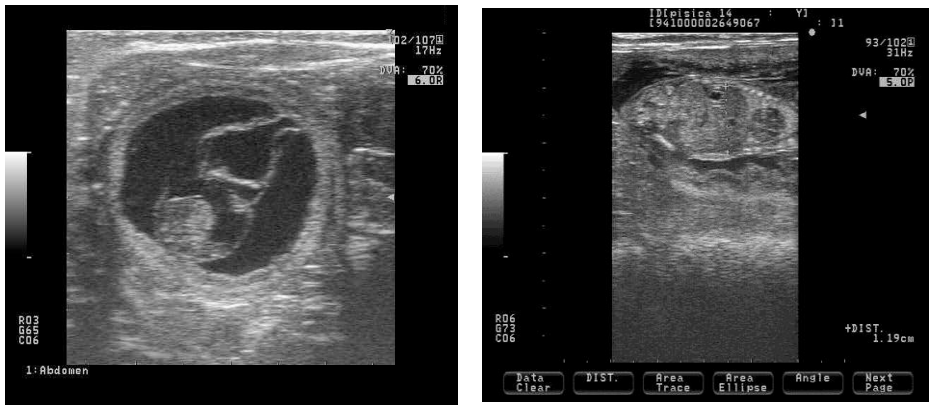


Fig. 3. Pregnancy diagnostic and monitoring

Results

1. Treatment with hCG induced ovulation in 83% of the cases (5 queens). All queens diagnosed pregnant with ultrasonography (30–40 days after AI) subsequently delivered kittens (except one with abortion in day 50 of pregnancy- 3 fetuses).
2. Pregnancy rate was 50%, with 3, 4 and 6 kittens.
3. Considering the average total number of sperm varies among males and ejaculates, as well the fertility in individual queens, we achieved acceptable pregnancy rates.
4. We believe this method has considerable potential in small felids as nonsurgical AI routine used method.

Acknowledgements

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References

1. Sojka NJ, Jemings LL, Hamner CE. Artificial insemination in the cat (Felis catus L.). Lab Anim Care 1970;20:198–204.
2. Platz CC, Wildt DE, Seager SWJ. Pregnancy in the domestic cat after artificial insemination with previously frozen spermatozoa. J Reprod Fertil 1978;52:279–82.
3. Howard JG, Barone MA, Donoghue AM, Wildt DE. The effect of pre-ovulatory anesthesia on ovulation in laparoscopically inseminated domestic cat. J Reprod Fertil 1992;96:175–86.
4. Zambelli D, Buccioli M, Castagnetti C, Belluzzi S. Vaginal cervical anatomic modification during the oestrus cycle in relation to transcervical catheterization in the domestic cat. Reprod Dom Anim 2004;39:76–80.
5. Tsutsui T, Tanaka A, Takagi Y, Nakagawa K, Fujimoto Y, Murai M, et al. Unilateral intrauterine horn insemination of fresh semen in cats. J Vet Med Sci 2000;62:1241–5.
6. Tanaka A, Takagi Y, Nakagawa K, Fujimoto Y, Hori T, Tsutsui T. Artificial intravaginal insemination using fresh semen in cats. J Vet Med Sci 2000;62:1163–7.

SEROEPIDEMIOLOGICAL STUDY REGARDING RUMINANT PARATUBERCULOSIS IN THE EAST OF ROMANIA

Ina Iuliana MACOVEI, Gheorghe SAVUŢA
USAMV Iaşi; macovei_ina@yahoo.com

Abstract
Mycobacterium paratuberculosis (MAP) is the causative agent of ruminant paratuberculosis, characterized by chronic hypertrophic enteritis in different species of ruminants. Some studies have demonstrated a link between MAP and Crohn's disease. Data on the epidemiology of paratuberculosis in our country are not complete and often missing. We started the study with a retrospective approach to paratuberculosis data from Romania then we turned to the seroepidemiologic data of Iasi and Neamt counties. Using two kits for detection of antibodies anti-M.paratuberculosis we conducted in the eastern area of Romania a serological screening in three species of ruminants. The bovine paratuberculosis seroprevalence showed a rate of 6.25%, for sheep the seroprevalence was estimated at 19.54% and for goat 32.35%. The animals found positive had an average age between 3 and 4 years.
Keywords: paratuberculosis, ruminants, epidemiology

Introduction

Paratuberculosis (also known as John's disease) is a chronic bacterial infection of ruminants characterized by excessive weight loss, diarrhea and death from cachexia. The disease is caused by *Mycobacterium avium ssp.paratuberculosis* (MAP), classified in the *Mycobacterium avium-intracellulare* complex [2], known as the "bacillus of John". Diagnosis is difficult because the immune response in MAP is difficult to detect. An animal serologically positive for paratuberculosis is an epidemiological risk and is a candidate for the statute of an active excretory. Antibodies are detectable after about 10-17 months after infection and serological testing can be done after the age of 15-18 months.

Though paratuberculosis has an increasing economic impact worldwide, and some zoonotic relevance in association with Crohns disease in humans has been discussed [5], very little is known about the biology of MAP and the pathogenesis of this disease[3,4].

Material and method

Material and method used: retrospectively epidemiology and seroepidemiology - serological test ELISA.

With retrospective cohort epidemiology we tried a presentation of ruminant paratuberculosis in Romania since 2006. The data about MAP in Romania presented in this study comes from articles published in Romanian Veterinary Journals, the databases of two county veterinary laboratories and the database of the official site OIE.

The descriptive epidemiological study was conducted using a serological examination, therefore we made an immunoassay test for detection of anti-paratuberculosis antibodies from 138 cattle,87 sheep and 39 goats from 8 counties. The cattle studied were selected from nine farms in Iasi, Suceava and Botosani. The sheep studied were from four counties: Braila, Iasi, Vaslui and Tulcea. The majority of tested goats were from Iasi. Blood samples were collected, conditioned and stored according to a well-established protocol, and serological testing was performed using kits for detection of *Mycobacterium paratuberculosos* antibodies producted by Pourquier Institute, Elisa paratuberculosis

Antibody Verification and detection kit of antibodies produced by LSI France, LSIVET ruminant paratuberculosis serum "ADVANCED".

Results and discussion

Data on the epidemiology of paratuberculosis in Romania are not complete and often missing, as confirmed by official data recorded in the database OIE [6].A study on this subject, conducted by Savuţa et al. stresses that in 1996 only 3 cases of paratuberculosis in sheep were reported, and for the subsequent three years, from 1997 to 1999, the national data are missing [8].Between 2007 and 2008, for the Cluj county the annual prevalence of MAP estimated was of 1.28% in 2007 and 1.72% in 2008, following a serological screening performed with ELISA kit KIT LSI of sheep and goats[7].For 2009, DSVSA Satu Mare has the animal health situation for the entire county and recorded the following data for paratuberculosis: blood harvesting cattle: 16,409; no. outbreaks:1 ; affected animals: 4 cattle.

According to data published on the official site of the OIE, the situation of ruminant paratuberculosis in Romania for the period 2008 - 2010 was diagnosed in 10 counties: Bistrita-Năsăud, Constanţa, Gorj, Harghita, Iaşi, Ilfov, Maramureş, Mureş, Prahova andSatu-Mare. The year with the highest number of cases was 2009, with 30 animals infected in Maramures only. As for the monitoring and control of paratuberculosis in Romania, the disease is included in the veterinary strategic program, it follows the serological control of ruminants and application of appropriate internal and external transport of animals[1].

Data for *Mycobacterium paratuberculosis* infection in ruminants in the two counties studied are from the databases of Departments County Sanitary Veterinary of Iasi and Neamt from 2005 to 2010.

Regarding the epidemiology of paratuberculosis in ruminants, the Iasi county includes data on the number of animals in households and those in communities (farms) serologically tested. According to the serological screening study of bovines in Iasi county, the positive number is the highest in 2006, with a progressive decrease until 2010 (fig. 1). As a statistical value, it can be estimated that the annual incidence of bovine paratuberculosis in this county for the years 2006 - 2010 is 11.

Paratuberculosis serological screening of tested sheep in Iasi found an increase in the number of positive animals for immunoassay test for detection of antibodies in 2007 and 2009 (fig. 2) with a decrease in 2008.

For the year 2010, according to data from the Department of Veterinary Science, the cases of caprine paratuberculosis include a total of 14 households and a farm which was taken under supervision. Subsequent serological tests were performed. ELISA detected a total of 37 goats positive for infection with Mycobacterium paratuberculosis.

For the period between 2005-2010, in the Neamt county 11 cattle were found positive for infection with *Mycobacterium paratuberculosis,* in 2010.Unlike the rate in cattle, the sheep infection for MAP is much higher. In 2009 a peak of ovine MAP infection is observed, with a total of 189 serologically positive sheeps. The decrease in serologically positive cases in the following year, 2010, is explained by effectively eliminating the positive animals.

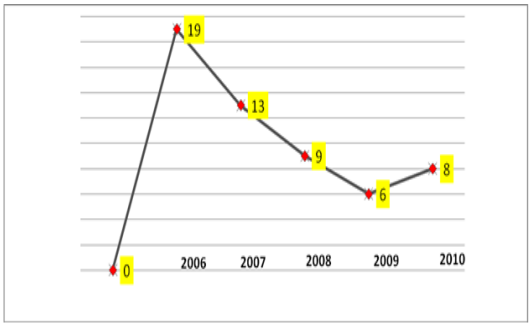

Fig.1 Bovine paratuberculosis seroprevalence in Iasi County, 2006 - 2010

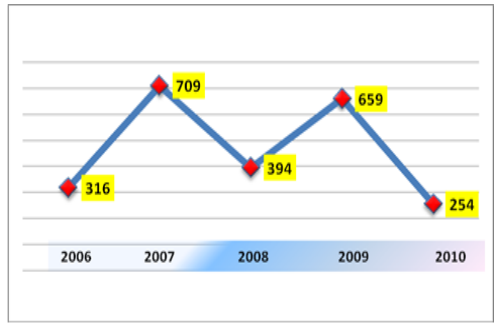

Fig. 2 Sheep paratuberculosis seroprevalence in Iasi County, 2006 - 2010

For all animals ELISA positive, cattle, sheep and goats, an examination of faeces for bacterioscopic by Ziehl-Neelsen strain was made. However, although this exam is one certainty in the diagnosis of paratuberculosis outcomes, bacterioscopic relevance is lower for negative smears, since the elimination of mycobacteria from the intestinal tract is intermittent in ruminants.

During the years 2010-2011, we studied 317 animals, respectively 144 cattle, 133 sheep and 39 goats from eight counties.The cattle studied were selected from five counties: Botosani, Iasi, Ilfov, Neamt and Suceava, out of which 9 farms and 2 households(tab1; fig3).The sheeps studied were from 4 counties'sheepfolds: Braila, Iasi, Vaslui and Vrancea(tab1; fig4). Serological screening of goats has considered a number of 39 goats from the Iasi and Vrancea counties (tab1; fig 5).

Table 1. Ruminants serologically tested during 2 years of study, 2010-2011

No.	County	No. cattle tested	No. of positive cattle samples	No. sheep Tested	No. of positive sheep samples	No. goats tested	No. of positive goat samples
1	Botoşani	21	0	0	0	0	0
2	Brăila	0	0	41	11	0	0
3	**Iaşi**	**56**	**1**	**64**	**9**	**31**	**10**
4	Ilfov	14	2	0	0	0	0
5	Neamţ	5	0	0	0	0	0
6	Suceava	48	6	0	0	0	0
7	Vaslui	0	0	18	2	0	0
8	Vrancea	0	0	12	6	8	1
Number of animals tested serologically		**144**	**9**	**135**	**28**	**39**	**11**

As described in the Methods section, we used two serological tests: Paratuberculosis Antibody Elisa Verification, Pourquier, France and LSIVET ruminant paratuberculosis serum "ADVANCED", France.

With the serological test paratuberculosis Antibody Elisa Verification, Pourquier, France we tested 138 cattle and seven sheep, and with the 2nd test, serum ruminant paratuberculosis LSIVET "ADVANCED", France, we tested six cattle, 126 sheep and 39 goats.

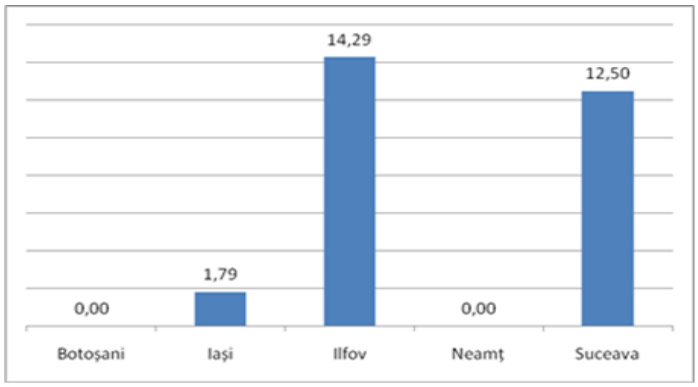

Fig.3 Paratuberculosis seroprevalence for cattle tested in the counties studied

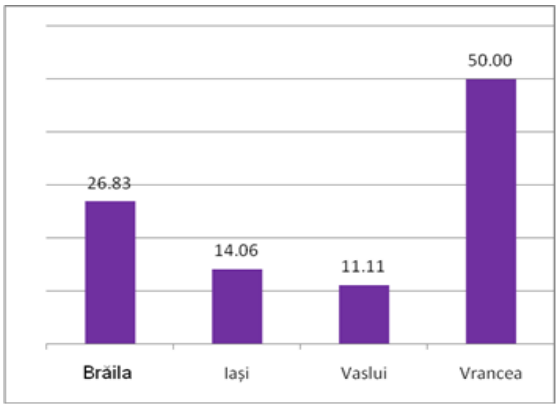

Fig.4 Seroprevalence for paratuberculosis sheep tested in the counties studied

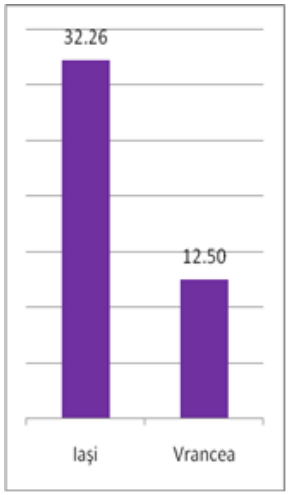

Fig.5 Seroprevalence for paratuerculosis in goats tested in the counties studied

From a total of 144 cattle tested serological for paratuberculosis a percentage of 6.25% had a positive response, 90.97% had a negative response and 2.77% had a dubious response. For the Iasi county, 48 sheep were tested in retrospective epidemiological investigation conducted by the Veterinary Directorate Food Safety Science's study database. Thus, deepening the serological study allowed the detection of a greater number of animals serologically positive for paratuberculosis in sheepfolds. The travel possibilities and indulgence of field colleagues allowed setting up a sample of 24 and 21 sheeps from two different sheepfolds in the same locality. Sheep samples, chosen randomly from the two

sheepfolds were a 10% of each population and the average for MAP infection was 14,89%. The age average of sheeps seropositive for MAP infection is about 4 years.

Most goats tested serologically (fig.5) for paratuberculosis are from Iasi. It should be noted that they come from a single fold. The population of goats tested was chosen after consultation with the Veterinary Directorate Food Safety Science database of Iasi county. Average age of positive serological paratuberculosis goats is about 3 years.

Conclusions

1. The importance of infection caused by *M. avium subsp. paratuberculosis* resides in the fact that after installation of clinical signs of disease and have significant economic losses are becoming more of a zoonotic nature through correlations with Crohn's disease.
2. Based on serological examination results may indicate that paratuberculosis is present in the herds of cattle, sheep and goats in the areas of the eight counties studied, namely Botosani, Suceava, Neamt, Iasi, Vaslui and Vrancea, Braila, Ilfov, the subclinical infection was detected by serological, ELISA.
3. For the two years of serological study, paratuberculosis showed seroprevalence rates of 6.25%, 19.54% for sheep and 32.35% for goats.
4. The serological testing of 10% of the animals from two sheepfolds shows a seroprevalence average of about 1,48% for each one.
5. The age of the sheep and goats found positive serological for paratuberculosis has an average of 3-4 years.

Bibliography

1. www.ansvsa.ro
2. Biet Frank, Boschiroli Maria Laura, Thorel Marie Françoise, Guilloteau Laurence A., 2005. *Zoonotic aspects of Mycobacterium bovis and Mycobacterium avium-intracellulare complex (MAC)*. Review article,Vet. Res. 36 ; 411–436 411© INRA, EDP Sciences
3. Chiodini R.J., Van Kruiningen H.J., Merkal R.S., 1984. *Ruminant paratuberculosis (Johne's disease): the current status and future prospects*. Cornell Vet, 74: 218-262
4. Chiodini R.J., *Crohns disease and the mycobacteriosis: a review and comparison of two disease entities*. Clin. Microbiol. Rev. 2 (1989) 90–117.
5. Duchmann R. Zeitz M., 2005. *Mucosal Immunology: Crohn's Disease*. Medical Department, Charité Universitätsmedizin Berlin, Campus Benjamin Franklin, Free University of Berlin, Berlin, Germany Hardbound, ACADEMIC PRESS
6. www. oie.int
7. Papastergiu D., Răpuntean Gh., Oleleu AnaMaria, Răpuntean S., Pivaru N., 2009. *Aspecte epidemiologice privind paratuberculoza la ovine şi caprine în arealul judeţului Cluj*. Lucr. Stiinţifice – vol 52, seria med. Vet, pp 1036-1042
8. Savuţa Gh., Irina Matei, Selma Murat, D. Aniţă, 2006. *Epidemiologia paratuberculozei rumegătoarelor în România*. lucr. Stinţifice – vol. 4 seria Med, Vet, pp843 – 847

CLINICAL, COMPUTER TOMOGRAPHYCAL AND CEREBROSPINAL FLUID ASPECTS IN BRAIN TUMOURS OF DOGS

Mihaela ARMAŞU, M. MUSTEAŢĂ, Adina ZBÂNGU, Gh. SOLCAN
Facultatea de Medicină Veterinară Iaşi, Aleea M. Sadoveanu 8
armasummihaela@yahoo.com

Abstract
The aim of the study was to describe clinical and imagistic aspects of cerebral tumours in dogs in conjunction with cerebrospinal fluid changes. Nine geriatric dogs were diagnosed with cerebral tumours. For all dogs neurological exam and computer tomography has been made. For 3/9 CSF exam has been performed. On CT examination all tumours were surrounded by a peritumoral oedema and most of them have enhancing with iodine contrast in different degrees.
Key words: computer tomography, brain tumour, dog

Introduction

The diagnosis of intracranial tumors has been greatly improved since advanced techniques such as magnetic resonance imaging (MRI) and computed tomography (CT) have become more available in veterinary medicine (Kraft 1999; LeCouteur 2007). Although, the definitive diagnosis of a brain tumor usually requires biopsy and histopathological examination, not all cases can be done so due to this many times it can be only presumptive diagnosis of the brain tumor. This study tries to describe intracranial tumors based on data obtained at neurological examination, tumor characteristics on CT images and CSF analyze.

Materials and method

The study was performed on 9 dogs with brain tumors, which were presented at the consultation at the Faculty of Veterinary Medicine Iasi. All dogs admited in the study underwent neurological examination and computer tomography. Computer tomography (CT) was performed using Toshiba 16 slice CT scan, patients being previously submited to general anesthesia, using for this precedure a combination of medetomidine 0.03 mg / kc and ketamine 3 mg / kg administered iv.The images were taken before and after administration of the contrast substance(Lux Scan 750 mg / kg iv).

The CSF sampling was performed in 3 patients, in their case CT images did not show the increasement of intracranial pressure. CSF puncture was performed under general anesthesia (by the protocol described above) at the cerebellomedullary cisternal level. CSF examination was considered normal when cell number was up to maximum 5/µl. Through the biochemical examination of CSF have been pursued the abnormal variations of the protein, glucose, lactate and calcium values.

In the case of 3 dogs, the owners have agreed with the euthanasia (using commercial preparation T61), followed by histopathological examination.

Results and discussion

Data on the breed, sex, age, clinical and histological diagnosis of the tumors identified in those nine dogs are presented in table 1.

Table 1. The breed, age, sex, clinical and histological diagnosis of dogs with brain tumors

Crt no	breed	Age (years)	sex	Clinical manifestations	Histological diagnosis
1	Caniche	11	M	Epileptic-like seizures	-
2	Boxer	10	F	Epileptic-like seizures	-
3	Caniche	12	M	Epileptic-like seizures, aggression, head pressing, modified nutritional behavior, disorientation	meningioma
4	Small Schnauzer	7	M	Epileptic-like seizures	-
5	Golden Retriever	10	F	Ataxia, lack of vocalization, walking in wide circles towards the right side, compulsive walking, anisocoria with larger right pupil, discrete protrusion of the 3rd eyelid at the right eye, discret ptosis of inferior eyelid at the right eye	Cystic meningioma
6	Bichon	9	M	Epileptic-like seizures, confusion, decreased hemilocomotion on right side, menace and pupillary reflexes void at right eye, the facial nerve paralysis, decreased response to stimulation of both nostrils, left side circles, hemiinatention	hemangiosarcoma
7	Pekingese	8	F	Epileptic-like seizures	-
8	Fox Terrier	14	F	Epileptic-like seizures	-
9	Boxer	13	F	Epileptic-like seizures	-

The predominant clinical manifestation was represented by of epileptiform seizures, which were found in 8 dogs. In 6 cases, epileptic-like seizures were the only clinical sign of brain disease. Although in a lower percentage (47-61.76%) than the one identified by us (88.88%), the occurrence of epileptic form seizures as a sign of brain tumors is known in medical literature (Schwartz, 2011, Snyder 2006, Platt 2002), some specialists considering this aspect the main reason for presenting the animal to doctor (Bagley 1999). We believe that in our case, the large number of dogs with epileptic seizures consecutive to the brain tumors are due to the small number of examined cases and to the presentation of the most serious cases, at our clinic.

In a case of a patient the status epilepticus installed when it has awaken from general anesthesia. The cause of epileptic seizures upon waking up from anesthesia might be due to simultaneous activation of all nervous centers of the brain, at that precise moment the excitation- inhibition ratio being sometimes in favor of excitation.

In addition to epileptic seizures, in 3 dogs, there were observed other neurological manifestations. Thus, in the case of the dog with meningioma that affected olfactory and frontal lobes, other clinical signs have consisted in aggression, head pressure, modified nutritional behavior and disorientation. The modifying of nutritional behavior is after Lahunta (2009) due to impaired smell and subsequent aggression is frontal lobe lesions. In the dog with cystic meningioma, the neurological changes consisted instability in walking, ataxia, lack of vocalizing, walking in wide circles towards the right side, compulsive walking, anisocoria with larger right pupil, discrete protrusion of the third eyelid at the right eye, discret ptosis of inferior eyelid at the right eye.

Interesting was the fact that the facial nerve examination was normal and there was no temporal muscle atrophy. The reason of this fact was encounter at De Lahunta (2009) which states that unilateral cerebellar lesions, particularly the ones that affect the medial cerebellar nuclei, may cause protrusion of the third eyelid and ipsilateral or contralateral mydriasis according to the nucleus involved. In this case, the mydriatic eye was on the opposite side of tumoral lesion.

In the dog with hemangiosarcoma were found next to epileptic-like seizures, confusion, decreased hemilocomotion on the right side, absent menace and pupillary reflexes in the right eye, facial nerve paralysis, decreased response to stimulation of both nostrils, walking in tight circles towards the left side and hemiinatention on the right side, clinical signs that have indicated the damage of forebrain and brainstem.

The changes seen in neurological exam gave us important clues on the location of the tumor process, information on tumor extension, number of tumors, or if the neurological changes observed are due to tumor process tumor or to mass effect induced by this being obtained from CT examination.

After performing CT and abdominal ultrasonography and radiography of the lung, in 8 cases the brain tumors were primary tumors, only in one case the tumors being metastatic at the level of the lung. In literature, the data on the incidence of primary and secondary tumors are conflicting (Moore 1996, Snyder 2008). One explanation for the small number of brain metastases diagnosed is the fact that the cranial vault was not routinely examined as metastatic site and because of the fact that sometimes brain tumors have a subclinical evolution.

Regarding the number of brain tumors per patient, in the case of primary tumors, in most patients was seen a tumor (6 cases), and in 2 dogs were observed 2 tumors. In the case of secondary tumor, the metastases were in number of 10. Our observations are similar to findings made by Lecouteur, (1999) and Patnaik (1986) that have claimed that the most primary brain tumors of dogs are solitary although multiple primary brain tumors have been reported.

The location of primary tumors in the brain was different, the frontal and temporal lobes being mainly affected (in every 6 cases), while parietal and occipital lobes were affected in one case. Regarding the metastasis, they have been occupying both posterior fossa (cerebellum, bulb) and being located supratentorial, as well.(Fig. 1).

Making a connection between the appearance of epileptiform seizures and affected brain region, we observe that they occur in 5/6 patients with frontal and temporal lobes damage. Thus, we state that in the present case series, epileptogenesis was more likely in

dogs with neoplasms located in the frontal and temporal lobes, similarly reported previously (Bagley 1999, Schwartz 2011).

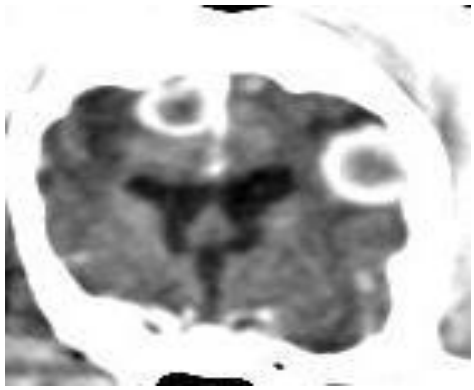

Fig.1. Caniche. CT post contrast. Isodense areas surrounded by one hyperdense ring. Brain metastases of lung tumors

Regarding the tumor characteristics determined on the basis of CT imaging after the application of contrast substance, the enhancement of tumors image was found in 7 cases, for the other two dogs these have remained isodense with the surrounding parenchyma. In 5 patients hiperintensity was uniform distributed, and in two cases it was in the form of a ring that surrounding the hypo or isodens tumor tissue with surrounding brain tissue (Fig. 2, 3).

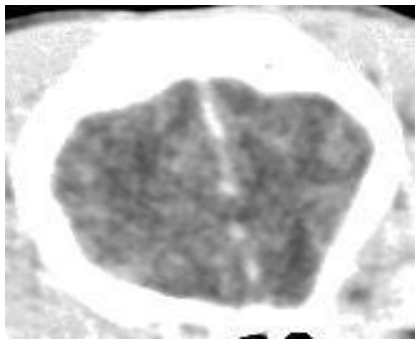

Fig. 2. Small Schnauzer. Post-contrast CT. Section in the frontal lobe. Isodense area surrounded by a ring of hypodense oedema

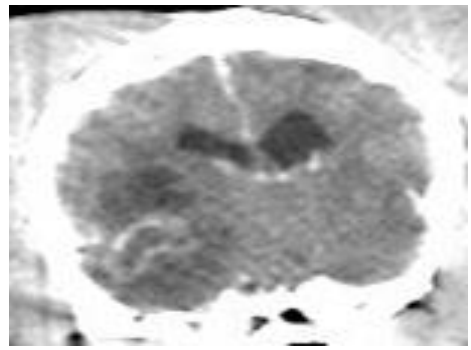

Fig.3. Boxer. Post-contrast CT. Section in the frontal lobe. Hypodense area surrounded by a hyperdense ring

In all cases the tumors were surrounded by a hypodense halo (11-19 UH) of different sizes corresponding to the area with perilezional oedema. Also, in 7 dogs the mass effect was present consisting of compression and deformation of brain ventricles (6 cases), blocking drainage of cerebrospinal fluid with secondary obstructive hydrocephalus (1 case) and midline shift (2 cases).

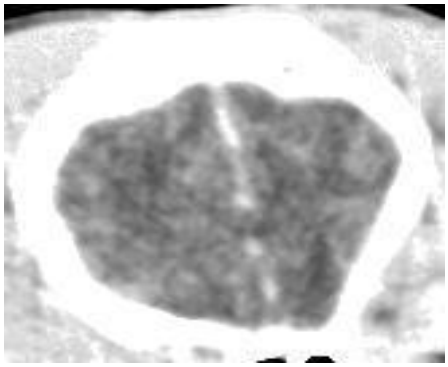

Fig. 2. Small Schnauzer. Post-contrast CT. Section in the frontal lobe. Isodense area surrounded by a ring of hypodense oedema

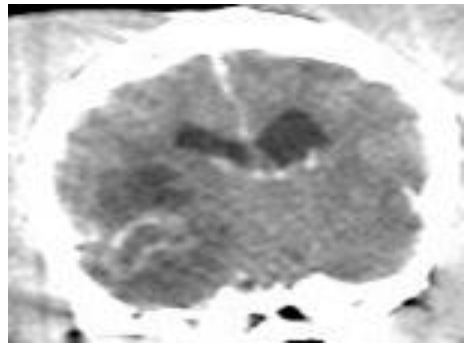

Fig.3. Boxer. Post-contrast CT. Section in the frontal lobe. Hypodense area surrounded by a hyperdense ring

For three cases submmited to histological exam, CT images could be compared with those found by other authors. Thus, meningiomas were characterized by broad-based, extra-axial attachment, exhibit distinct tumor margins and enhance contrast uniformily. They tended to displace rather to invade the parenchyma tissue (Fig. 4, 5). In the case of cystic meningioma (Fig. 2), cystic component was extended from the main tumor mass and it had dimenssions larger than the tumour itself. Although, after Kitagawa (2002) in most cases cystic meningioma is located in the olfactory lobe, in our case it was located infratentorial extending to the left temporal lobe. The reccurence causes of cystic component are, after Oakley RE, 2003, the tumor necrosis, CSF isolation or fluid producted by the tumor.

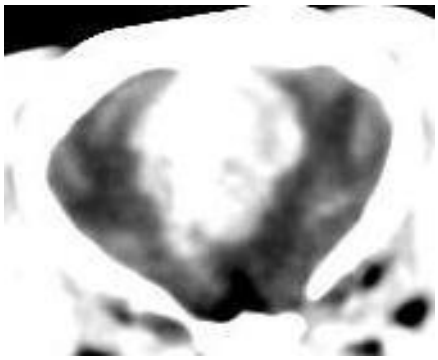

Fig.4. Caniche. Post-contrast CT. Section in the frontal lobe. Hyperdense area located in the frontal lobe of the center area. Meningioma

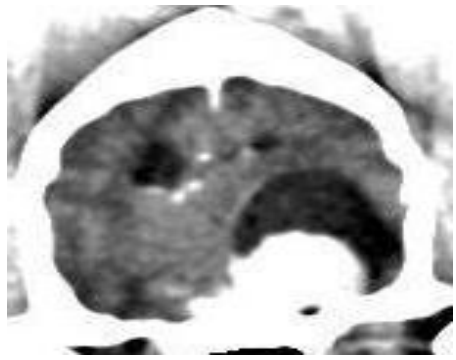

Fig.5. Golden Retriever. Post-contrast CT. Cortico-diencefalic section. Hyperdense mass associated with a hypointense well defined area by the surrounding parenchyma. Cystic meningioma

In terms of hemangiosarcoma, it is usually described in the literature as to hyperattenuated areas surrounded by oedema that is increasing with administration of contrast substance (Akutsu H. 2004, Guode Z. 2006, Dennler M. 2007). CT images captured by us are atypical for this type of tumor, where hemangiosarcoma was presented as 2 isodense zones surrounded by a hypodense halo suggestive for an oedema, without intensification at contrast enhancement (Fig.6). These particular aspects of the hemangiosarcoma neuroimaging were occasionally reported in the medical literature. Thus, Renukaswamy (2009) could not diagnose a hemangiosarcoma of 1 year old child using CT imaging technique because of its isodensity with the cerebral parenchyma, but discovered it later using RMN.

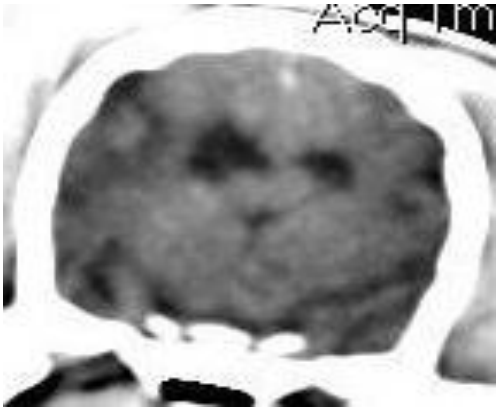

Fig.6. Bichon. Post-contrast CT. Section in the temporal lobes. Two bilateral isodense areas surrounded by an isodense halo. Hemangiosarcoma

CSF was collected from three dogs, none of the patients being identified with any tumor cells. The incidence of tumor cells in CSF is low, a negative cytology for tumor cells not excluding the presence of intra cerebral tumors , usually intraparenchimal, that does not include the piamater or ventricular system (Grevel 1992, Bichsel 1984).

The pleocytosis (12 cells/µl) was found only for the dog with meningioma, the cells being mainly neutrophils. The predominance of neutrophils in CSF after Di Terlizzi (2009) and Kaneko (1997) can be explained by necrosis and inflammation associated to the tumor. More, Bailey (1986) and Grevel (1992) have described CSF's changes associated to intracranial meningiomas in the dog, noting a neutrophilic pleocytosis in most of the cases.

Regarding the biochemical examination of CSF, abnormal variations were observed only in the case of dog with hemangiosarcoma. So, it was observed the increase of protein values (0.1g/dl), lactate and calcium (14.8 mg / dl). The literature states that high level of protein in CSF may be due to the increased permeability of blood-brain barrier (allowing the passage of serum proteins in CSF), intrathecal production of globulin and disruption of CSF flow or its absorption. (Kaneko 1997, Di Terlizzi 2006). Also, after Kaneko (1997), the increase of lactate occurs in diseases that are causing severe ischemia and the high level of calcium in the CSF may indicate the increased excitability in the CNS, dog having epileptic seizures.

Conclusions

1. Epileptic seizures occur in most patients with intracranial tumors, these being sometimes their only clinical manifestation. On CT imaging, the tumors have a polymorphic nature (hypo, iso or hyperdense) compared with surrounding parenchyma, being associated with variable-sized areas of cerebral edema.
2. In the case of tumor diseases, the CT aspects being various and CSF examination, in most cases, could not be achieved or not providing reliable information, accurate diagnosis of brain tumor can be established only after histopathological examination.

Acknoledgements

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References

1. Akutsu H., Tsuboi K, Sakamoto N., Nose T., Honma S., Jikuya T., 2004 - Cerebral metastasis from angiosarcoma of the aortic wall: casereport., Surg Neurol;61:68 –71.
2. Bagley R.S., Gavin P.R., Moore M.P., Silver G.M., Harringhton M.L., Conors R.L., 1999 - Clinical signs associated with brain tumours in dogs: 97 cases (1992-1997) J.of.American Medical Association, 215 (6), 818-819
3. Bichsel P., Vandevelde M., Vandevelde E., Affolter U., Pfister H., 1984. - Immunoelectrophoretic determination of albumin and IgG in serum and cerebrospinal fluid in dogs with neurological diseases. Research in Veterinary Science 37, 101–107.
4. De Lahunta A., 2009 - Veterinary neuroanatomy and clinical neurology , Third edition, W.B. Saunders Company, Philadelphia
5. Dennler M., Lange Eva Maria, Schmied O., Kaser-Hotz Barbara 2007- Imaging diagnosis-Metastatic Hemangiosarcoma causing cerebral hemorrhage in a dog, Veterinary Radiology &Ultrasound vol.48, issue 2, pg.138-140.
6. Di Terlizzi Roberta, Platt R.S., 2009 - The function, composition and analysis of cerebrospinal fluid in companion animals: Part II – Analysis. The Veterinary Journal 180, 15–32.
7. Grevel V., Machus B., Steeb C., 1992 - Cytology of the cerebrospinal fluid in dogs with brain tumors and spinal cord compression. Part 4. Tierarztliche Praxis 20, 419–428.
8. Kaneko J.J., Harvey J. W., Bruss M.L., 1997 - Clinical biochemistry of domestic animals, Fifth Edition, Academic Press, USA.
9. Kitagawa M., Kanayama K., Sakai T. 2002 - Cystic meningioma in a dog, J. Small Anim. Pract; 43(6): 272-274
10. Kraft S.L., Gavin P.R., DeHaan C., Moore M., Wendling L.R., Leathers C.W., 1997- Retrospective review of 50 canine intracranial tumours evaluated by magnetic resonance imaging. Journal of Veterinary Internal Medicine 11, 218–225.
11. LeCouteur R.A., 1999 - Current concepts in the diagnosis and treatment of brain tumours in dogs and cats, Journal of Small Animal Practice 40. 411-416
12. LeCouteur, R.A., Withrow, S.J., 2007 - Tumours of the nervous system. In: Withrow, S.J., Vail, D.M. (Eds.), Withrow and MacEwens´s Small Animal Clinical Oncology, fourth ed. Saunders–Elsevier, St. Louis, MO, USA, pp. 659–685
13. Moore M. P., Bagley R. S., Harrington M. L., Gavin P. R. 1996 - Intracranial tumors. Veterinary Clinics of North America: Small Animal Practice 26,759-777
14. Oakley R.E., Patterson J.S., 2003 - Tumors of the central and peripheral nervous system. In: Slatter D, ed Textbook of Small Animal Surgery, Philadelphia, Elselvier Science: 2405-2425
15. Patnaik A. K., Kay W . J., Hurvitz A. I., 1986 - Intracranial meningioma: a comparative pathologic study of 28 dogs. Veterinary Pathology 23, 369-373
16. Platt S. R., Haag M., 2002 - Canine status epilepticus: a retrospective study of 50 cases. Journal of Small Animal Practice 43, 151-153

17. Renukaswamy G. M., Boardman Simone J,. Sebire J.N., Hartley B.E.J., 2009 -Angiosarcoma of skull base in a 1-year-old child—A case report, International Journal of Pediatric Otorhinolaryngology 73, pg. 1598–1600
18. Schwart M., Lamb, C.R., Brodbelt D.C., Volk H.A., 2011 - Canine intracranial neoplasia: clinical risk factors for development of epileptic seizures Journal of Small Animal Practice, DOI: 10.1111/j.1748-5827.2011.01131
19. Snyder J. M., Shofer, F. S., Van Winkle T. J., Massicotte C., 2006 - Canine intracranial primary neoplasia: 173 cases (1986-2003). Journal of Veterinary Internal Medicine 20, 669-675
20. Snyder J.M., Lipitz,L., Skorupski K.A., Shofer F.S., Van Winkle T.J., 2008 - Secondary Intracranial Neoplasia in the Dog: 177 Cases (1986–2003) Background: J Vet Intern Med; 22:172–177
21. Zhai Guode, Pang Qi, Guo Hua, Xu Shangchen, Wang Hanbin -Primary cerebellopontine angle angiosarcoma , Journal of Clinical Neuroscience, vol.15, issue 8, 2008, pg.942-946

PRELIMINARY DATA REGARDING SUMMER PARASITIC DISEASES OF WILD AND CULTURED TROUT IN IZVORU-MUNTELUI BICAZ LAKE

Ramona ŞORIC, Liviu MIRON
Faculty of Veterinay Medicine Iasi
ramonas@uaiasi.ro, lmiron@uaiasi.ro

Abstract
This study is based on field and laboratory diagnosis of parasitic diseases in floating cages cultured and free rainbow trout (Oncorhynchus mykiss), from a mountain man-made reservoir, for a period of three month in the summer of 2011 (June to August). Parasitological diagnosis was done by microscopic examination of skin and gills scraping, wet squash-smear preparation of internal organs, artificial digestion of fish muscle and by histological methods. Our investigation, leaded to the identification of some ciliates, trematodes and crustacean parasites like Ichthyophthyrius multifiliis, Tricodina spp, Diplostomum sphataceum, and Ergasilus sieboldi. Variation of the physical and chemical water parameters, the artificial reef effect of floating cages and the water flow are influencing the evolution and transmission of the parasitic diseases from free living fish to cultured fish and reverse.

Key words: artificial reef, parasitic diseases, rainbow trout

Introduction

Izvoru-Muntelui Bicaz Lake is a mountain man–made reservoir with a high bioproductive potential in aquaculture (Miron, 1983) due to the high self purifying capacity. In 1996, Bicaz Lake was classified like oligo-mesotrophic, dimictic reservoir with three different periods: one with a direct thermic stratification, one with reverse thermo-stratification and two homothermic periods (Miron, 2011). In the period of thermal stratification there are three distinct layers, epilimnion (surface layer), metalimnion (middle layer) and hypolimnion (bottom layer).

In the last three decades, the parasitic fauna, of the free and cultured trout, in Bicaz Lake, was represented by: *Trichodina* spp. in 1980 and 1987; *Argulus foliaceus* in 1989 and 1990; *Ergasilus sieboldi* in 1899; *Ichthyophthyrius multifiliis* in 1989 and 1993; *Chilodonella* spp. in 1994, *Ichthyobodo necator* (Miron L., 1999);

In trout breeding in Bicaz Lake, the summer time (June–August) is considered the critical period, because of the high water temperature (21-25°C) which represents a stress for the trout and being favorable for parasitic, bacterial or viral agents development. Also, over the summer, dissolved oxygen quantity, is under trout lower tolerance of 10 mg/l, with difficulties in fish breathing, but is not considered a hypoxic environment. If in this period algal bloom may be encountered, fish respiration becomes more difficult than already is (Soric *et al*, 2011).

The purpose of this paper was to establish the summer parasitic fauna of cultured and free living trout from Bicaz Lake. We chose this period because of the existing water qualities alteration, with great influence, both in fish and parasite life cycle.

Material and method

We examined different categories of cultured rainbow trouts (4 months, 7 months, 18 months and 24 months) and also, free living trout collected with fish-nets (16, 18, 20, 22, 25 and 30 mm), disposed in 6 work stations, around the existing farm.

After the clinical examination, fishes with clinical signs were examined by microscopic examination of skin and gill scraping, wet squash-smear preparation of internal organs. Some fragments of the organs with macroscopic lesions were fixed in 4% buffered formalin and processed by histological methods. Histological sections (5µm in thickness) were stained with haematoxylin – eosin (HE), and Giemsa.

Because of the water qualities importance in trout breading, and its effect on parasites life cycle, in this period it was also monitored water temperature, pH, dissolved oxygen variation, turbidity and chlorophyll, in the epilimnion layer (surface layer). We used an Environmental monitoring system 6600 V2, Multiparameter Water Quality Sonde YSI 2, in one work station, between 2 floating trout farms.

Results

After microscopic examination of skin and gill scraping and wet squash-smear preparation of internal organs, we identify: two genres of ciliates: *Ichthyophthyrius multifiliis and Trichodina spp*; mataceracarial infection with *Diplostomum sphataceum; and* the crustacean copepod *Ergasilus sieboldi.*

Ichthyophthyrius infestation was registered from July to August, in 4 months and 7 months fry trout, but also in 18 months old rainbow trout. Diseases associated with *Ergasilus sieboldi,* was observed in all three groups, in different percentage: 50% of 4 months old trout, 12,5% of 7 months old trout and 33% of 18 months old trout. Both parasites were encountered on the same gills. Infections intensity was around 30 parasites/fish. Clinical signs observed were: atypical swimming positions, inappetence, lethargy and emaciated. This disease was associated with: denudation of the operculum localised haemorrhagic lesion of dermal and gills epithelium, pustules on the skin, necrotic lesions and haemorrhagic infiltration with serious alteration of gills integrity, as sited in the literature (Ferguson, 2006). Microscopic examination of skin and gill scraping, and wet mount of gill biopsy lead to the identification of *Ichthyophthyrius multifiliis* trophonts (adult stage), tomits (invasive stage) and trophozoite (feeding stage), the last one being confirmed by histological methods.

Ergasilus sieboldi is a parasitic copepod that was found in cultured trout, at the age of 4 months, 7 months, 18 months and 24 months. All free living rainbow trout's, examined were positive for ergasilosis with an intensivity of 4 parasites/fish. At the clinical examination we observed: swimming at the surface of the water and in abnormal positions, hypoxia with increase respiratory movements. Necropsy methods, revealed haemorrhagic lesion, rupture of the gills epithelium and necrotic lesions with a yellow deposit. *Ergasilus sieboldi* identification was done in wet mount of gills, using key of identification by Kabata 1979

Infestation with metacercariae of digean trematode *Diplostomum sphataceum* was signalled for the first time in salmonid from Izvoru-Muntelui Bicaz Lake. It is well known parasite in fishes where it occurs as metacercaria in the eye lenses of the host and often causes parasitic cataract (Shariff et al., 1980). The helminth has been recorded from rainbow trout in a large number of countries and is still known to occur prevalently in some modern

trout farms (Buchmann et al., 1994). It has been stated in the last few decades that the parasite can exert a negative influence on the production of fish in aquaculture.

Metacercarial stages were identified in the eye lens of all 24 months old trout's examined and also in trout's captured by net fishing, with a maximum intensity of 23 parasites/fish. Digeneans are usually asymptomatic infections, but the localisation of the mataceracariae in the lens and other ocular tissue, can cause exophthalmia, blindness and finally undernourishment, by the impossibility of founding the feed. At the necropsy, we observed serious modification of the internal organs like: kidney hypertrophy with white nodules, splenic hypertrophy with haemorrhagic infiltrations, dystrophic lesions of the liver and pale gills. Histological methods were used for metacercariae identification in the lens and also the necrotic lesions consecutive to their migration throughout the organs.

The ectocommensal parasite, *Trichodina spp. (Oligohymenophorea, Ciliophora)* was identified by microscopic examination of nostrils mucous, stained with silver nitrate. Because, only one specimen was found we can't declare as an evolving disease, but must be mentioned as existing in an atypical place, as usually it is found on the gills, skin and fins of fishes, though some species parasitise the urogenital system.

In the summer period the epilimnion (surface layer) was of 34 m in depth. Analysing the variation of water physicochemical parameters from this layer, we registered: water temperature was from 21°C - 23°C, the optimum value of 15 °C was found around the depth of 25 m; dissolved oxigen was between 8 and 10 mg/l; water pH was around 7,7; high chlorophyll charge of the epilimnion.

Discussions

In fish breeding, parasitic diseases alone, cause important economic losses. We chose to do this study in the summer period because of the major impact of water qualities on pathogens life cycle, altering parasites range, virulence and transmission rate but, also, because of the negative effect on host immunocompetence (Marcogliese, 2008), on fish metabolism and physiology (Noga, 2010).

In the summer of 2011, water temperature was higher than rainbow trout maximum tolerable ranges 19°C (Noga, 2010) optimal temperature of 15 °C, being registered much under the migration area of cultured trout, at the depth of 25 m. Ichthyophthyriosis is a serious and common disease that affects freshwater fish between 3 and 6 months old (Matthews, 2005) and cane produce, high mortality (80 – 100%) in the period of tomites multiplication (Dickerson, 2006). At 25°C, parasites complete their life cycle in 3-6 day and many epidemics in salmonids are registered in summer (Noga, 2010).

In Bicaz Lake this parasitosis was diagnose, for the first time, in the summer of 1994, in fingerlings (Miron L., 1999) and caused mass mortality. Our study leaded to the identification of the three stages of *Ichthyophthyrius multifiliis* in 18 month old trout is the result of fish severe hyperthermia stress, and added to the high density of cultured fish. Another reason is represented by the major impact of high water temperature on pathogens life cycle, altering parasites range, virulence, transmission rates, host immunocompetence and host range. (Marcogliese, 2008; Woo, 2007). Water mediated but permanent contact between free and cultured fish and also between fish from the same floating farm, allows parasite transitions.

All samples of trout, captured by net fishing, were more than 12 month old and tested negative for ichthyophthyrosis. This confirms the role of fish density this disease. *Ichthyophthyrius multifiliis* pathology was aggravated by increased phytoplanktonic biomass, consecutive to algal bloom, registered in this period by Aoncioaie *et al*. (2011).

High water temperature, algal bloom, and high density of cultured fish lead to the rapid formation of a periphyton on the submerged cages (Miron I, 1991), a perfect place for *Ergasilus sieboldi* and snails (*Diplostomum sphataceum*, first intermediary host) to complete their life cycle.

The artificial reef effect of the immersed surface of the salmonid floating cage farms, creates new reproduction territories and oviposition substrate areas for play and shelter and increase periphyton development (Miron I., 1991). Identification of *Ergasilus sieboldi* in all trout's captured by net – fishing, in the area surrounding of the existing salmonid farms and also in all categories of cultured rainbow trout, supports the roll of artificial reef in increase parasites transmission rate.

Floating fish farms are a continuous source of nutrients for lake, with consequences on eutrophication process, starting with the periphyton formation, on the immersed surface. In the periphyton intermediated host or stages of parasites can develop, fact confirmed by the presence of *Diplostomum* metacercariae and *Ergasilus sieboldi* females on cultured trout.

Ergasilosis was mentioned for the first time on Bicaz Lake, at caged cultured fingerlings in June 1989, when water temperature was between 19 and 22°C (Miron L., 1999). In the same year Miron L, (1999) identifies a branchiuran infestation *Argulus foliaceus* in the 3 year old rainbow trout, arthropod infestation that we didn't encountered. In the summer of 1993 another episode of infestation with *Ergasilidae* was registered at rainbow trout fry (Miron 1993). In our study we observed the parasites in all age categories examined, but with a severe pathology on small fish.

Metaceracarial infestation with *Diplostomum sphataceum* in cultured rainbow trout and in free fish, confirm the roll of piscivorous birds in the biological pollution of the water. Extermination of *Diplostomum sphataceum* definitive host in the Izvoru-Muntelui Bicaz area is very difficult, so prevention method are targeted on the elimination of the first intermediated host, represented by snails.

Conclusions

1. Identification of all stages of *Ichthyophthyrius multifiliis* in fish older than 6 months, may be the result of favourable factors as: high temperature level and high fish density.
2. Gills lesions in Ichthyophthyrosis were aggravated by water hyperthermia, by algal bloom of *Cyanophiceae* and the simultaneous development of *Ergasilus sieboldi.*
3. Copepode and mataceracarial invasion of both free and cultured trout reveals the importance of prophylactic actions in eliminating the periphyton from the surface of the submerged cages.
4. In the summer of 2011 it was identified for the first time mataceracariae of *Diplostomum sphataceum* in salmonids from Bicaz lake.

5. In trout breading the water quality variation have importance, because of their impact on pathogens life cycle, parasites range, virulence, transmission rates and host physiology and metabolism.

References

1. Aoncioaie Carmen, Erhan M. G., Miron Manuela, (2011), New observation on biological features of Izvoru Muntelui Bicaz reservoir. *Anal. St. Univ. Cuza.* Tom LVII.
2. Buchmann K, Uldal A., (1994)- Effects of eyefluck infections on growth of rainbow trout *Oncorhynchus mykiss* in a mariculture system.Bull Eur Ass Fish Patol. 14: 104-107
3. Ferguson H.W., (2006) - *Systemic Pathology of Fish: A Text and Atlas of Normal Tissues in Teleosts and their Responses in Disease*: 2nd edition, Editura Scotian Press, Londra, UK, 368 pp;
4. Marcogliese D.J., 2008, *The impact of climate change on the parasites and infectious diseases of aquatic animals,* Scientific and Technical Review Off. int. *Epiz.*, 27 (2), 467-484.
5. Noga E. J., (2010), *Fish diseases – Diagnosis and Treatment*, 2nd Edition, Wiley – Blackwell, 378.
6. Miron I., (1983), *Lacul de acumulare Izvoru-Muntelui Bicaz,* Editura academiei socialiste Romania, 224.
7. Miron I., (1991), *The effect of the reef of the first floating salmonid farm on the Bicaz Reservoir,* Verh. Internat. Verein Limnol, 24, 2528 -2530.
8. Miron L., (1999), *Tratamente antiparazitare la salmonidele crescute intensiv pe lacurile montane din România*, Lucrările simpozionului Lacurile de Acumulare din România, 2, 107-117:
9. Miron L, (1999), *Parazitofauna Salmonidelor crescute intensiv în lacurile de acumulare montane şi în unele păstrăvării clasice din România*, Lacurile de acumulare din România, 2, 43-48;
10. Miron I., Anca-Narcisa Neagu, Mihai Erhan, Carmen Aoncioaie, Maria-Magdalena Fusu, 2011, *Succesiunea ecologică (Râul Bistriţa – Lacul de acumulare Bicaz). Monografie limnologică II,* Ed. Universităţii Alexandru Ioan Cuza, Iaşi, 2009.
11. Miron L., (1993), *An episode of Ergasilus infestation on Trout fry in the Bicaz reservoir*, Lacurile de acumulare din România, tipologie, valorificare, protecţie, 42-46.
12. Soric Ramona, Liviu Miron, Dumitru Acatrinei (2011)The monitoring of water temperature, ph, and dissolved oxygen variation, in Izvoru-Muntelui Bicaz man-made lake, between 2009-2010, 54: 274 - 278.
13. Woo P.T.K., (2007), *Protective immunity in fish against protozoan diseases,* Parasitologia, 49, 185-191.

HYPERTROPHIC PULMONARY OSTEOPATHY (HPO)

Cristina BARBAZAN, Vlad TIPIŞCĂ, Constantin DARABAN, Vasile VULPE
USAMV-Iaşi, Facultatea de Medicină Veterinară
cristina_serbanmv@yahoo.com

Abstract
Hypertrophic pulmonary osteopathy (HPO), also known as Marie's Disease or acropachia, is a rarely seen condition in dogs and humans and extremly rare in cats, consisting of the periosteal proliferations on the long bones of the body (members) as a response to chronic lung disease such as abscesses or tumors. Since october 2010 until may 2012, 3 cases of HPO were diagnosed in Radiology Laboratory of FMV Iaşi. Two cases were presented to control with lameness and swelling of all four limb extremities. The third case was diagnosed initially with a large, ulcerated brest tumor and pulmonary metastases, subsequently developing hypertrophic osteopathy.
Keywords: lameness, pulmonary metastases, hypertrophic osteopathy.

Hypertrophic pulmonary osteopathy (HPO), also known as Marie's Disease or acropachia, is a rarely seen condition in dogs and humans and extremly rare in cats, consisting of the periosteal proliferations on the long bones of the body (members) as a response to chronic lung disease such as abscesses or tumors. It has also been reported in association with bladder tumors.

The ethiology is still unknown, it is suposed to be associated with circulatory disturbances – intrathoracic compression on blood or limphatic vessels.

Usually the patients claim bilateral swellings and lameness both in front and hind limbs, which are tumefied in distal portion and deformed.

Radiological signs include periosteal new bone formation along the phalanges, metacarpal and metatarsal bones (affecting at the begining the medial aspects of the second and lateral aspects of the fifth digits). As the affection progreses, the new bone formations extend to the radius, ulna and tibial bones, and finally to humerus and thigh-bone.

The new bone is laid down at right angles to the long axis of the bone, in „palisade" fashion. As the periostal deposits progreses, the new bone tends to become smooth. The lung disease is also radiologicaly diagnosed.

The treatment of the pulmonary affection leads to regression of the bony lesions. In case of inoperable thoracic neoplasia, intrathoracic vagothomy on the side of the lesion also results in regression of bone changes.

Materials and methods

Investigations took place between October 2010 and May 2012 in the Radiology Laboratory of the Veterinary Science Faculty in Iaşi, where 3 cases of HPO were diagnosed.

The first case - common breed dog, male, 13 years, with amputation following a rear member osteosarcoma, presented clinical acropodiale swelling in all 3 states, lameness, plod, decreased appetite and overall ill. Following radiological examination of the limbs, HPO specific periosteal proliferation was found.

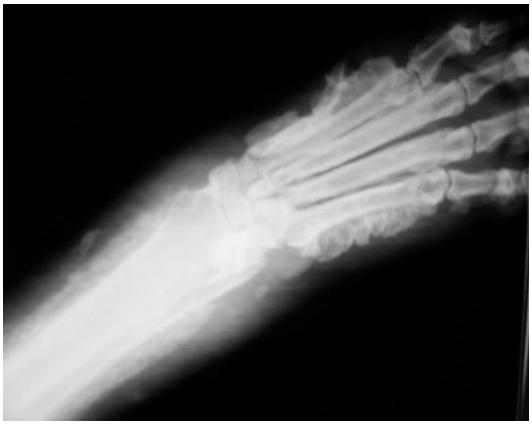


Fig. 1. Periosteal proliferation perpendicular to the bone - metacarpals 2 and 5. Proliferation continues at the radius and ulna - HPO

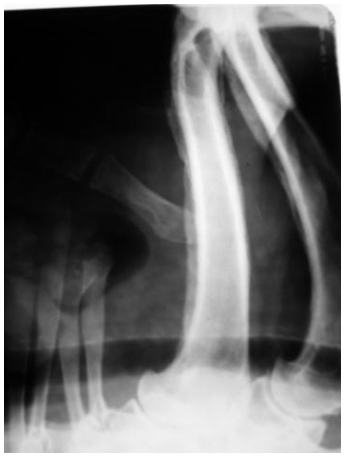


Fig. 2. Periosteal proliferation looking smoother though irregular in the long bones - humerus (HPO)

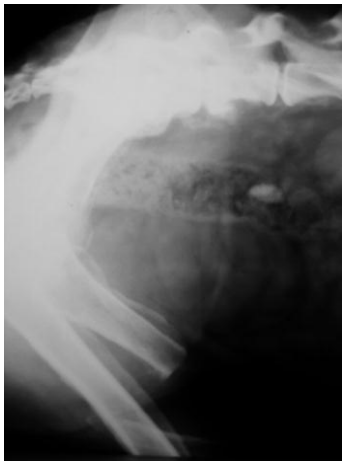


Fig. 3. Periosteal proliferation in the long bones - femur (HPO)

Radiographs of the thorax and abdomen revealed multiple radioopaque, round areas, spreaded in the entire lung, some well differentiated and some with a difuse, fluffy aspect.

HPO was diagnosed, associated to a pulmonary cancer. The owner decided to euthanise the dog and agreed to a necropsic examination.

On the necropsic examination, multiple nodular areas were found, with grease consistance. Pulmonary carcinoma was the diagnose.

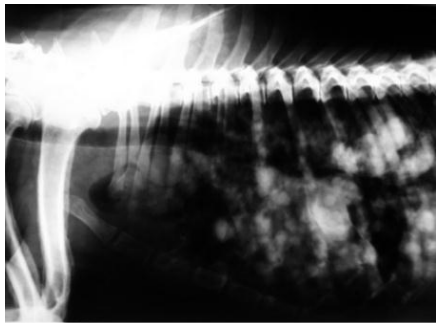


Fig. 4 Multiple areas of radiopacity, disseminated throughout the lung tissue, multiple lung tumors. Lateral view

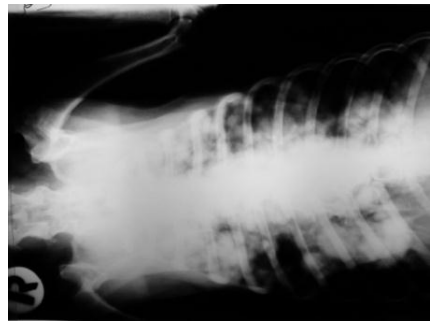


Fig. 5 Multiple areas of radiopacity, disseminated throughout the lung tissue, multiple lung tumors. Ventrodorsal view

The second case - cocker, female, 9 years - was brought in clinical accusing swellings all 4 states, with increasing local temperature, pain, lameness and partial loss of appetite. We proceeded to the limbs and chest radiography. The pacient was diagnosed with HPO.

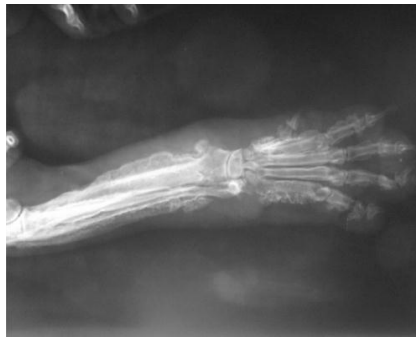


Fig. 6 Periosteal stockade proliferations on the metacarpals 2 and 5. - HPO

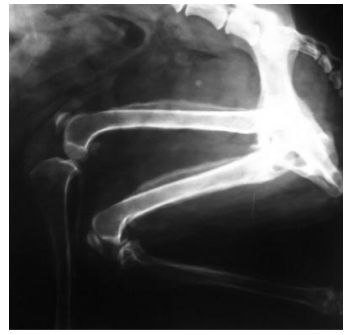


Fig. 7 - Periosteal proliferation looking smoother though irregular in the long bones - humerus (HPO)

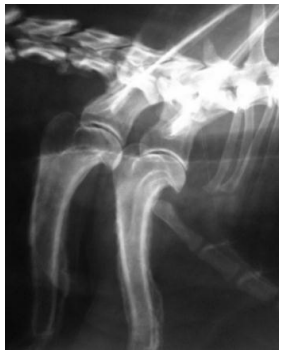


Fig. 8 Periosteal proliferation looking smoother though irregular in the long bones - femur (HPO)

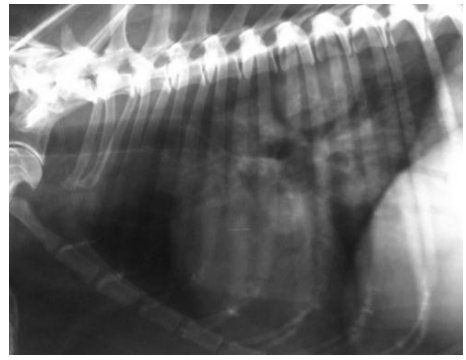


Fig. 9 Circumscribed areas of increased radiopacity - lung formations, possibly tumor

The third case was a common breed dog, female, 12 years old, with a large, ulcerated breast tumor, who came to the radiological examination to verify the integrity of the lung, in the event of tumor ablation operations. Chest radiographs in lateral and ventrodorsal incidence showed the presence of a large formation in the lung, near the heart. The patient was monitored for two months and a half, during which other diagnostic methods were applied, (CT, ultrasound) and an antitumor therapy was given, subsequently moved to a palliative and supportive care.

During the last weeks of life, the clinical signs of HPO began (lameness, swollen legs and increased local temperature). At the last radiological examination, the loss in consistance of the lung tumor was seen, as well as the incipient periostal reaction, perpendicular on the long bones of the metacarpal and metatarsal and also on the distal end of the radius and tibia.

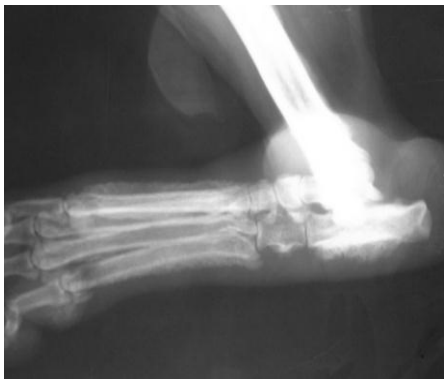


Fig. 10 Early periosteal stockade proliferations on the metatarsals 2 and 5 and the calcaneus - HPO

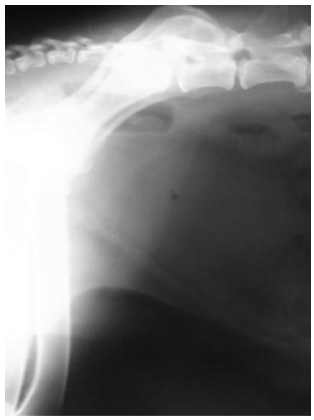
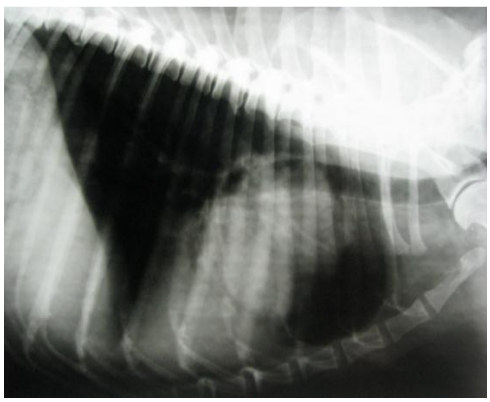


Fig. 11 Periosteal proliferation missing in the long bones - femur. Incipent HPO



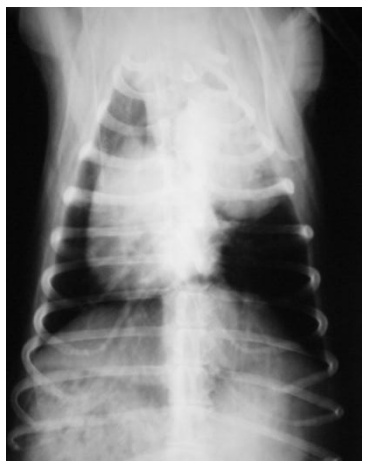


Fig. 12 Well defined, large area of increased radiopacity - pulmonary formation, possibly tumor (A – lateral view, B – ventrodorsal view)

After the death of the pacient, at the necropsy, besides the large lung tumor, small dimensions methastases were found on the lung, liver and urinary bladder.

Results and discussions

This study shows the fact that despite the large number of cases of lung disease - particularly tumors, the number of cases of HPO was only 3 within one year and eight months, since this is a very rare disease, a complication of the primary lung disease.

Two of the studied cases were females, one was a male. Although the number is very small for statistical study, it corresponds to the descriptions in the literature. However, the females are most commonly affected by this disease, because they are more susceptible to developping lung tumors, due to the high frequency in brest tumors.

In one case a small tumor of the bladder was found. Unfortunately the causality between that aspect and HPO couldn't be established, since the tumor was in early stage and it was considered as methastases from the lung tumor.

We could not find a correlation between the stage of the lung disease and the development of HPO, the first case presented numerous lung tumors, scattered throughout the lung field, compared with the second case, where there was a small number of lung formations, of lower consistency. However, periosteal deposits were most important in the second case. The third case had the greatest pulmonary tumor, however it began to develop HPO shortly before death, therefore the periostal deposits were not so well represented. Location of lung formations was also different.

Conclusions

1. HPO is a rare disease, only a total of 3 cases were diagnosed within about two years.
2. Of the 3 cases, 2 were females and one was a male, this might translate into a percentage of 66% females and 33% males, although the small number of cases prevents any statistics.
3. One of the three cases was diagnosed with bladder tumor, although this may be considered more a consequence of the lung tumor rather than a cause of HPO.
4. The disease was found in animals with lung formations of different sizes and location, and there cannot be established a correlation between cancer stage and HPO. The location of the tumor probably plays the most important role.

References

1. C. Guillermo Couto – Oncology. Manual of Small Animal Internal Medicine (2005, second edition), Ed. Mosby, Inc, SUA.
2. Eleanor. C. Hawkins – Respiratory Sistem Disorders. Manual of Small Animal Internal Medicine (2005, second edition), Ed. Mosby, Inc, SUA.
3. Kevin J. Kealy – Diagnostic rdiology of the dog and cat, (1987) Ed. W. B. Saunders Company, Philadelphia, SUA.
4. R. Dennis, R. H. Kirberger, R. H. Wrigley, F. J. Barr – Handbook of Small Animal Radiological Differential Diangnosis (2001), Harcourt Publishers.

IDENTIFICATION OF SIBLING SPECIES OF THE ANOPHELES MACULIPENNIS COMPLEX (DIPTERA: CULICIDAE) BY A POLYMERASE CHAIN REACTION ASSAY

Larisa PARASCA[1], Tatiana SULESCO[2], Liviu MIRON[1], Lidia TODERAS
[1] Faculty of Veterinary Medicine Iasi
[2]Institute of Zoology, Academy of Sciences of Moldova
lparasca@uaiasi.com; lmiron@uaiasi.com

Abstract
Until the eradication of malaria from Europe, members of the Anopheles maculipennis complex had been the major vectors for plasmodial parasites. With the possible reintroduction of malaria due to climate change and increased tourism to and from countries where malaria is endemic,is necessary to indentify the species of mosquitoes belonging to the species considered potential vectors. Interest in European Anopheles mosquitoes has revived and modern molecular biology techniques are now available for their investigation. The most identification is by PCR. The second internal transcribed spacer (ITS2) of the ribosomal DNA was amplified and sequenced for each species. Based on literature references, the nucleotides sequences were ordering primers specific for each species in the complex and made PCR mix.
Keywords: Anopheles maculipennis complex, malaria

Introduction

Malaria is a mosquito-borne infectious disease of humans and other animals caused by eukaryotic protists of the genus Plasmodium. The disease is widespread in tropical and subtropical regions in a broad band around the equator, including much of Sub-Saharan Africa, Asia and Americas. Malaria is prevalent in tropical regions because of the significant amounts of rainfall, consistently high temperatures and high humidity, along with stagnant waters in which mosquito larvae readily mature, provide them with the environment they need for continuous breeding.

The WHO has estimated that malaria annually causes over 200 million cases of fever, in 2010, around 655.000 people died from the disease, most of whom were children under the age of five (World Malaria Report, 2011). The definitive hosts for malaria parasites are female mosquitoes of the Anopheles genus, which act as transmission vectors to humans and other vertebrates, the secondary hosts. (Talman A. 2004). The presence of malaria in an area requires a combination of high human population density, high mosquito population density and high rates of transmission from humans to mosquitoes and from mosquitoes to humans. If any of these is lowered sufficiently the parasite will eventually disappear from that area as happened in North America, Europe and much of the Middle East. (World Health Organization, 1958). Many countries are seeing an increasing number of imported malaria cases owing to extensive travel and migration. Members of the *Anopheles maculipennis* complex together with *An. superpictus* (Diptera: *Culicidae*) were principally responsible for the transmission of the malaria. These mosquioto vectors are still widely distribution.

Anopheles maculipennis the historic vector of malaria in Europe and the Middle East was the first sibling species complex to be discovered among mosquitoes. The *Maculipennis* complex formally comprised 12 Palearctic members including *An. atroparvus, An.*

beklemishevi, An. labranchiae, An. maculipennis, An. martinus, An. melanoon, An. messeae, An. sacharovi, An. perisiensis, An. daciae, An. lewisi and An. artemievi. *An. sacharovi* is the main vector in Turkey and is, together with *An. superpictus* and *An. pulcherrimus*, the most important vector, of malaria in the former Soviet Union, although *An. messeae* has been implicated in the resurgence of malaria in Russia and the Ukraine.

The first attempt to map the distribution of Romanian mosquitoes included the malaria vectors of the *Anopheles maculipennis* complex recorded at the time in 284 localities in the endemic area (Martini and Zotta 1934, Zotta et al. 1940).

Seven species of *Anopheles* have been recorded from Romania. The four species of the *An. maculipennis* complex recorded in Romania, namely *An. maculipennis, An. messeae, An. sacharovi* and *An. atroparvus* have been identified in over seven hundred and thirty localities of the malaria endemic area (Ecological zones: Oriental Carpathians, South Carpathians, Occidental Carpathians, Banat Mountains, Oriental Subcarpathians, South Subcarpathians, Subcarpathians of Transylvania, Western Transylvania, North-western Romanian Plain, South-eastern Romanian Plain, The Danube Delta, Moldavian Tableland). Characteristically for species complexes, the *A. maculipennis* sibling species are very closely related and al-most indistinguishable by morphological characters.The identification of the species of *Anopheles* was performed on eggs obtained from females collected in animal shelters. Modern molecular biology techniques are now available for their investigation. The internal transcribed spacer(ITS) region is widely used in taxonomy and molecular phylogenetics. The internal transcribed spacer (ITS) region located between the 5.8S and 2.8S gene is highly conserved and species specific. The second internal transcribed spacer (ITS2) of the ribosomal DNA was amplified and sequenced for each species (Cornel et al, 1996).

Materials and methods

Mosquitoes

The *Anopheles maculipennis* complex specimens used for this study originated from Danube Delta, branches Chilia, Sulina and Sf. Gheorghe. Material was collected from June in 2010. The field-collected specimens were pre-identified as belonging to the *Anopheles maculipennis* group using the determination key by Norbert Becker, 2003. Adults of both sexes were examined. Mosquitoes were captured with a trap equipped with ultra-violet light source, fan, nylon and a container with soapy water. Male mosquitoes are not attracted by CO_2 traps, but can respond to light traps, they are best collected in the field when they are swarming.

Mosquitoes, attracted by the light, enter through nylon and are sucked downward by airflow created by the fan and collected in a container with soapy water placed at the lower end of the nylon. A total of 125 specimens of adults of both sexes were examined by PCR assay, preliminary stored in 70% ethanol until DNA extraction. The trap was set up in the late afternoon at 08PM and removed the next morning at 05AM.

Sequencing and selection of primers

The whole individuals were used for DNA extraction by Fermentas DNA purification kit. The ITS2 region of the rDNA was entirely amplified by PCR using primers described by Jana Proft (1999).The sequence of the forward primer was complementary to a conserved region of the 5.8S r DNA (5'-TGTGAACTGCAGGACACATG-3') whereas the reverse

primer annealed to a conserved 28S rDNA region (5'-ATGCTTAAATTTAGGGGGTA-3') (Jana Proft, 1999).

PCR was carried out in a 50µl volume containing 10mM 10xTrueStart Taq Buffer, 4mM $MgCl_2$, 0.2mM dNTP mix, 0.6 mM Forward primer, 0.3 mM of reverse primer and 1.5 units of True Start Taq DNA polymerase and distilled water to give a total volume of 50µl, using the Applied Biosystems 9700. Amplification was performed in a programmable thermal cycler for 35 cycles with DNA denaturation at 94°C for 1 min, primer annealing at 53°C for 30 sec, and primer extension at 72°C for 1 min. For checking PCR results, 10µl of product in addition to 2.5µl of loading buffer (bromphenol blue Sigma) was loaded onto a 2% agarose gel. The gel was stained with ethidium bromide and run at 80 V for 1.5h. DNA bands were visualized by illumination with short-wave ultraviolet light.

Table 1. Characters of universal and species-primers described by Jana Proft

Species (primer code)	**Primer nucleotide sequence (5' - 3')**	**m(°C)**	**Length of specific PCR product (bp)**
Complex (universal) (5.8 –UN)	TGTGAACTGCAGGACACATG	7	±
An. maculipennis s.s. (AMA)	TATTTGAGGCCCATGGGCTA	6	410
An. atroparvus (AAT)	CGTTTGGCTTGGGTTATGA	4	117
An. messeae (AMS)	GACGCCTCACGATGACCTT	8	305
An. melanoon (AML)	TGCAAGTTGAAACCTGGGGC	9	224
An. labranchiae (ALA)	GTATCTCTGCTGCTATGGTC	6	374
An. sacharovi (ASA)	CAAGAGATGGATGTTTTACG	3	180

Results and discussion

Primers used are listed in Table 1. Due to the selected primer annealing sites, every ITS2 sequence is flanked by a 5.8S rDNA region of 91 bp and a 28S rDNA region of 39 bp. Using 1±2% of the total extracted DNA from a single mosquito, PCR products of species-specific length were produced after empirical optimization of the reaction conditions.

DNA used was extracted from whole mosquito. Jana Proft (1999) protocol used was modified, obtaining results with mix:10mM 10xTrueStart Taq Buffer; 4mM $MgCl_2$; 0.2mM dNTP mix; 0.6 mM Forward primer; 0.3 mM of reverse primer and 1.5 units of True Start Taq DNA polymerase and distilled water to a total volume of 50µl.

Optimal PCR conditions for concentrations of reaction compounds and amplification thermoprofile were determined empirically following basic protocols. To set the ideal primer annealing temperature, a mean value of the melting temperatures of the different primers was

calculated and then optimized empirically.

Sequencing of ITS2 rDNA from six sibling species of the *An. maculipennis* complex was carried out to identify interspecific variation that could be utilized for a species-diagnostic PCR assay.

Length of specific PCR product was the same for all captured mosquitoes. DNA bands were visualized by illumination with short-wave ultraviolet light and all had length of 305 bp, corresponding to *An. messeae* Fall.

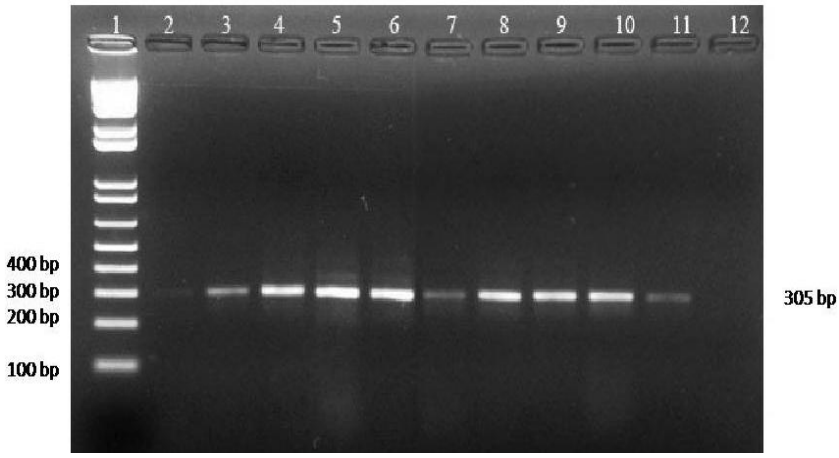


Fig.1. Specific PCR product for *Anopheles messeae* species

1 — O'Range Ruler 100bp DNA marker; 5, 6, 7 — ♀ *An. messeae* from Danube Delta;
8, 9 — ♀ *An. messeae* from Sulina;10,11 — ♀ *An. messeae* from Sf. Gheorghe;
12 – negative control.

Conclusions

Mosquitoes captured in Danube Delta and pre-identified as *An. maculipennis* sensu lato using the determination key of Norbert Becker, 2003, were identified as *An. messeae* according to PCR assay.

This species is considered vector in malaria transmission, feeding mainly on cattle and domestic animals and sometimes on people.

With climate change and increased travel to and from countries where malaria is endemic, is possible reintroduction of malaria in Romania. A very favorable climate with high temperatures and frequents rainfall, may favor increase of this species density and can become a real danger in malaria transmission. Accurate identification of mosquito species will be essential for the attempt to preventer introduction of malaria in Romania.

References

1. Ahmed S.I. Aly, Ashley M. Vaughan, 2009- Malaria Parasite Development in the Mosquito and Infection of the Mammalian Host, Seattle Biomedical Research Institute.
2. Ashraf M.Ahmed, Hilary Hurd, 2006-Immune stimulation and malaria infection impose reproductive costs in Anopheles gambiae via follicular apoptosis, Microbes and Infection 8 308-315.
3. Daniel Voytas 2000-Preparation and Analysis of DNA-Resolution and Recovery of Large DNA Fragments, Agarose Gel Electrophoresis, Current Protocols in Molecular Biology, 2.5A. 1-2.5A.9.
4. Jana Proft,Walter A. Maier,Helge Kampen,1999-Identification of six sibling species of the Anopheles maculipennis complex (Diptera:Culicidae) by polymerase chain reaction assay, Parasitol Res 85:837-843.
5. John Wiley,2009 - The Polymerase Chain Reaction, Current Protocols in Molecular Biology,15.0.1-15.0.3.
6. Kenneth D. Bloch,Barbara Grossmann 1995: Restriction Endonucleases.Digestion of DNA with Restriction Endonucleases. Enzymatic Manipulation of DNA and RNA 3.1.1-31.21. Supplement 31.
7. Norbert Becker, 2003-Mosquitoes and their control, Kluwer Academic/Plenum Publishers.
8. Martha F. Kramer , Donald M.Coen 2001: The Polymerase Chain Reaction. Enzymatic Amplification of DNA by PCR:Standard Procedures and Optimization 15.1.1-15.1.14.Supplement 56.
9. Michael Finney,Paul E., Nisson Ayoub Rashtchian,2001-Molecular Cloning of PCR Products, Current Protocols in Molecular Biology, 15.4.1-15.4.11.
10. Rhonda Feinbaum 1998: Vector Derived from Plasmids.Introduction to Plasmid Introduction to Plasmid Biology.Current Protocols in Molecular Biology, 15.4.1-15.4.11.
11. Silvieo, Mota Mm, Matuschewski K, Prudencio M, 2008: Interactions of the malaria parasite and its mammalian host. Current opinion in microbiology 11(4):352-359.
12. Talman A., Domarle O., Mc Kenzie F., Ariey F.,Robert V., 2004-Gametocy to genesis the puberty of Plasmodium falciparum" Malaria Journal.
13. World Health Organization(1958): "Malaria". The First Ten Years of the World Health Organization. World Health Organization pp.172-87.
14. World Malaria Report 2011 summary World Health Organization.

THE OCCURRENCE OF ESBL IN *E. COLI* STRAINS ISOLATED FROM LIVESTOCK DUE TO INCORRECT TREATMENTS WITH B-LACTAM ANTIBIOTICS

Ramona MORARU, V. NASTASA, Mariana GRECU, G. SAVUTA, M. MARES
USAMV Ion Ionescu de la Brad, Facultatea de Medicină Veterinară Iași
rmoraru82@gmail.com

Abstract
Antimicrobials represent an important category of active substances used for prophylaxis and treatment of clinical diseases in order to keep healthy status and high productivity of the animal. Despite these facts, their increased and wide usage has been considered the trigger factor of the emergence, selection and occurrence of antibiotic-resistant microorganisms in both veterinary and human medicine. The double discs diffusion method, described by the CLSI M100 S-18 standard, identified the presence of ESBLs in some of the E. coli strains. From the E. coli strains (n=53) isolated in swine, only 2 (3.77%), were resistant thus confirming the presence of ESBLs. Within the E. coli strains (n=28) isolated from poultry, was identified a resistant strain belonging to poultry from extensive system indicating the presence of ESBLs. Four strains (23.52%) of E. coli (n=17) isolated in cattle from the intensive farming system were resistant indicating the presence of carbapenemases from A class. Two E. coli strains isolated in cattle from intensive farming system were resistant to all of the tested cephalosporins emphasizing the presence of ESBLs, CTX-M type. Two strains (12.52%) of E. coli (n=16) isolated in cattle from extensive farming system were resistant, confirming the presence of ESBLs, AmpC type. One E. coli strain (6.25%) isolated in cattle from the extensive farming systems was resistant to all cephalosporins, indicating the presence of CTX-M type ESBLs. The results obtained in CLSI disk diffusion method of E. coli strains, indicate the fact that antibiotic resistance represents an emergence linked to the clinical use of antibiotics, against which the resistance is directed.

Key words: antimicrobials, ESBL, E.coli, livestock

Increasing use of antibiotics is a worldwide trend and more efforts are made to control the emergence of the bacterial resistance. The susceptibility determinations using disk diffusion method and MIC are the most important factors that must be taken into consideration in the choice of therapeutic protocol. Must be specified, though, that the list of bacterial specie, that are included in the official action spectrum of the antimicrobial agent, are generally limited to those species involved in the pathology selected by the recognized therapies. Action spectrum brings information for a better understanding of a certain drug.

Antimicrobial action spectrum categorizes bacterial species in three classes, related to their behavior towards antibiotics (susceptible, intermediary, and resistant). Thus, the action spectrum is related to natural resistance and the prevalence of acquired resistance, which changes in time and space, in various ways for each country, but also differently inside a country by regions, cities, hospitals, age or pathology of the patient. Action spectrum gives information of both potency of a drug and its limitations.

It is the poultry and pigs livestock that receive the majority of antibiotics used for therapy in veterinary medicine field (Martinez-Carballo et al, 2007). The consumption of antibiotics in livestock farms and agriculture implies dramatic medical consequences and environmental hazards, as both agriculture and veterinary use determine resistance reservoirs, persistent residues of antibiotics and thus selective pressure for bacteria, opening new transmission pathways (Ghosh and LaPara, 2007; Kemper, 2008; Martinez, 2009).

Material and Method

The researches were conducted by analyzing the strains isolated from 144 samples (n=144) that were taken from livestock from both intensive and extensive farming system. From intensive farming system, samples were harvested from swine (n=60), cattle (n=18), and poultry (n=10); from extensive farming system, samples were harvested from cattle (n=32), poultry (n=20) and pigeons (n=4).

The bacterial strains (*E. coli)* have been purified, identified and preserved at -80º C for further testing.

For the identification of the ESBLs in *E. coli* strains, the double disk diffusion method from CLSI M100 S-18 standard was used as it was described by Babic et al., (2006) and Sundin (2009).

The samples from livestock collected with sterile swabs and cultivated on Lauryl sulphate broth at 44.5°C 24 h for *E. coli*. After overnight incubation period, the samples were passed on selective agar, specific to the *Enterobacteriaceae* (Levine and TBX for *E. coli)* and incubated again at 36±2°C for 24 hours.

After obtaining the pure strains, these were identified to species level by using conventional methods. The *E. coli* strains can be distinguished from other coliforms by its growth and color reaction on certain types of culture media. Thus, when cultured on Levine agar plate, a positive result for *E. coli* is metallic green colonies on a dark purple media or blue color on TBX agar.

Results and Discussions

From the total number of samples (n=144), 118 *E. coli* strains (81.94%) have been isolated.

For the *E.coli* strains, by using the CLSI M100 S-18 disk diffusion method, there were identified multidrug resistant (MDR) strains, 30 strains in swine, 5 in cattle from the extensive farming system and 9 in cattle from intensive farming system, 6 in poultry from the intensive farming system and 10 in poultry from the extensive farming system.

It should be underlined the fact that the resistance in the strains of *E. coli* is generally encountered to the antibiotics frequently used in therapy.

The bacterial strains isolated in pigeons, as negative control-sample, had proved to be susceptible to all of the tested antibiotics, thus confirming the theory of selective pressure of antibiotic treatments on the occurrence of resistance determinants.

Once ceftazidime and cefotaxime was introduced in therapy in 1983, the *E. coli* and *Klebsiella sp.* developed resistance to them, by "new beta-lactamases", the ESBLs (Extended Spectrum Beta Lactamases) (Babic et al., 2006). These are the first example of resistance to a new class of antibiotics resulted from mutation of a single amino acid. The penicillinases that are normally found in *E. coli* strains (TEM-1 and SHV-1, respectively) do not give resistance to ceftazidime or cefotaxime (Babic et al., 2006). Bacteria that have β-lactamases *AmpC* type are resistant to penicillins, β-lactamases inhibitors, cefoxitin, cefotetan, ceftazidime, ceftriaxone and cefotaxime (Jacoby and Munoz-Price, 2005; Babic et al., 2006; Sundin, 2009).

The double discs diffusion method, described by the CLSI M100 S-18 standard was used to identify the presence of ESBLs in some of the *E. coli* strains. Interpretation of results using double discs method for identification of ESBL, was made as described in table 1.:

Table 1. Interpretation of the results of the disk diffusion method used for ESBL identification (Babic et al., 2006)

β-lactamase	Susceptibility substrate				Inhibitor	
	Ceftazidime	Cefotaxime	Cefoxitin	Imipenem	Clavulanic Acid	EDTA
ESBL	R	R	S	S	yes	no
AmpC	R	R	S, R	S	no	no
Carbapenemases						
Class A	R	R	S	R	yes, no	no
Class B	R	R	S, R	R	no	no
Class D	R	R	S, R	R	no	no

From the *E. coli* strains (n=53) isolated in swine, only 2 (3.77%) were resistant to aztreonam, cefpodoxim, cefotaxim, ceftazidim and all the strains being susceptible to amoxicillin + clavulanic acid (fig. 1.). By correlating these data with the fact that these strains had been also susceptible to imipenem and resistant to cefoxitin, the presence of ESBLs is confirmed in the two strains.

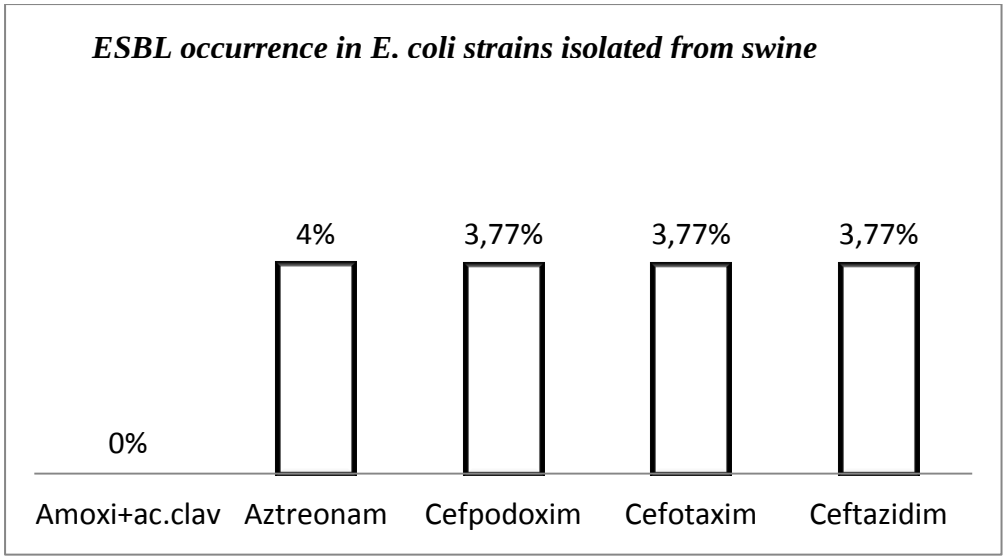


Fig. 1. Resistance occurrence for *E. coli* strains, double discs method for ESBL identification from swine samples

Within the *E. coli* strains (n=28) isolated from poultry, was identified a strain belonging to chickens from extensive system, resistant to aztreonam, cefpodoxim and to ceftazidim, while being susceptible to amoxicillin + clavulanic acid, imipenem, cefotaxim and cefoxitin, indicating the presence of ESBLs (fig. 2.).

Others studies identified decreased susceptibility to newer compounds (cefepime, cefotaxime) for *E. coli* and *Salmonella* strains from chickens (de Jong et al., 2009). There was no *E. coli* strain resistant to amoxicillin + clavulanic acid, aztreonam cefpodoxime, ceftazidime, cefoxitin or imipenem, in the strains isolated from farm chickens (n=10) (fig. 2.). Six strains were resistant to ampicillin and ticarcillin, indicating the presence of penicillinases. For the strains isolated from farm chickens, there was identified by CLSI disk diffusion method and confirmed by EUCAST method for determination of MIC, a high resistance level to fluoroquinolones. Though, resistance or susceptibility to β-lactamic antibiotics is not interfering with the activity of fluoroquinolones (Appelbaum and Hunter, 2000; Srinivasan et al., 2007; Arisoy et al., 2008), it is considered that in those farms were used only antibiotics from this class, avoiding the selective pressure for resistance mechanism to β-lactams.

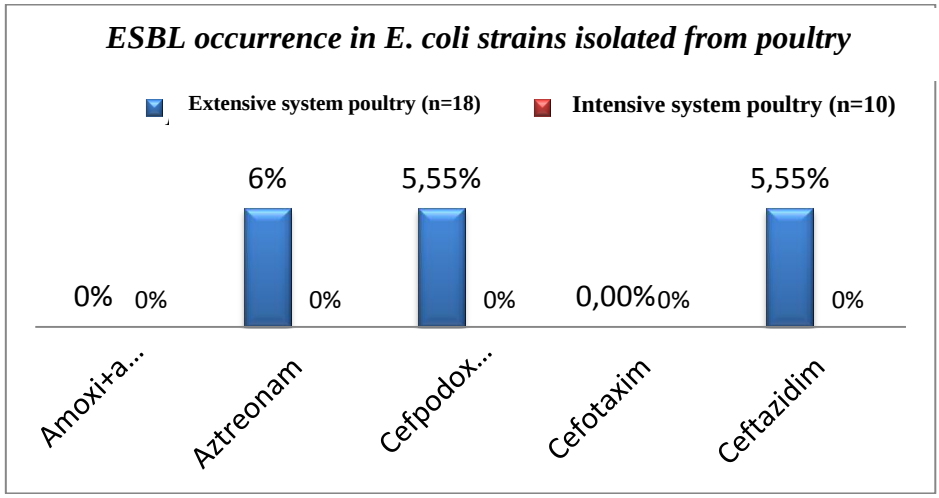


Fig. 2. Resistance occurrence for *E. coli* strains, double discs method for ESBL identification from chicken samples

Four strains (23.52%) of *E. coli* (n=17) isolated in cattle from the intensive farming system were resistant to aztreonam, cefpodoxim, cefotaxim, ceftazidim, cefoxitin and imipenem, while being susceptible to amoxicillin + clavulanic acid (fig.3.). The resistance to these antibiotics and the fact that the strains had proved sensible to β-lactamase inhibitors indicate the presence of class A carbapenemases (Jacoby and Munoz-Price, 2005; Babic et al., 2006; Sundin, 2009; Veldman et al., 2011).

Two *E. coli* strains isolated in cattle from intensive farming system were resistant to all of the tested cephalosporins, including cefoxitin, but proved susceptible to imipenem and β-lactamase inhibitor, amoxicillin + clavulanic acid, emphasizing the presence of ESBLs, CTX-M type.

In another study, the *Enterobacteriaceae* with CTX-M resistance enzyme, or another ESBL type, were highly resistant to ciprofloxacin (Denton et al., 2007), similarly with the

results obtained in this study. The explanation is given by the fact that the plasmid which codes CTX-M-15 β-lactamase determines also *aac(6`)-Ib-cr*, which acetylates ciprofloxacin and other FQ, leading to resistance (Denton, 2007).

Two strains (12.52%) of *E. coli* (n=16) isolated in cattle from extensive farming system were resistant to aztreonam, cefpodoxim, cefotaxim, ceftazidim, cefoxitin, amoxicillin + clavulanic acid and susceptible to imipenem, thus confirming the presence of ESBLs, *AmpC* type.

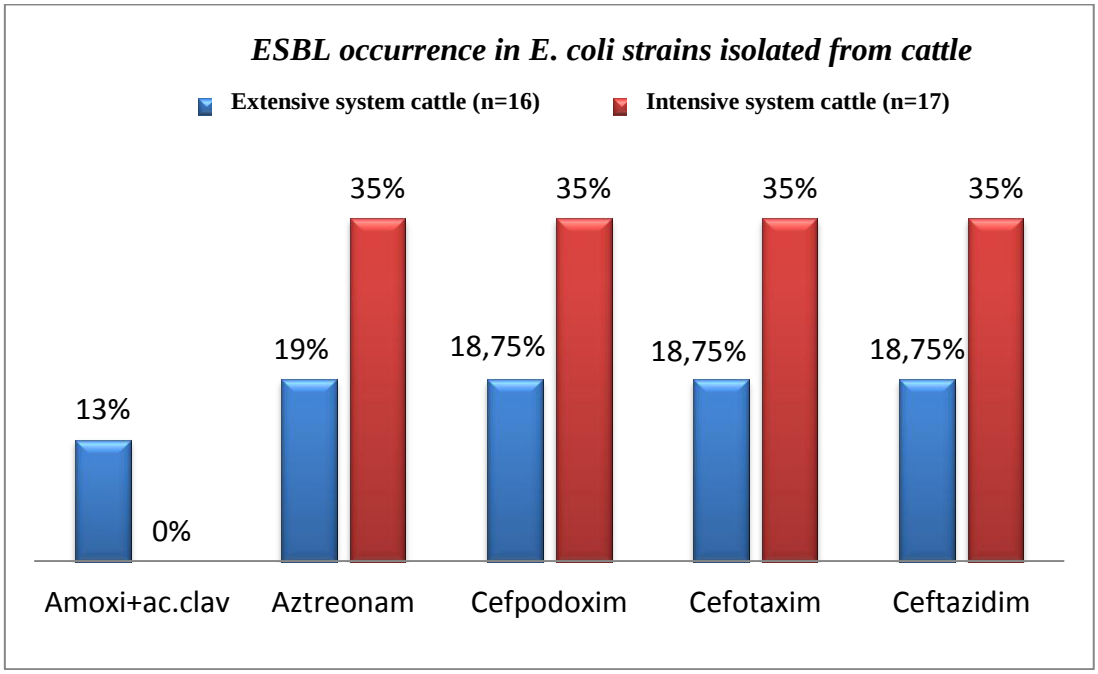


Fig. 3. Resistance occurrence for *E. coli* strains, double discs method for ESBL identification from cattle samples

One *E. coli* strain (6.25%) isolated in cattle from the extensive farming systems was resistant to all cephalosporins, including cefoxitin, but proved susceptible to imipenem and to β-lactamase inhibitor amoxicillin + clavulanic acid, indicating the presence of CTX-M type ESBLs (fig.3.).

Brănescu and Ştirbu (2007) emphasize the fact that some *E. coli* and *Salmonella sp*. Strains which may contaminate the carcasses during slaughtering process are surviving in foods of animal origin (cattle and chicken meat) after thermal treatment.

Conclusions

1. Present study underlined the presence of major antibiotic resistance markers from the analysed samples, to a decreased but significant level for human health.
2. ***E. coli*** strains producing **Class A carbapenemases** were identified in cattle from farms (4/17).
3. ***E. coli*** strains producing **CTX-M type ESBLs** were identified in cattle from farm (2/17) and cattle from extensive farming system (1/16).
4. ***E. coli*** strains producing ***AmpC* type ESBLs** were identified in cattle from extensive farming system (2/16).
5. ***E. coli*** strains producing **ESBLs** were identified in swine (2/53), in poultry from extensive farming system (1/18).
6. ***E. coli*** strains producing **narrow spectrum β-lactamases** were identified in swine (34/53), in cattle from intensive farming system (7/17), in cattle from extensive farming system (5/16), in poultry from intensive farming system (6/10) and extensive farming system (15/18).

Repherences

1. Appelbaum P. C., Hunter P. A., 2000 - *The fluoroquinolone antibacterials: past, present and future perspectives.* International Journal of Antimicrobial Agents, vol. 16, p. 5-15;
2. Arisoy M., A. Y. Rad, A. Akin, N. Akar, 2008 – *Relationship between susceptibility to antimicrobials and virulence factors in paediatric Escherichia coli isolates*. International Journal of Antimicrobial Agents 31S, p. S4-S8;
3. Babic M., Hujer Andrea M., Bonomo R. A., 2006 – *What's new in antibiotic resistance? Focus on beta-lactamases.* Drug Resistance Updates 9, p. 142 – 156.
4. Brănescu C, C. Ştirbu, 2007 - *Curbele de creştere ale bacteriilor din genurile Escherichia şi Staphylococcus în medii cu concentraţii diferite de clorură de sodiu The growth patterns of Escherichia and Staphylococcus bacterial genre in varied concentrations of sodium chloride cultures*. Revista Română de Medicină Veterinară vol. 18, Nr.1;
5. Clinical Laboratory Standard Institute M100 – S18, Performance Standards for Antimicrobial Susceptibility, 2008 - Testing Eighteenth informational Supplement (CLSI) M100 S18.vol 28.
6. Denton M., 2007 – *Enterobacteriaceae*. International Journal of Antimicrobial Agents 29, supl. 3, p. S9-S22;
7. Ghosh S., LaPara T. M., 2007 - *The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria*. Isme J. 1:191-203.
8. Jacoby G. A., Munoz- Price L. S., 2005 - *The new beta- lactamases.* The New England Journal of Medicine 352, p. 380-391;
9. de Jong A., Bywater R., Butty P., Deroover E., Godinho K., Klein U., M. Hervé, Simjee S., Smets K., T. Valérie, Vallé M., Wheadon A., 2009 – *A pan-European survey of antimicrobial susceptibility towards human-use antimicrobial drugs among zoonotic and commensal enteric bacteria isolated from healthy food-producing animals.* Journal of Antimicrobial Chemotherapy, 63, p. 733-744;
10. Srinivasan, Velusamy, Barbara E. Gillespie, Lien T. Nguyen, Susan I. Headrick, Shelton E. Murinda, Stephen P. Oliver, 2007 – *Characterization of antimicrobial resistance patterns and class 1 integrons in Escherichia coli O26 isolated from humans and animals*. International Journal of Antimicrobial Agents 29, p. 254-262;
11. Sundin Daniel R., 2009 – *Hidden beta-lactamases in the Enterobacteriaceae – Dropping the extra disks for detection, Part I.* Clinical Microbiology Newsletter, vol. 31, no.6, p. 41 - 44.
12. Sundin Daniel R., 2009 – *Hidden beta-lactamases in the Enterobacteriaceae – Dropping the extra disks for detection, Part II.* Clinical Microbiology Newsletter, vol. 31, no.7, p. 47 - 52.

13. Veldman Kees, Lina M. Cavaco, Dik Mevius, Antonio Battisti, Alessia Franco, Nadine Botteldoorn, Mireille Bruneau, Agne`s Perrin-Guyomard, Tomas Cerny, Cristina De Frutos Escobar, Beatriz Guerra, Andreas Schroeter, Montserrat Gutierrez, Katie Hopkins, Anna-Liisa Myllyniemi, Marianne Sunde, Dariusz Wasyl, Frank M. Aarestrup, 2011 – *International collaborative study on the occurence of plasmid-mediated quinolone resistance in Salmonella enterica and Escherichia coli isolated from animals, humans, food and the environment in 13 European countries*. Journal of Antimicrobial Chemotherapy vol. 66, p. 1278-1286;
14. Martinez J. L., 2009 - *Environmental pollution by antibiotics and by antibiotic resistance determinants.* Environmental Pollution, vol. 157, p. 2893-2902 ;
15. Martínez-Carballo E., González-Barreiro C., Scharf S., Gans O., 2007 - *Environmental monitoring study of selected veterinary antibiotics in animal manure and soils in Austria.* Environmental Pollution, Volume 148, Issue 2, July 2007, Pages 570-579;
16. Kemper N., 2008 - *Veterinary antibiotics in the aquatic and terrestrial environment.* Ecological Indicators, vol. 8, p. 1-13;

SPONTANEOUS AND MITOGEN-INDUCED REACTIVITY OF LYMPHOCYTES FROM LAYING HENS IN RELATION TO *SALMONELLA* INFECTION

Grigore BIANU, Mihaela NICULAE, Carmen Dana ŞANDRU, Marina SPÎNU
University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Faculty of Veterinary Medicine, Discipline of Infectious Diseases, Cluj-Napoca, Mănăştur street no 3-5, 400372, Romania

Abstract
Salmonella enterica serovars associated pathological entities in poultry represent a world-wide major concern due to the economic impact, the zoonotic potential and the emergence of multiple antibiotic-resistant strains. Effective vaccination protocols, required to control and prevent such infections, are based on relevant data regarding immunological mechanisms induced by Salmonella. The present study was focused on the evaluation of the cellular immune response in case of asymptomatic carriers compared with those vaccinated against Salmonella Gallinarum. Two immunological techniques, leukocyte blast transformation test and carbon particle inclusion test, were used to investigate the cell mediated immune response in laying hens. Values obtained for the stimulation indices were significantly higher in the case of the carrier group ((1) = 78,05±4,263 and (2) = 76,9±3,053) compared to both control ((1) = 11,985±3,868 and (2) = 17,646±3,3177) and vaccinated groups ((1) = 17,592±3,247 and (2) = 30,864±4,9249) ($p<0,05$). As for the phagocytic activity, Salmonella antigen induced an inhibitory effect on the ability to engulf carbon particles. The statistically significant increase observed in lymphocytes reactivity for the Salmonella carrier hens when compared to live attenuated vaccine injected group may suggest that the low dose combined with sustained stimulation induced by the carrier state is more efficient than high dose and of short duration challenge (vaccination).

Keywords: cell-mediated immunity, poultry, Salmonella

The infections determined by *Salmonella enterica* serovars in poultry represent a world-wide major problem due to the economic impact, the zoonotic potential and the emergence of multiple antibiotic-resistant strains. These aspects emphasize the importance of effective vaccination protocols needed to control and prevent this infection. Furthermore, one of the most important features of Salmonella infection, particularly in case of Pullorum serovar, is the prolonged persistence in convalescent birds in the absence of clinical disease (9, 10) or the carrier state. Thus, studies on the associated immunological mechanisms are required (2, 7, 8).

The immune response of chickens against Salmonella infections has been studied mainly for the development of immuno-diagnosis methods rather than through the role of humoral and cellular immunity effectors. An integrated approach allowing a better understanding of the immunological defense mechanisms should include investigations on the functionality of protective effectors towards *Salmonella* in laying hens (1, 4, 5).

The present study was focused on the evaluation of the cellular immune system response in case of asymptomatic carriers compared with those vaccinated against *Salmonella gallinarum*.

Materials and methods

The biological material was represented by three experimental groups:

(I) control group: *Salmonella*-free laying hens (n=10), at the age of 80 weeks, vaccinated against Marek's disease, infectious bronchitis, Gumboro disease, Newcastle disease and fowl pox;

(II) vaccinated group: laying hens (n=10), at the age of 37 weeks, that received the same vaccination protocol and also a live attenuated vaccine against *Salmonella gallinarum* (9R strain commercial vaccine);

(III) carrier group: laying hens (n=10), at the age of 37 weeks, (n=10); the carrier state was established using both direct and indirect methods (microbial isolation and identification and evaluation of the specific agglutinins) as recommended by OIE manual (12).

The cell mediated immune response was evaluated based on two techniques: leukocyte blast transformation test and carbon particle inclusion test previously described by Khokhlova et al. (6). The first method estimates the *in vitro* reactivity of mononuclear cells to sensitizing substances, in this case phytohaemagglutinin M as a mitogen, quantifying cell growth by means of the glucose consumption determination. The 1:4 heparinized blood - RPMI 1640 culture medium mixture was distributed in 96-sterile-well-plates (100 µl per well), considering two variants for each individual blood sample, namely (1) untreated control culture and (2) phytohaemagglutinin M (PHA-M) (1 µl per well) stimulated culture. These cultures were incubated for 48 h at 37.5°C and 5% CO_2. Glucose concentrations were measured at the end of the incubation period, using a standard (100 mg dl^{-1}) glucose solution, by means of an orto-toluidine colorimetric test and the stimulation index (SI) was calculated as follows: SI%=[(MG–SG)/MG]×100, where SI=blast transformation index, MG=glucose concentration in the initial culture medium and SG=glucose concentration in the sample after incubation.

The second mentioned method, carbon particle inclusion test, measures the phagocytic activity; this technique consists of mixing 50 µl of heparinized blood with 1,5 µl of India ink, followed by the transfer of 15 µl of this mixture to 3 ml of saline and the incubation of the rest for 15 min at 37°C. Another 15 µl from the sample were transferred to saline, the rest of the samples being incubated for another 30 min, repeating the previous operations. All resulting tubes containing saline, blood and ink were centrifuged at 800 rpm and the supernatants were read spectrophotometrically (λ=535 nm, d=1 cm). Based on the difference between the natural logarithms of the optical densities of the phagocytosis at 0–15 min and 15–30 min divided by time (15 min), the phagocytic activity index was determined for the carrier group in two experimental variants: (1) control and (2) *Salmonella* antigen (6 µl) stimulated cells.

Statistical analyses of the results were performed using *Analyse-it* Microsoft Excel software, with the level of statistical significance set at $p<0.05$.

Results and discussions

Values obtained for the stimulation indices were significantly greater in case of carrier group ((1) = 78,05±4,263 and (2) = 76,9±3,053) compared to both control ((1) = 11,985±3,868 and (2) = 17,646±3,3177) and vaccinated groups ((1) = 17,592±3,247 and (2) = 30,864±4,9249) ($p<0,05$) (figure 1).

These results indicated an elevated reactivity of *Salmonella* carrier hens lymphocytes compared to the other two experimental groups. Also, for the last mentioned groups, superior stimulation indices were observed for the PHA-M stimulated variant (figure 1); this increase was found statistically significant for the vaccinated group ($p<0.05$). Still, a paradoxically reduced response in case of T cells challenges with PHA-M was noticed for the carrier group. Normally, PHA-M should induce an augmentation of T cell response.

As for the phagocytic activity, Salmonella antigen induced an inhibitory effect on the ability to phagocyte carbon particle as suggested by the higher values of the optic density. Analyzing the dynamics of this immune function, statistically significant reduced activity was determined after 15 minutes of incubation (figure 2).

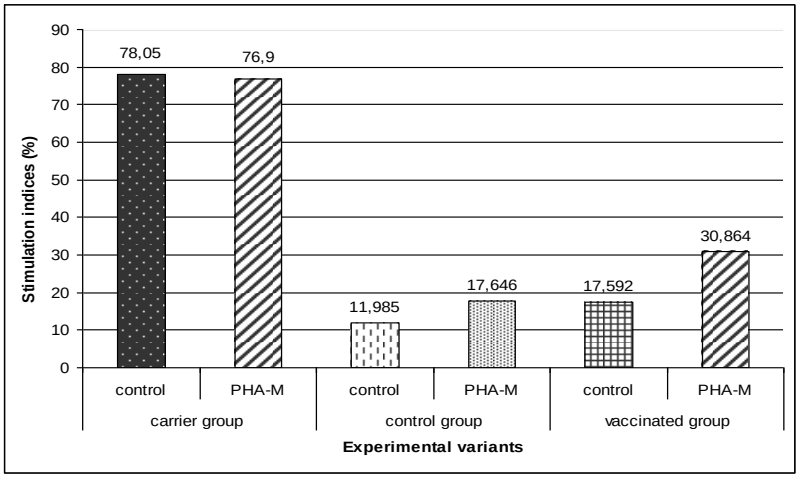


Fig.1. Spontaneous and PHA-M induced reactivity of laying hens lymphocytes

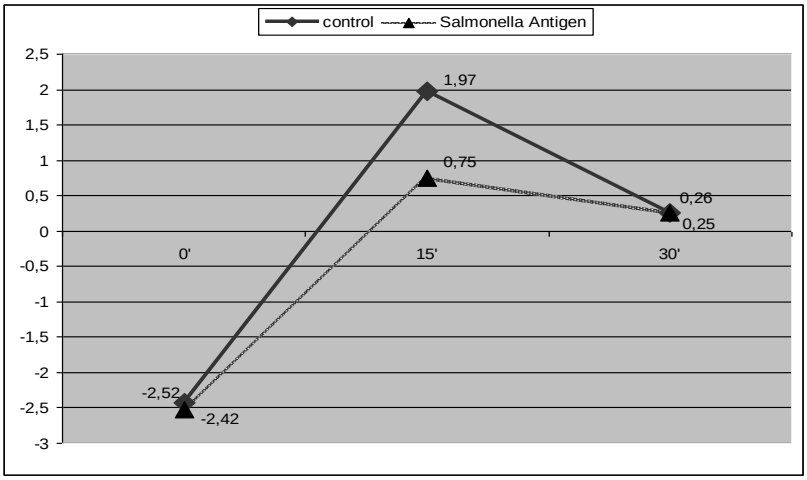


Fig.2. Dynamics of phagocytosis in case of carrier group

Results of several *in vitro* and *in vivo* studies established that *Salmonella enterica* serovar Pullorum is able to cause persistent infections in laying hens with the main site of persistence represented by the splenic macrophages (3, 5, 11).

Salmonella's presence induces a complex immune reaction that includes both cellular and humoral (2, 4) mediated responses that present fluctuations during several physiological periods and the most important seems to be the onset of egg laying. Regarding this aspect, Wigley et al. demonstrated that at the onset of laying both the T-cell response to *Salmonella* and nonspecific responses to mitogenic stimulation fall sharply in both infected and noninfected birds (11). Also, the decreased T-cell responsiveness coincided with the increase in numbers of *Salmonella* serovar Pullorum and its spread to the reproductive tract. Only after three weeks, this immune response was restored with a consecutive reduction of the bacterial number. The general conclusion of this study was that the nonspecific suppression of cellular responses occurring at the laying onset plays a major role in the ability of *Salmonella* serovar Pullorum to infect the reproductive tract and also has implications for *Salmonella* enterica serovar Enteritidis infection and vertical transmission (3, 11).

The asymptomatic carrier state associated immune mechanisms were investigated in poultry also for *Salmonella* enterica serovar Enteritidis, a pathogen with severe consequences on food safety and public health and molecular methods pointed out interferon-gamma (IFN-gamma) as the factor to consider in the development of prophylactic measures for the reduction of Salmonella carrier state (9). This observation was suggested by the significantly low expression of IFN-gamma gene recorded in infected chickens after oral inoculation of Salmonella enterica serovar Enteritidis at 1 week of age in comparison to resistant ones (9).

Using flow cytometric analysis of crop and cecal samples obtained from White Leghorn hens challenged with *Salmonella* enterica serovar Enteritidis, Holt et al. reported the impact of infection on the cellular dynamics of these lymphoid tissues (5). T cell populations suffer variations, with a persistently increased CD4/CD8 ratio in both Peyer's patches and cecal tonsils (5).

An intense cellular immune response mediated by T-cell subsets against *Salmonella* enterica serovar Enteritidis (SE) was demonstrated also in the hen ovary (1). By the use of a double immunostaining method, the ovarian immunity against Salmonella infection in laying hens was pointed out based on the significantly increased number of cells belonging to the populations of CD3+, CD4+, and CD8+ T cells in the ovarian stroma and the follicular tissues as a protective response against SE invasion within 12 h of challenge (1).

Conclusions

The statistically significant increase observed in lymphocytes reactivity, as determined using the leukocyte blast transformation test, for the *Salmonella* carrier hens compared to live attenuated vaccinated group may suggest that the low dose sustained stimulation induced by the carrier state is more efficient than high dose but short duration challenge (vaccination).

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Bibliography

17. Barua A, Yoshimura Y, Ovarian Cell-Mediated Immune Response to *Salmonella enteritidis* Infection in Laying Hens (Gallus domesticus), *Poultry Science* 2004; 83(6):997–1002;

18. Beal RK, Powers C, Barrow A., Davison FT, Smith AL, Clearance of Enteric *Salmonella* enterica Serovar Typhimurium in Chickens Is Independent of B-Cell Function, Infection And Immunity 2006; 74(2):1442–1444;
19. Chappell L, Kaiser P, Barrow P, Jones MA, Johnston C, Wigley P, The immunobiology of avian systemic salmonellosis. *Vet Immunol Immunopathol.* 2009;128(1-3):53-59;
20. Erf GF, Cell-Mediated Immunity in Poultry, *Poultry Science* 2004;83(4):580–590;
21. Holt PS, Vaughn LE, Gast RK, Flow cytometric characterization of Peyer's patch and cecal tonsil T lymphocytes in laying hens following challenge with Salmonella enterica serovar Enteritidis, *Vet Immunol Immunopathol.* 2010;133(2-4):276-281;
22. Khokhlova IS, Spinu M, Krasnov BR, Degen AA, Immune response to fleas in a wild desert rodent: effect of parasite species, parasite burden, sex of host and host parasitological experience*. J. Exp. Biol.* 2004; 207(Pt 16):2725-2733;
23. Mastroeni P, Menager N, Development of acquired immunity to Salmonella. *J.Med.Microbiol.* 2003;52:453-459;
24. Nagaraja TR, Raghu TK, Anand Kumar P, Mishra SC, Mitogenic reactivity of chicken intestinal intraepithelial lymphocytes to concanavalin A and its potentiation by muramyl dipeptide and immune lymphokynes during salmonella enteriditis infection.*Online Journal of Veterinary Research* 2001;5(1):180-199;
25. Sadeyen JR, Trotereau J, Velge P, Marly J, Beaumont C, Barrow PA, Bumstead N, Lalmanach AC, Salmonella carrier state in chicken: comparison of expression of immune response genes between susceptible and resistant animals. *Microbes Infect.* 2004;6(14):1278-1286;
26. Wigley P, Berchieri AJr, Page KL, Smith AL, Barrow PA, *Salmonella enterica* serovar Pullorum persists in splenic macrophages and in the reproductive tract during persistent, disease-free carriage in chickens, *Infection and Immunity* 2001; 69(12):7873–7879;
27. Wigley P, Hulme SD, Powers C, Beal RK, Berchieri A Jr, Smith A, Barrow P, Infection of the reproductive tract and eggs with Salmonella enterica serovar pullorum in the chicken is associated with suppression of cellular immunity at sexual maturity. *Infect Immun*. 2005;73(5):2986-2990;
28. http://www.oie.int/international-standard-setting/terrestrial-manual/access-online

DIFFERENCES IN PHAGOCYTIC ACTIVITY OF SMALL AND LARGE RUMINANTS INDUCED BY *IN VITRO* VEGETAL EXTRACT TREATMENT - SHORT COMMUNICATION

Gheorghiţă DUCA, Marina SPÎNU, Carmen Dana ŞANDRU, Mihaela NICULAE, Daniel CADAR

Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine, Mănăştur Street no 3-5, Cluj-Napoca, România, marinaspinu@yahoo.com

Abstract

Currently, a growing interest in the therapeutic potential of medicinal plants as immunomodulators is noticed. The present experiment was employed to determine the in vitro phagocytic activity of ethanolic extracts obtained from Echinacea angustifolia and Taraxacum officinale, assessing them as potential stimulators of the innate cell-mediated response in two farmed herbivores: bovine and sheep. A statistically significant increase in the phagocytic activity was established in case of Echinacea angustifolia variant ($p<0.005$); compared to the spontaneous variant the ability to engulf carbon particle was augmented during the first 35 minutes of incubation, but reduced for the following 15 minutes, as the phagocytic index values became negative. Monitoring the influence of the Taraxacum officinale ethanolic extract on blood cells from healthy Ţurcana sheep, during the first reading period (15 minutes), a stimulating effect was reported. Both ethanolic extracts were demonstrated to influence to a certain extend a very complex and important immunological process – phagocytosis in case of two farmed animal species (bovine and sheep). Further in vitro and in vivo studies are needed to reveal the complex mechanisms of herbal-derived products with immunomodulating properties.

Key words: phagocytosis, bovine, sheep, *Echinacea angustifolia, Taraxacum officinale*

Currently, a growing interest in the therapeutic potential of medicinal plants as antiviral, antibacterial, wound healing, anti-inflammatory, antitumoral, antioxidants, antidiabetic, hepatoprotective, etc is noticed. Also, as the aim of several studies, the immunomodulating properties of whole plants and their corresponding isolated compounds against infections and neoplastic diseases were investigated, providing now the scientific base for medical applications. As documented by numerous researchers, several types of products have proven their efficacy on modulating both specific and non-specific immunity.

In this regard, two of the most important species are represented by *Echinacea angustifolia* (narrow-leaved purple coneflower, blacksamson echinacea) and *Taraxacum officinale* (dandelion), both belonging to *Asteraceae* (*Compositae*) family and well known for beneficial effects on the immune functions (1, 10). Along with *Echinacea pallida* and *Echinacea purpurea*, *Echinacea angustifolia* is one of the most important Echinacea species with a long history of medicinal use for a variety of conditions, particularly in the prevention and treatment of upper respiratory tract infections based on the immunostimulant (or immunomodulatory) properties (1).

There is little scientific evidence for the efficacy of these herbal products in animals (3,4,8), thus the aim of this study was to determine the *in vitro* phagocytic activity of ethanolic extracts obtained from *Echinacea angustifolia* and *Taraxacum officinale*, assessing them as potential stimulators of the innate cell-mediated response in two farmed herbivores: bovine and sheep.

Materials and methods

The study was performed on blood samples collected from two groups of animals: (1) adult Romanian Spotted cows (n=18) and (2) adult Ţurcana sheep (n=9); the blood was sampled on heparine (50 IU/ml) and subjected to *in vitro* treatment with ethanolic extracts obtained from *Echinaceea angustifolia* and *Taraxacum officinale* (10µl), testing also 70° ethanol as a control. In order to estimate the innate cell-mediate response in sheep and bovine the carbon particle clearance test was carried out using three experimental variants: control (or spontaneous phagocytosis), 70° ethanol treated blood as solvent control, *Echinaceea angustifolia* ethanolic extract treated samples, and *Taraxacum officinale* ethanolic extract treated samples. The selected technique consists on several steps as follows: 0.50·ml heparinized blood were mixed with India ink supernatant (2·µl, obtained by centrifugation at 6000 rpm for 40·min), 0.15·ml of the mixture were transferred immediately to 2·ml of saline and the rest was incubated at 37°C; another 0.15·ml sample were transferred to saline and the incubation was continued, repeating the operation. The incubation periods differed for the studied species, being of 35 and 60 minutes for bovine, and 15 and 30 minutes for sheep, respectively. All tubes containing saline, blood and ink were centrifuged at 800 rpm and the supernatants were read spectrophotometrically (λ=535·nm, d=0.5·cm). There was a decrease in absorbance with time as carbon was phagocytized. A phagocytic activity index was determined as the difference between the natural logarithms of the optical densities of the phagocytosis at the two times of incubation for each species, divided by the difference between the incubation intervals. The average values, standard deviation, standard error and the statistical significance of the differences were calculated.

Results and discussion

The *in vitro* treatment of the bovine blood samples with the quoted vegetal extract resulted in the phagocytic values presented in table 1.

Table 1. Phagocytic activity for the monitored periods of time in bovine

Phagocytic activity index	**Experimental variants**		
	Spontaneous	**Solvent control**	***Echinacea angustifolia***
ln0-ln35/35	-0.0092± 0.0226	0.0031±0.0215	0.0156±0.0307
ln35-ln60/15	0.0135±0.0316	-0.0002±0.0144	-0.0051±0.0337

Considering the values, a statistically significant increase in the phagocytic activity can be reported in case of *Echinacea angustifolia* variant ($p<0.005$); compared to the spontaneous variant the ability to engulf carbon particle was augmented during the first 35 minutes of incubation (table 1). Still, an intense negative influence is observed for the following 15 minutes, as the phagocytic index values became negative.

In sheep, the phagocytic values observed for the reading period were presented in table 2.

Table 2. Phagocytosis in sheep

Phagocytic activity index	**Experimental variants**		
	Spontaneous	**Solvent control**	***Taraxacum officinale***
ln0-ln15/15	0.0003±0.0114	0.0008±0.0307	0.0009±0.0050
ln15-ln30/15	0.0013±0.0133	0.0089±0.0099	0.0019±0.0076

Monitoring the influence of the *Taraxacum officinale* ethanolic extract on blood cells from healthy Ţurcana sheep, during the first reading period (15 minutes), a stimulating effect was noticed too, but compared to *Echinacea angustifolia*, this effect was present for the next 15 minutes, determining higher values of the phagocytic index (table 2).

Such beneficial influence of herbal derived products and/or their active principles on immune parameters level and functionality is well documented in the scientific literature. Thus, by inducing the secretion of TNF-alpha an aqueous extract obtained from *Taraxacum officinale* was able to restore the inhibition of nitric oxide (NO) production in mouse peritoneal macrophages pretreated with cadmium (5). Rebalancing the level of NO, an important effector molecule involved in immune regulation and defense, this herbal extracts was suggested to enhance cell-mediated, humoral, and nonspecific immunity (5).

A related mechanism was proposed also for the antiviral activities of extracts derived from four different *Echinacea* species (*E. angustifolia*, *E. purpurea*, *E. tennesseensis*, *E. pallida*). In the experimental conditions of a herpes simplex viral infection in the murine macrophage cell line RAW264.7 model, all these extracts increased the amount of inducible nitric oxide synthase (iNOS) protein and had no influence on the secretion of interferons alpha or beta as compared to control cells (10).

Investigated by flow cytometry, the immunostimulant activity of distinct extracts produced from *E. purpurea*, *E. pallida*, and *E. angustifolia* leaves, stems, flowering tops, and seems to be associated with polysaccharides (9). Such *Echinacea spp*. polysaccharides tested with analogues from *Astragalus*, wolfberry, and kelp were considered as immunopotentiators/adjuvants of a veterinary rabies vaccine based on the results indicating significantly elevated lymphocyte proliferation and cytokines level and also an accelerated and enhanced effect on rabies-neutralizing antibody responses in mice and dogs (6).

Conclusions

1. The values obtained for the spontaneous *in vitro* phagocytic activity were higher in bovine.
2. Both ethanolic extracts were demonstrated to influence at a certain extend a very complex and important immunological process – phagocytosis in case of two farmed animal species (bovine and sheep);
3. Further *in vitro* and *in vivo* studies are needed to reveal the complex mechanisms of herbal-derived products with immunomodulating properties.

References

1. Barnes J, Anderson LA, Gibbons S, Phillipson JD, Echinacea species (*Echinacea angustifolia* (DC.) Hell., *Echinacea pallida* (Nutt.) Nutt., *Echinacea purpurea* (L.) Moench): a review of their chemistry, pharmacology and clinical properties. *J Pharm Pharmacol.* 2005;57(8):929-954;
2. Barrett B, Review Medicinal properties of Echinacea: a critical review. *Phytomedicine*. 2003;10(1):66-86;
3. Gurbuz E, Balevi T, Kurtoglu V, Coskun B, Oznurlu Y, Kan Y, Kartal M, Effects of Echinacea extract on the performance, antibody titres, and intestinal histology of layer chicks.*Br Poult Sci*. 2010;51(6):805-810;
4. Hermann JR, Honeyman MS, Zimmerman JJ, Thacker BJ, Holden PJ, Chang CC, Effect of dietary *Echinacea purpurea* on viremia and performance in porcine reproductive and respiratory syndrome virus-infected nursery pigs. *J Anim Sci.* 2003;81(9):2139-2144;
5. Kim HM, Lee EH, Shin TY, Lee KN, Lee JS. *Taraxacum officinale* restores inhibition of nitric oxide production by cadmium in mouse peritoneal macrophages. I*mmunopharmacol Immunotoxicol*. 1998;20(2):283-297;
6. Liu Y, Zhang S, Zhang F, Hu R.Adjuvant activity of Chinese herbal polysaccharides in inactivated veterinary rabies vaccines. *Int J Biol Macromol.* 2012;50(3):598-602;
7. Morazzoni P, Cristoni A, Di Pierro F, Avanzini C, Ravarino D, Stornello S, Zucca M, Musso T, *In vitro* and *in vivo* immune stimulating effects of a new standardized *Echinacea angustifolia* root extract (Polinacea). *Fitoterapia*. 2005; 76(5):401-411;
8. Orengo J, Buendía AJ, Ruiz-Ibáñez MR, Madrid J, Del Río L, Catalá-Gregori P, García V, Hernández F, Evaluating the efficacy of cinnamaldehyde and *Echinacea purpurea* plant extract in broilers against *Eimeria acervulina. Vet Parasitol*. 2012;185(2-4):158-163;
9. Pillai S, Pillai C, Mitscher LA, Cooper R. Use of quantitative flow cytometry to measure ex vivo immunostimulant activity of Echinacea: the case for polysaccharides. *J Altern Complement Med.* 2007;13(6):625-634;
10. Senchina DS, Martin AE, Buss JE, Kohut ML.Effects of Echinacea extracts on macrophage antiviral activities. *Phytother Res*. 2010;24(6):810-816.

THE EFFECT OF UV LIGHT ON CERTAIN *STAPHYLOCOCCUS SPP.* STRAINS ISOLATED FROM CANINE DERMATITIS

Mircea TĂUŢAN, Marina SPÎNU, Bogdan Sebastian FERŞEDI
Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine, Str. Manastur no.3-5, Cluj-Napoca, Romania, tautan_mircea@yahoo.com

Abstract
The effect of UV radiation was studied in Staphylococcus spp. strains isolated from six dogs with staphylococcal-like dermatitis. The strains were inseminated on solid growth medium and incubated for 24h at 37°C to obtain 24h old strains. Each strain was exposed to UV radiation for1 to 5 minutes, then re-inseminated on broth and inactivated. For each sample, the optical density was read using a spectrophotometer. In order to assess the length of the effect of the UV radiation, each sample was then re-incubated for another 24h at 37°C. After that another reading was made for each sample. The results showed that the UV radiation had either a static or bactericidal effect on isolated Staphylococcus spp. strains and that the effect was more intense with the increasing length of exposure.
Keywords: UV radiation, *Staphylococcus spp*., dog, dermatitis

Wound infections produced by antibiotic-resistant bacterial strains are particularly difficult to manage, and canine recurrent dermatitis is one of the frequent diseases encountered in patients with a problematic thepary. UV radiation can be best described as invisible radiation. In order to kill microorganisms, ultraviolet rays must reach bacterial cells. The energy from UV rays penetrates the outer membrane of the cell, passing through the body and breaks the bonds in the DNA, preventing bacterial reproduction (3).

Ultraviolet germicidal irradiation (UVGI) is a sterilization method that uses ultraviolet (UV) at a wavelength short enough to kill microorganisms and prevent their multiplication (5).

For more than one hundred years it is known that the effect of ultraviolet radiation is mutagenic at cell level. At a wavelength of 253.7 angstroms (253.7 nm) UV break molecular bonds in the DNA of microorganisms, producing modified thymine molecules in their DNA, thereby destroying them, making them harmless or stopping their growth and multiplication (4).

The degree of inactivation achieved by ultraviolet radiation is directly related to the UV dose applied. The dosage, the intensity and the exposure time to UV rays is measured in microwatt-seconds per square centimeter (mws/cm2) (3).

Material and methods

The method of exposure of the *Staphylococcus spp*. strains to UV radiation chosen for this experiment was the direct method in micro plates, thus avoiding any contamination that may occur through the manipulation of biological material on the blade. A Germicidal UV lamp was used (15 Watt UV Bulb, emitting wave length 253.7nm).

Each strain isolated from the six study cases was inseminated Mueller-Hinton agar, and incubated at 37°C for 24 h. The inoculum was prepared by dilution of the culture with 3 ml of sterile broth, according to the optical density of the first tube on the McFarland scale.

The initial dilution was distributed in sterile plates, 200 μl/each well. All tests were done in triplicate. The first plate was exposed to UV (254 nm) for 1 minute, the second plate for 2 minutes, the third plate for 3 minutes, the fourth and fifth plate for 4 and 5 minutes, respectively. 30 μl of each well's content was transferred to a tube containing 3 ml of sterile broth. The unexposed culture, grown under the same conditions represented the control. All the tubes were incubated at 37 ° C for 24 h.

The assessment of the UV effects on bacteria was quantified by spectrophotometry (SUMAL PE2, Karl Zeiss, Jena), at a wavelength of 535 nm, d=0.5cm. 150 μl of each bacterial culture, inactivated with 50 μl of 1% formalin for 1 h, were placed in 96 well plates and red. In order to determine the intensity of UV rays on the growth potential of different strains of *Staphylococcus spp*. , previously cultured strains were re-inseminated on fresh media, incubated for another 24h at 37^{o}C and the optical densities were red after the inactivation with formalin, as described before. The bacterial growth expressed by optical density units (ODU) as compared to that of untreated controls, was considered better when the density was higher and lesser at lower optical densities.

Results and discussion

Ultraviolet C (UVC) light has long been known to be highly germicidal, but has not been much developed as a therapy for infections. In an experiment carried out on antibiotic-resistant strains of *Staphylococcus aureus* and *Enterococcal faecalis*, researchers indicated that the latter bacteria was more susceptible to the killing effects of UV. The UV light at 254 nm was bactericidal for of *S. aureus* and *E. faecalis* at times as short as 5 seconds. The *in* vitro findings suggested that, with recommended patient treatment times for infected wounds being significantly longer than 5 seconds, patient treatment exposure times needed to be re-examined (1).

UVC at a single radiant exposure of 2.59 J/cm(2) reduced the bacterial burden in the infected wounds in mice by approximately 10-fold in comparison to the non-treated ones ($p<0.00001$). Furthermore, UVC increased the wound healing rate in mice infected with *S. aureus* by 31.2% ($p<0.00001$). The DNA lesions that were observed in the UVC-treated mouse wounds were extensively repaired by 48 h after UVC exposure. These results suggested that UVC may be used for prophylaxis of cutaneous wound infections (2).

After different strains of *Staphylococcus spp*. were isolated from dogs with dermatitis and incubated for 24h at 37^{o}C, they were exposed at UV rays for 1 to 5 minutes. The optical density of the culture medium was determined for each strain and exposure time and they were shown in *Table 1*. The only sharp decrease in growth after UV exposure was observed in the *Staphylococcus spp.* strain isolated from case 1, where the differences against control were more than 50% (*Table 2*). The other isolated strains were less sensitive to exposure. In the present protocol, the only exposure times were from 1 to 5 minutes maximum. In order to obtain a more explicit dynamics scheme of bacterial growth of the strains isolated from canine dermatitis after UV exposure, the experiments need to be expanded, including more numerous exposure periods. The history of the isolated strains concerning the potential use of UV light in dermatitis treatment was unknown.

Table 1. Optical densities of different isolates of *Staphylococcus spp.* exposed to UV (ODU)

Exposure time (min)		Control	1	2	3	4	5
Case no.	1	0.0790	0.0520	0.0450	0.0480	0.0470	0.0370
	2	0.0850	0.0860	0.0790	0.0840	0.0780	0.0640
	3	0.0480	0.0510	0.0460	0.0480	0.0440	0.0380
	4	0.0790	0.0720	0.0700	0.0670	0.0650	0.0650
	5	0.0400	0.0370	0.0300	0.0360	0.0360	0.0320
	6	0.0960	0.0940	0.0950	0.0945	0.0933	0.0930

Comparing the growth results of the unexposed controls with exposed strains, depending on the exposure time, the average and standard deviations were calculated (*Table 2*).

Table 2. Degree of growth or inhibition of different isolated strains of Staphylococcus spp. exposed to UV (% versus control)

Exposure time (min)		1	2	3	4	5
Case no.	1	-34.18	-43.04	-39.24	-40.51	-53.16
	2	1.18	-7.06	-1.18	-8.24	-24.71
	3	6.25	-4.17	0.00	-8.33	-20.83
	4	-8.86	-11.39	-15.19	-17.72	-17.72
	5	-7.50	-25.00	-10.00	-10.00	-20.00
	6	-2.08	-1.04	-1.56	-2.86	-3.13
	x	-7.53	-15.28	-11.19	-14.61	-23.26
	s	14.19	15.96	14.98	13.56	16.43

The majority of the isolated strains exposed to UV light had a lower optical density when compared to the unexposed controls. In most of the strains, the decrease in optical densities was more pronounced with the increase of the exposure period. An exposure time of 5 minutes seemed to be the most efficient in decreasing the post exposure growth of staphylococci (*Table 2*).

The optical density of the culture medium was determined for each strain and time and they are shown in *Table 3*. Differences between the growth pattern of untreated controls and exposed staphylococci were calculated in inhibition percentages (*Table 4*). In one case there was a stimulating effect of the exposure after re-insemination (case 2, *Table 4*), while the rest of the percentages were negative. The inhibiting effect of the exposure was persistent after the re-insemination, but at a lower extent, suggesting the bacteria would potentially

come back to the growth speed encountered before exposure.

Table 3. Optical densities of different isolates of *Staphylococcus spp*. exposed to UV following re-incubation

Exposure time (min)		M (0)	1	2	3	4	5
Case no.	1	0.1050	0.0570	0.0650	0.0780	0.0690	0.0770
	2	0.0610	0.0580	0.0610	0.0570	0.0470	0.0880
	3	0.0780	0.0600	0.0450	0.0450	0.0470	0.0560
	4	0.0540	0.0540	0.0450	0.0440	0.0400	0.0400
	5	0.0100	0.0200	0.0130	0.0150	0.0110	0.0070
	6	0.0875	0.0760	0.0855	0.0915	0.0830	0.0795

Table 4. Percentage of growth inhibition of different isolates of *Staphylococcus spp*. exposed to UV following re-incubation

Exposure time (min)		1	2	3	4	5
Case no.	1	-45.71	-38.10	-25.71	-34.29	-26.67
	2	-4.92	0.00	-6.56	-22.95	44.26
	3	-23.08	-42.31	-42.31	-39.74	-28.21
	4	0.00	-16.67	-18.52	-25.93	-25.93
	5	100.00	30.00	50.00	10.00	-30.00
	6	-13.14	-2.29	4.57	-5.14	-9.14
	x	2.19	-11.56	-6.42	-19.67	-12.61
	s	50.58	26.92	31.97	18.75	28.87

UV rays produced an inhibition of bacterial development. On most of the strains (1, 2, 3, 4 and 6) ultraviolet light had a bacteriostatic effect, confirmed by a lower optical index after passage. Nevertheless, the growth dynamics maintains its overall picture of increased inhibition with increasing exposure time.

Conclusions, acknowledgements

The effect of UV light on *Staphylococcus spp*. strains isolated from dogs with staphylococcal-like dermatitis was rather bacteriostatic than bactericidal. The effect was enhanced by a prolonged exposure time.

References

1. Conner-Kerr TA, Sullivan PK, Gaillard J, Franklin ME, Jones RM. – 1998 - The effects of ultraviolet radiation on antibiotic-resistant bacteria in vitro. Ostomy Wound Manage.;44(10):50-6.
2. Dai T, Garcia B, Murray CK, Vrahas MS, Hamblin MR. – 2012 - Ultraviolet C prophylaxis for cutaneous wound infections in mice. Antimicrob Agents Chemother.[Epub ahead of print]
3. Dreeszen P. , 1997, Ultraviolet Disinfection, 1-2
4. Kowalski W.J.; Bahnfleth W.P.; Witham D.L.; Severin B.F.; Whittam T.S.- 2000 - Mathematical Modeling of Ultraviolet Germicidal Irradiation for Air Disinfection,.
5. *** -2008- NIOSH, (National Institute for Occupational Safety and Health), NIOSH eNews 12

IN VITRO ASSESMENT OF RESISTENCE TO ANTIBIOTICS AND UV RADIATION OF CERTAIN *STAPHYLOCOCCUS SPP.* STRAINS ISOLATED FROM DOGS WITH DERMATITIS

M. TĂUŢAN, Marina SPÎNU, B.S. FERŞEDI
Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine, Str. Manastur no.3-5, Cluj-Napoca, Romania, tautan_mircea@yahoo.com

Abstract
The effect of UV radiation and antibiotics was comparatively studied on Staphylococcus spp. strains isolated from six dogs with staphylococcal-like dermatitis. After preliminary isolation and identification, the strains were re/inseminated and the inoculum was prepared from 24h cultures. Each strain was exposed to UV radiation for 1 to 5 minutes in the presence of one of the following 7 antibiotics: enrofloxacin, amoxicillin potentiated with clavulanic acid, penicillin, amoxicillin, oxytetracycline, doxycycline and biseptol, then inseminated on broth for 24 h at 37℃ and subsequently inactivated for spectrophotometrical reading. The duration and intensity of the combined UV and antibiotic effects were estimated by re-nsemination of the strains for 24h at 37°C.The optical desnities were red by a spectrophotometer. The results showed that the UV radiation in combination with different antibiotics had a bacteriostatic and bactericidal effect on the Staphylococcus spp. Strains. The effect depends mainly on the antibiotic used and on the length of the exposure.
Keywords: *Staphylococcus spp*., UV radiation, antibiotics, dog, dermatitis

Staphylococcal infections are often treated with antibiotics and thus resistance to antibiotics and / or acquired resistance has developed (600). Antibiotic resistance in *Staphylococcus* strains isolated from dogs is common (2, 4, 5, 8).

The energy of UV light penetrates the outer membrane of the cell, passing through the body and breaks the bonds in the bacterial DNA, preventing multiplication (2).

It is known that at a cellular level the effect of ultraviolet radiation is mutagenic. At a wavelength of 253.7 nm UV breaks molecular bonds in the DNA of microorganisms, producing modified thymine molecules in their DNA thereby destroying them, making them harmless or stopping their growth and multiplication (3, 6).

The purpose of this study was to assess the changes in antibiotic resistence of strains isolated from dog dermatitis cases, after exposure to UV light.

Material and methods

To measure the sensitivity of bacterial strains to antibiotics subsequent to exposure to UV light, antibiograms were done using bacteria cultivated in tubes, on Mueller Hinton broth.

Five *Staphylococcus spp.* Strains isolated from dermatitis cases in dogs and seven antibiotics commonly applied in dog dermatitis therapies, namely: enrofloxacin (ENR), amoxicillin potentiated with clavulanic acid (AMC), penicillin (P), amoxicillin (AMX), oxytetracycline (T), doxycycline (DOX) and Biseptol (BIS) were used.

Two milliliters of sterile Mueller Hinton broth, in which antibiotic disks were immersed, were inseminated with 24 h cultures (30 μl) of staphylococcal strains, exposed for various periods to UV light. For each isolate, six series of eight tubes were used, one for each exposure period (1 to 5 min) and antibiotic (ENR, AMC, P, AMX, T, DOX and BIS),

including the unexposed control. of bacterial suspension was put and then it was subjected to UV, resulting for each bacterial strain 5 series, one for each exposure time and one for control. Afterwards, the tubes were incubated for 24 h at 37°C, and the readings of optical densities indicating growth were done in a 96 well microplate using a SUMAL PE2 (Karl Zeiss, Jena) multi channel spectrophotometer. For that, 150 µl of each tube were inactivated with 50 µl of a 1% formalin solution and the reading was performed at a wavelength of 535 nm, d=0.5 cm.

Results and discussion

Considerable efforts are being made to combat hospital and community acquired infections worldwide as well as reducing contamination within a range of settings. In human medicine, the incidences of *Staphylococcus aureus* demonstrate the requirement for an improved approach to preventing and rapidly controlling this and other important infections and sources of contamination (1).

The most studied plasmids of *S. aureus* have been the penicillinase plasmids which have been shown to carry the determinants conferring resistance to penicillin and to a number of metal ions. There are studies indicating that this UV-induced decrease in transduction rate, typical of plasmid borne markers, does not always occur and that the rate of transfer of some penicillinase plasmids may be increased rather than decreased after UV irradiation of transducing phage (7).

Due to the widespread use of antibiotics and the emergence of more-resistant and -virulent strains of microorganisms, there is an urgent need to develop alternative sterilization technologies. Visible light was used to enhance the bactericidal activity of various chemical compounds such as TiO2 (9).

The initial mean optical densities of the cultures, after exposure and insemination in antibiotic treated tubes were presented in *Table 1*.

Table 1. Optical densities of different isolated strains of *Staphylococcus spp*. exposed to UV (mean values)

Time of exposure (min)	Antibiotic							
	M	Enr	Amc	P	Amx	Dox	T	Bis
M(0)	0,0488	0,0047	0,0038	0,0166	0,0108	0,0079	0,0067	0,0317
1	0,0513	0,0052	0,0032	0,0159	0,0106	0,0103	0,0052	0,0285
2	0,0246	0,0051	0,0050	0,0113	0,0090	0,0055	0,0078	0,0264
3	0,0350	0,0052	0,0031	0,0159	0,0150	0,0116	0,0037	0,0283
4	0,0254	0,0048	0,0034	0,0134	0,0090	0,0033	0,0034	0,0240
5	0,0348	0,0048	0,0041	0,0179	0,0122	0,0071	0,0062	0,0203

The lowest rate of growth was recorded for most of the tubes exposed to UV for 3 minutes. The antibiotic treatment further decreased the growth capacity of the cultures. The

group of tetracyclins seemed to be the most efficient at various exposure times.

The percentages of inhibition when compared to the unexposed control were presented in *Table 2*. The majority of the strains exposed to UV light had a lower optical density when compared to the unexposed culture. The results indicated that, in combination with UV light, the most efficient antibiotics were, in decreasing order: amoxicillin potentiated with clavulanic acid, enrofloxacin, oxytetracycline, doxycycline, amoxicillin, penicillin and biseptol.

In order to evaluate the static or cidal effect of the UV light combined with antibiotics on different strains of *Staphylococcus spp*., each strain was re-inseminated and incubated for 24h at 37^{o}C. The optical densities of the cultures were red as in the previous trial and they were shown in *Table 3*.

Table 2. Percentage of the growth or inhibition of different isolated strains of *Staphylococcus spp*. exposed to UV

Time of exposure (min)	Antibiotic						
	Enr	Amc	P	Amx	Dox	T	Bis
M(0)	-89,90	-93,83	-68,99	-79,38	-79,87	-89,94	-44,48
1	-79,19	-79,70	-54,31	-63,45	-77,66	-68,53	7,11
2	-85,24	-91,14	-54,52	-57,10	-66,90	-89,52	-19,29
3	-97,69	-98,36	-93,49	-95,62	-98,42	-98,36	-88,31
4	-86,36	-88,28	-48,76	-65,07	-79,62	-82,30	-41,87
5	-90,44	-92,32	-66,04	-77,99	-83,79	-86,35	-35,15
x	-87,78	-89,96	-63,42	-71,84	-81,28	-85,01	-35,50
s	6,85	6,81	17,94	15,30	11,43	10,95	35,03

Table 3. Optical density of different isolated strains of Staphylococcus spp. exposed to UV, after reincubation for 24h at 37^{o}C

Time of exposure (min)	Antibiotic							
	M	Enr	Amc	P	Amx	Dox	T	Bis
M(0)	0,0483	0,0111	0,0162	0,0253	0,0276	0,0178	0,0228	0,0485
1	0,0554	0,0045	0,0154	0,0280	0,0293	0,0199	0,0238	0,0573
2	0,0481	0,0089	0,0240	0,0388	0,0345	0,0161	0,0291	0,0469
3	0,0473	0,0052	0,0149	0,0296	0,0305	0,0318	0,0259	0,0533
4	0,0493	0,0061	0,0126	0,0394	0,0221	0,0160	0,0264	0,0415
5	0,0463	0,0249	0,0075	0,0316	0,0223	0,0157	0,0368	0,0403

The combined UV light and antibiotic use produced an inhibition of bacterial development in most of the tubes, but this effect was milder than in the innitial cultures. The bacteria succeeded to grow after the re-insemination, indicating a static effect of the combined treatment. No other re/insemination attempts were done, to further monitor the bacterial growth and establish the potential recovery dynamics of the bacteria.

The lowest optical density, compared with the control, was obtained for the tubes exposed to UV rays for 1 minute in combination with enrofloxacine, therefore this antibiotic was considered the most efficient (*Table 4*).

Table 4. Percentage of the growth or inhibition of different isolated strains of Staphylococcus spp. exposed to UV and after reincubation for 24h at 37°C

Time of exposure (min)	Antibiotic						
	Enr	Amc	P	Amx	Dox	T	Bis
Control	-77,07	-66,55	-47,76	-42,90	-63,28	-52,76	0,34
1	-91,88	-72,30	-49,47	-47,22	-64,06	-57,14	3,46
2	-81,56	-50,13	-19,48	-28,31	-66,49	-39,48	-2,60
3	-89,03	-68,43	-37,46	-35,45	-32,80	-45,15	12,70
4	-87,56	-74,37	-20,05	-55,08	-67,51	-46,45	-15,74
5	-46,13	-83,78	-31,71	-51,89	-66,09	-20,36	-12,97
x	-79,23	-69,80	-31,64	-43,59	-59,39	-41,72	-3,03
s	18,89	12,36	12,59	11,33	14,92	13,54	11,73

Conclusions

The effect of UV rays and antibiotics on different strains of *Staphylococcus spp*. isolated from dogs with Staphylococcic-like dermatitis were rather bacteriostatic than bactericidal. The effect of the combination on different strains of *Staphylococcus spp*. depended mainly on the antibiotic used and not on the exposure time.

References

1. Holloway A.C., Gould S.W.J., Fielder M.D., Naughton D.P., Kelly A. F., 2011, Enhancement of antimicrobial activities of whole and sub-fractionated white tea by addition of copper (II) sulphate and vitamin C against Staphylococcus aureus; a mechanistic approach, BMC Complementary and Alternative Medicine, 11:115
2. Holm B.R., Petersson U., Mörner A., Bergström K., Franklin A., Greko C., 2002, Antimicrobial resistance in staphylococci from canine pyoderma: a prospective study of first-time and recurrent cases in Sweden, Vet. Rec., 151, 600-605.
3. Kowalski W.J.; Bahnfleth W.P.; Witham D.L.; Severin B.F.; Whittam T.S., 2000, Mathematical Modeling of Ultraviolet Germicidal Irradiation for Air Disinfection.
4. Lloyd D.H., Lamport A.I., Feeney C., 1996, Sensitivity to antibiotics amongst cutaneous and mucosal isolates of canine pathogenic staphylococci in the UK, 1980-1996. Vet. Dermatol, 7, 171-175.

5. Noble W.C., Kent L.E., 1992, Antibiotic resistance in *Staphylococcus intermedius* isolated from cases of pyoderma in the dog, Vet. Dermatol., 3, 71-74.
6. Normand, E.H., Gibson, N.R., Reid, S.W., Carmichael, S. and Taylor, D.J., 2000, Antimicrobial-resistance trends in bacterial isolates from companion animal community practice in the UK. Preventative Veterinary Medicine 46, 267–278
7. Rubin SJ, Rosenblum ED.,1971, Effects of the recipient strain and ultraviolet irradiation on transduction kinetics of the penicillinase plasmid of Staphylococcus aureus. J Bacteriol.;108(3):1192-9.
8. Werckenthin C., Cardoso M., Martel J.-L., Schwarz S., 2001, Antimicrobial resistance in staphylococci from animals with particular reference to bovine Staphylococcus aureus, porcine Staphylococcus hyicus and canine Staphylococcus intermedius, Review article, Vet. Res., 32, 341-362.
9. Wong MS, Chu WC, Sun DS, Huang HS, Chen JH, Tsai PJ, Lin NT, Yu MS, Hsu SF, Wang SL, Chang HH. 2006, Visible-light-induced bactericidal activity of a nitrogen-doped titanium photocatalyst against human pathogens. Appl Environ Microbiol.;72(9):6111-6.

INVESTIGATION ON THE CYTOTOXIC POTENTIAL OF *LAVANDULA ANGUSTIFOLIA* MILL. DERIVED PRODUCTS

Mihaela NICULAE[1], Marina SPÎNU[1], Emoke PALL[1], Olga SORITAU[2], Piroska VIRAG[2], Carmen Dana ŞANDRU[1], Mihai CENARIU[1]

[1]University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Faculty of Veterinary Medicine, Mănăştur Street no 3-5, Cluj-Napoca, Romania; [2]"Prof. Dr. Ion Chiricuţă" Oncology Institute, Cluj-Napoca, Romania
niculaemihaela1@gmail.com

Abstract

The aim of this study was to investigate the cytotoxic potential of three herbal products derived from lavender (Lavandula angustifolia Mill.) previously demonstrated with complex and strongly expressed antimicrobial efficacy against several bacterial strains isolated from animal clinical cases (7,8). The cytotoxicity was estimated in terms of quality and quantity, using two experimental models that allow prediction of standard cellular response to potentially toxic product with greater confidence than using only one method: evaluation of biocompatibility on human fibroblasts (HFL-1) cell culture by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and evaluation of morphology and cell attachment level respectively. The results pointed out both dose and type extract dependence of tested herbal extracts-induced effects with lower concentrations stimulating cell viability, while higher concentrations and mostly in case of essential oil inducing moderate or highly expressed cytotoxicity.

Keywords: *Lavandula angustifolia*, cytotoxic effect, human fibroblasts

Lavender (*Lavandula angustifolia* Mill.) derived products are well know for therapeutic qualities; lavender oil is mentioned for sedative, anti-depressive, carminative, anti-inflammatory, wound healing, antimicrobial, antifungal (5), acaricidal (6) properties. Still, to consider this herbal extracts for medical applications, evaluation of the toxicity is mandatory, including in this category several types of *in vitro* and *in vivo* assays aimed to fully characterize this potential. In this regard, the objective of one of the most important class of screening methods for the toxic potential refers to the study on the cytotoxicity.

Materials and methods

The present study was intended out to investigate the *in vitro* toxic potential of essential oils and ethanolic extracts obtained from *Lavandula angustifolia* Mill. The MTT results, expressed as optic density (absorbance), were calculated as viability percentages by dividing the absorbance reading of cells under different herbal extracts concentrations by the absorbance reading of cells under normal growth (assumed 100% viability). The difference between the control and herbal extracts dilutions treated cells were analysed using ANOVA post hoc, followed by Dunnett test (against the control) or by Bonferroni test (against ten different dilutions).

Results and discussions

MTT test results indicated the dose dependence of tested herbal extracts-induced effect as some dilutions stimulated cell viability, while higher concentrations had moderate or highly expressed cytotoxicity, depending on herbal species and also on the investigated extract type (essential oil or ethanolic extract).

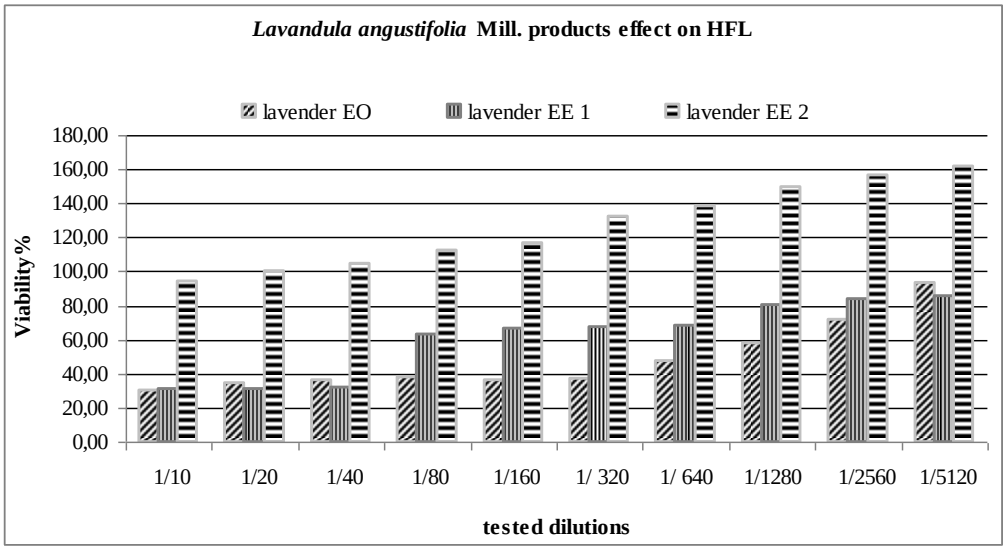

Fig. 1. *Lavandula angustifolia* Mill. products impact on the tested cells

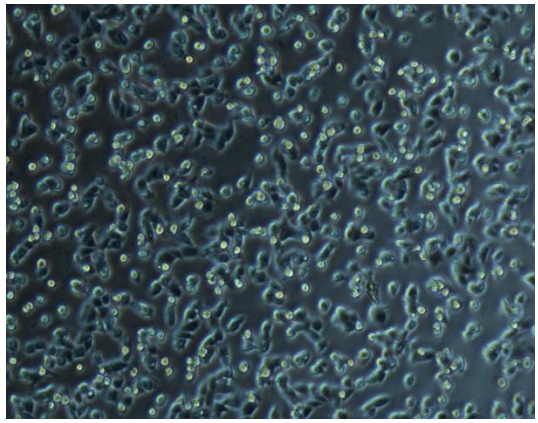

Fig. 2. Aspect of HLF-1 1 h after the *Lavandula angustifolia* Mill. EE1 treatment

In case of ethanolic extracts an *in vitro* stimulatory effect on HFL viability was noticed.

Pooled analysis pointed out no adverse effects on the adhesion molecules, as the cells attachment degrees were unchanged. Cell viability was not affected except for cells treated with thyme essential oil dilution 1/1280, where one hour after exposure, viability percentage was lower compared with untreated control cultures.

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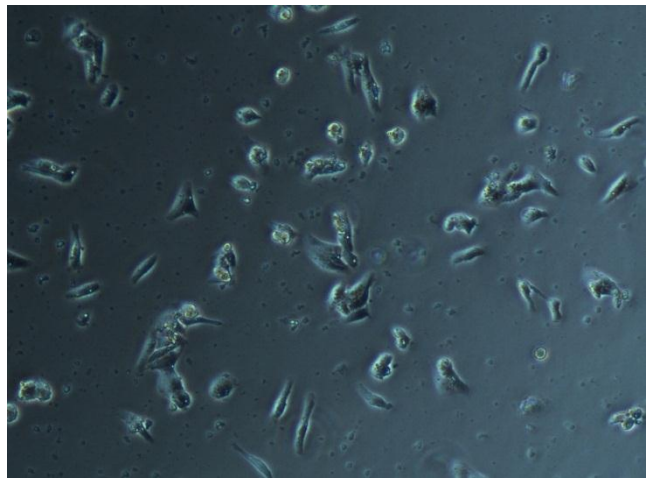

Fig. 3. Aspect of HFL cells 24 h after the *Lavandula angustifolia* Mill. EO treatment dilution 1/5120

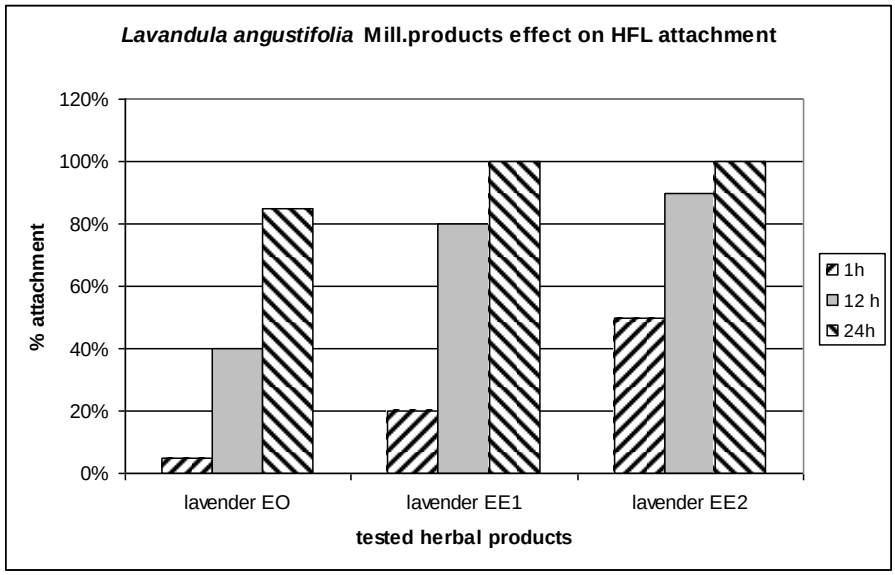

Fig. 4. Graphic reprezentation of HFL cells attachement level

The obtained data suggested cytotoxic potential mostly in case of essential oil, consistent with the findings of other similar studies, and less intense for alcoholic extracts, particularly for lower concentrations.

Cytotoxicy of lavender oil and its major components, linalyl acetate and linalool, were demonstrated when tested *in vitro* on human skin cells (endothelial cells and fibroblasts) for the concentration of 0.25% (v/v) in case of all tested cell types (HMEC-1, HNDF and 153BR). Linalyl acetate cytotoxicity was higher than that of the oil itself, suggesting

suppression of its activity by an unknown factor in the oil. Membrane damage was proposed as the possible mechanism of action (9).

Lavender oils displayed inhibitory activity against three fungi, two bacteria and Chinese hamster cells by both gaseous and solution contact. A relatively higher cytotoxicity was observed in case of solution contact compared with gaseous contact (5).

Toxicity of lavender essential oil and its major components, linalool, and linalyl acetate, was also investigated on a molecular level and the findings suggested a mutagenic activity due to the linalyl acetate that displayed the profile of an aneugenic agent (3). In case of this research, the potential genotoxicity was evaluated *in vitro* using the micronucleus test on peripheral human lymphocytes. Non-toxic concentrations of linalyl acetate (0.5-100 μg/ml) significantly augmented in concentration-dependent manner the frequency of micronuclei, while the lavender oil presented similar activity only at the highest concentration tested, whereas linalool was devoid of genotoxicity. None of the tested substances led to an increase in nucleoplasmic bridges or nuclear buds frequency (3).

Conclusions

These observations on the cytotoxicity displayed by three products derived from lavender indicate that such extracts should be considered for further *in vitro* and *in vivo* studies aimed to evaluate their complex therapeutical potential in terms of efficacy and safety.

Bibliography

1. Askin CT, Aslanturk OS, Cytotoxic and genotoxic effects of *Lavandula stoechas aqueous* extracts. *Biologia* 2007;62(3):292-296;
2. Cavanagh HM, Wilkinson JM, Biological activities of lavender essential oil. Phytother Res.2002;16(4):301-308;
3. Di Sotto A, Mazzanti G, Carbone F, Hrelia P, Maffei F. Genotoxicity of lavender oil, linalyl acetate, and linalool on human lymphocytes *in vitro*. *Environ Mol Mutagen*. 2011;52(1):69-71;
4. Evandri MG., Battinelli L, Daniele C, Mastrangelo S, Bolle P, Mazzanti G. The antimutagenic activity of *Lavandula angustifolia* (lavender) essential oil in the bacterial reverse mutation assay. *Food and Chemical Toxicology* 2005;43(9):1381-1387;
5. Inouye S, Abe S, Yamaguchi H, Asakura M. Comparative study of antimicrobial and cytotoxic effects of selected essential oils by gaseous and solution contacts, *International Journal of Aromatherapy* 2003;13(1):33–41;
6. Khodadad Pirali-Kheirabadi, Jaime A. Teixeira da Silva. *Lavandula angustifolia* essential oil as a novel and promising natural candidate for tick (*Rhipicephalus* (*Boophilus*) *annulatus*) control. *Experimental Parasitology* 2010;126(2):184-186;
7. Niculae M, Spinu M, Sandru CD, Chirila F. The bioactivity of herbal essential oils and ethanol extracts against *Escherichia coli* of animal origin. *Planta Med*. 2010;76:P507;
8. Niculae M, Spînu M, Şandru CD, Brudaşcă F, Cadar D, Kobolkuti L, Ungvari A, Rindt I, Uricaru A, Kiss T. Lamiaceae essential oils and alcoholic extracts and their effects on zoonotic multi rug- resistant bacteria. *Planta Med*. 2009; 75:PJ86;
9. Prashar A, Locke IC, Evans CS. Cytotoxicity of lavender oil and its major components to human skin cells. *Cell Prolif* 2004;37(3):221-229.

THE INNATE CELL-MEDIATED IMMUNITY AS AN INDICATOR OF ANTIINFECTIOUS RESISTENCE IN EXTENSIVELY RAISED SHEEP

Marina SPÎNU, Carmen Dana ŞANDRU, Mihaela NICULAE, Silvana POPESCU, Daniel CADAR, Armela BORDEANU

Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine, Str. Manastur no.3-5, Cluj-Napoca, Romania, marinaspinu@yahoo.com

Abstract

Environmental factors act as stressors, changing the non-specific and adaptive immune responses in sheep, depending on their raising system (3, 4, 5). The aim of the study was to investigate the immune changes, other than antibody mediated response against anthrax vaccination and evaluate the extent to which this compulsory operation influences the level of immune reactivity of the host as an estimate of resistence to other potential pathogens. The investigations were carried out in a group of 30 animals and the stress impact on the immune function was assessed based on the results of the carbon particle inclusion test. Both taking the animals to the pasture and anti-anthrax vaccination exert a negative effect on spontaneous phagocytosis (negative values), but the use of Taraxacum officinale extract helps somewhat restoring these negative effects.

Key words: immunity, sheep, environment, vaccination

Environmental factors act as stressors, changing the non-specific and adaptive immune responses in sheep, depending on their raising system (3, 4, 5). The changes are different in intensity, depending on stress levels. Ie, the vaccination against anthrax represents a compulsive operation in extensively raised sheep, nevertheless is one of the stressing agents for this animal species.

The link between stress factors or stressful conditions and a decreased immunity is suggested by several authors in both human and animal subjects, but the associated mechanisms are not clearly pointed out (1, 2). As Dohms and Metz concluded:" Since the imposition of a stressor disrupts physiological homeostasis, understanding the capacity of the immune system to function under such conditions is of prime importance in predicting disease onset and outcome" (2), studies evaluating this aspect are needed.

The aim of the study was to investigate the immune changes, other than antibody mediated response against anthrax vaccination and evaluate the extent to which this compulsory operation influences the level of immune reactivity of the host as an estimate of resistence to other potential pathogens.

Material and methods

The investigations were carried out on a group of 30 animals, kept, fed and watered along with the other individuals in the flock (n=130 animals), and raised extensively. The animals belonged to Ţurcana breed, both sexes (3 rams and 127 lactating sheep) and were aged between 3 and 5 years. Blood was sampled in various moments of the technological cycle, as follows: Sampling I - year I, before taking the sheep to the pasture; Sampling II- two weeks before the anti-anthrax vaccination; Sampling III two weeks after the anti-athrax vaccination; Sampling IV year 2, before taking the sheep to the pasture; Sampling a V-a two

weeks after taking the animals to the pasture. With this protocole, both changes in the innate cell mediated immunity due to environmental and technological changes and those induces by the compulsory anti-anthrax vaccination could be monitored.

Blood was sampled on heparine (50UI/ml) and processed withing maximum 4 hours by means of carbon particle inclusion test . Phagocytic cells engulf inert particles such as carbon due to the defensive capacity of these cells. In 96 well plates, aliquots of 50 µl of heparinized blood were mixed with 2 µl of supernatant of India ink, which were obtained by centrifugation at 6000 rpm for 40 min (Hettich, Germany). 15 µl of the mixture were transferred immediately to 2 ml of saline and the plates was incubated for 15 min at 37°C. Another 15 µl sample were transferred to saline and the incubation was continued to 30 min, repeating the operation. All tubes containing saline, blood and ink were centrifuged at 800 rpm and the supernatants were read spectrophotometrically (λ=535 nm, d=1 cm). Simultaneously, variants with alcohol and *Taraxacum officinale* extract were performed. There was a decrease in absorbance with time as carbon was phagocytized. Phagocytic activity index was calculated as the difference between the natural logarithms of the optical densities of the phagocytosis at 0–15 min and 15–30 min divided by time (15 min).Carbon particle inclusion test *in vitro*: unstimulated, alcohol and alcoholic *Taraxacum officinale* extract stimulated variants, calculation of the reverse of the slope of phagocytosis in time. The statistical significance of the results was estimated by Student's t test.

Results and discussion

Following the *in vitro* challenge of the sheep blood samples with *Taraxacum officinale* ethanolic extract the corresponding phagocytic values were obtained and these are presented in figures 1, 2, 3, 4 and 5.

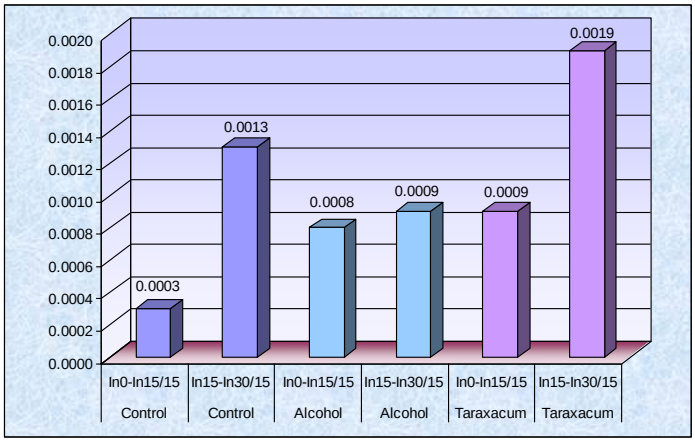

Fig. 1. Phagocytic activity during the first year, before the first grazing – sampling I

The first sampling indicated a phagocytic activity within the frame of physiological parameters, higher during the second period than during first one. The activity of the plant

extract proved to be strongly stimulative at this sampling, especially during the second period of evaluation.

The very low phagocytic values at the second sampling indicated a relatively high degree of stress, therefore the animals were more susceptibe during this period to other ubiquitous infections. There was no positive influence at all at this stage of the *Taraxacum* extract on phagocytosis. The vaccination somewhat stimulated the phagocytosis, more in the treated samples than in the untreated controls. Nevertheless, the influence of the *Taraxacum* extract was lower than expected. When comparing the first and second years and samplings before the first grazing, the results differ, leading to the conclusion of the intervening factors, others than grazing, that differed between the two years. Vaccination and taking the animals to the pasture seemed to exert similar stressful effects on non/specific cell mediated immunity, since the phagocytic indexes were almost similar (fig. 3 and 4).

Grazing as a technologica procedure in extensive raising somewhat restored the phagocytic capacity of the leucocytes and induced higher responses in controls variants during the second period, but lesser in the stimulated variants (fig. 5).

Comparing the obtained average values reflecting the phagocytic activity in case of the five sampling moments, a decreasing tendency may be noted, with two main exceptions: the alcohol variant (the solvent control) and less intense the *Taraxacum officinale* variant (figures 1 and 5).

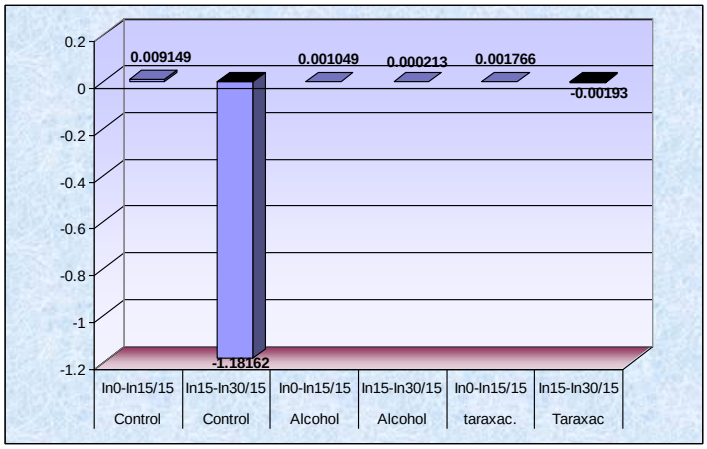

Fig. 2. Phagocytic activity two weeks before anti- anthrax vaccination – sampling II

Negative values were suggestive for the intensity of the negative impact exhibited by the environmental factors as indicated in figures 2, 3 and 4.

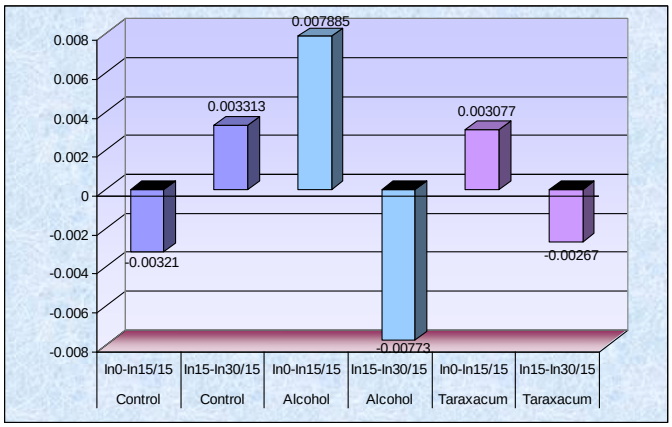

Fig. 3. Phagocytic activity two weeks after the anti anthrax vaccination– sampling III

The impact of stress factors on the immune parameters' level and functionality can not be overlookedd, especially because of a decreased immunity inducing high susceptibility towards infections (1), thus several restoring products are subjected to both in *vitro* and *in vivo* screening of their immunomodularory properties.

Such an example is represented by the melatonin. As the results of the subcutaneous coadministration of melatonin to sheep vaccinated against two stam of A1 and C strains of *Dichelobacter nodosus*, an enhanced antibody titer and serum IgG levels to A1 and C strains of *D. nodosus* compared to vaccinated animals not treated with melatonin was reported (3).

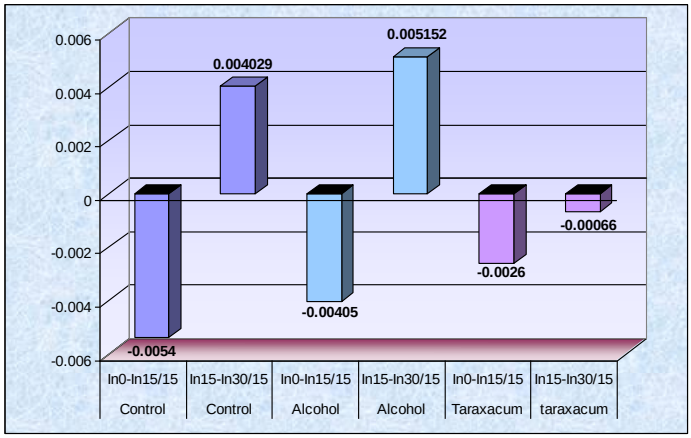

Fig. 4. Phagocytic activity in the second year two weeks before first grazing – sampling IV

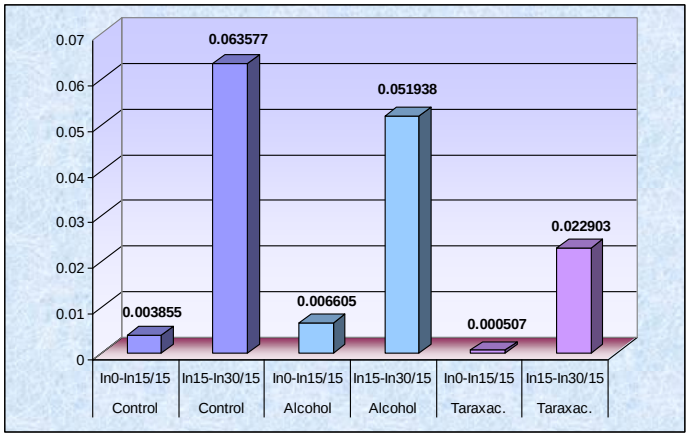

Fig. 5. Phagocytic activity two weeks after the first grazing – sampling V

Following a similar protocol a higher dose of melatonin (36mg/animal) was tested for immunomodulating potential in an oxidative/nitrosative stress model produced during the footrot immunization protocol and the results indicated a positive effect on the serum antibodies level and a neutralizing effect on the vaccinal oxidative stress (3).

Similarly, the effect of *Taraxacum officinale*, a plant commonly found on pastures was aimed to be monitored with the purpose of potential use in stimulating various immune responses of sheep to external stressors, vaccination included. In this experiment, the vegetal extract tested did not entirely fulfill the expectations.

Conclusions

Both taking the animals to the pasture and anti-anthrax vaccination exert a negative effect on spontaneous phagocytosis, but the use of *Taraxacum officinale* extract helps somewhat restoring these negative effects.

References

1. Dohms JE, Metz A, Stress--mechanisms of immunosuppression. *Vet Immunol Immunopathol*. 1991;30(1):89-109;
2. Kusnecov AW, Rabin BS, Stressor-induced alterations of immune function: mechanisms and issues. *Int Arch Allergy Immunol.* 1994;105(2):107-121;
3. Ramos A, Laguna I, de Lucía ML, Martín-Palomino P, Regodón S, Míguez MP, Evolution of oxidative/nitrosative stress biomarkers during an open-field vaccination procedure in sheep: effect of melatonin. *Vet Immunol Immunopathol*. 2010;133(1):16-24;
4. Roger PA, The impact of disease and disease prevention on sheep welfare. *Small Ruminant Research.* 2008;76(1–2):104-111;
5. Sevi A, Casamassima DV, Pulina G, Pazzona A, Factors of welfare reduction in dairy sheep and goats. *Ital. J. Anim. Sci*. 2009;8:81–101

USE OF CANINE ADENOVIRUS TYPE 2 (CAV-2) AS A POTENTIAL VACCINE VECTOR IN THE EQUINE RABIES IMMUNOPROPHYLAXIS – PRELIMINARY STUDIES

Remus Gabriel PLEŞCA, Bogdan-Ionuț OLĂERIU,Gheorghe SAVUŢA
USAMV Iasi, Aleea MihailSadoveanu nr. 6-8,
remus_plesca@yahoo.com

Abstract
Usually horse rabies has been reported occasionally in recent years and only in certain geographical areas. Although there are commercial inactivated vaccines in place to ensure protection against rabies virus in horses, but searching for a more economically viable formulation for use in developed countries is always a priority. Currently rabies vaccines produced by means of molecular biology are described. This study describes the testing of a canine adenovirus type 2(CAV-2) that expresses a rabies viral epitope (G glycoprotein) in an equine rabies model. Five horses were inoculated with the recombinant vaccine intramuscularly. We evaluated the quality of specific humoral response by ELISA anti-rabies and activity evaluation of cellular immunity was made by dosing IFNγ. Horses vaccinated with $10^8 TCID_{50}$/ml, developed a humoral response short and not intense that not protect the animals against rabies infection, and the IFNγ level remains constant the duration of the experiment. The recombinant virus stimulated an effective low level of antibody response in the immunized horses after a single intramuscular inoculation.

Keywords: CAV-2,rabies, vaccine, horse, vector

Introduction

Each year are 50000-55000 people dying from rabies worldwide, with 25000-30000 human deaths in India alone and over three billion people continuing to be a risk of rabies virus infection in over 150 countries in this century. The number of human deaths and the circumstances by which these deaths continue to occur are extraordinary, with over 95% of rabies victims reported residing Asia and Africa where the spread of canine rabies is not under control and is far from being eliminated. In other parts of the world, largely in developed countries where elimination of canine rabies has been achieved, there are models to be followed and lessons learned that will challenge epidemiologists and molecular virologists alike in the future as they apply new techniques to achieve the elimination of canine and human rabies worldwide (4).

Rabies in the horse is a relatively uncommon disease. In 1981 the number of rabies cases peaked in the horse industry at 88 and has leveled off to about 40 cases for the past few years. Although rabies in horse in United States is low (5%), the potential for human exposure makes it important to discuss its prevention and control (6). Usually fewer than 100 horses are infected in USA every year, prevention is key and is centered on vaccination of all animals residing on the farm and minimizing contact between wildlife and horses.

Avirulent canine adenovirus type-2 (CAV-2) is an effective tool for gene delivery and expression (1, 5). The biological featuresof adenoviruses show that they are able to infect a broad range of cell types, but their genomes do not become integrated into the host genome, therefore adenoviruses are considered to be safe vaccine vectors for humans and other animals (3).Research on thestructure, replication and transcription of adenoviruses has been extensive. When foreign genes are inserted into the non-essential E3 region of CAV-2, replication of the recombinant adenovirus is notimpacted, resulting in high virus titers and

high-level gene expression (2).

Materials and methods

Vaccines

CAV-G^+ vector was transfected in canine DKcré cell line where caused the appearance of characteristic adenoviruses citopatogen effect as shown in another study (data not available). RecCAV-G^+ purification was done using the CsCl method and titration CAV-2 was made on DKcré cells using formula Reed-Muench and RT-PCR leading to a titer of 2,5 x 10^{10} $TCID_{50}$/ml. Concentration used in this experiment was $10^8 TCID_{50}$/ml in 2 ml dose intramusculary.

Nobivac Rabies is an inactivated, adjuvated vaccine against rabies, prepared in cell culture. This vaccine is highly immunogenic and offers complete protection that lasts at least 3 years. Each dose contains ≥ 2 mouse potency I.U. of inactivated rabies virus, strain Pasteur, adjuvated with aluminiumphosfate. Every cat was received one dose of 1 ml Nobivac Rabies intramuscularz in a single point.

Horses

Was used 11 domestic horses (6 male and 5 females, 1-10 years age) were purchased from Faculty of Veterinary Medicine, Iasi and used in this study. The horses, randomly assigned in two experiment groups (five horses per group). The horses had previously disinfected externally and internally with preparate Telmin, but they had not been vaccinated against rabies, this is confirmed by specific neutralizing antibody assay of the sera. All horses were housed in stables located in practice center Vatra Dornei, Faculty of Veterinary Medicine in Iasi, Romania.

Vaccination of horses and bleeding

On day zero were vaccinated 10 horses, which were grouped in two groups of five horses per group. Group 1 of horses were vaccinated with $10^8 TCID_{50}$/ml CAV-G^+vaccine, group 2of horses each received a dose of inactivated vaccine Nobivac Rabies produced by Intervet and last group (one horse) was unvaccinated against rabies. The equine received intramuscular injection of 4 ml of vaccine CAV-G^+(group 1) and 1 ml of Nobivac Rabies (group 2) at side of the neck. Before inoculation all animals were bled for a pre-immunized control. All horses was bled in seven, 14, 21, 28, 35, 42, 49 and 112 day after the initial vaccination. All sera were separated and stored at -20°C.

Rabies neutralizing antibody assay

Rabies virus neutralizing antibody in cat sera were determined using ELISA Platelia Rabies II usum veterinarum and the results were expressed in International Units (UI)/ml of rabies virus neutralizing antibodies (protective titer 0.5 UI/ml) as described by (7).The unknown sera as well as the calibrated Positive Controls or the QuantificationStandards are distributed in the glycoprotein coated wells of the microplates. Duringincubation of one hour at 37°C, anti-rabies antibodies present in the sample bind tothe glycoprotein coated to the microplate wells. After incubation, unbound antibodiesand other serum proteins are removed by washings. The conjugate (protein A labeled with peroxidase) is added to the microplate wells. During a second incubation of one hour at 37°C, the labelled protein A binds to the anti-rabies-antibody-antigen complexes attached to the microplate wells. The unbound conjugate is removed by washings. The presence of immune complex is demonstrated by the addition of a solution containing a peroxidase substrate and a chromogen, initiating a color

development reaction. After 30 min. incubation at room temperature, the enzymatic reaction is stopped by addition of a solution H2SO4 1N. The optical density reading obtained with a spectrophotometer set at 450 - 620 nm is proportional to the amount of anti-rabies antibodies present in the samples. A standard curve is constructed using the Quantification standards (S1 to S6), obtained by serial dilutions of the R4b calibrated Positive Controls. The optical density values for the unknown samples are compared with the Positive Controls. Sera titres in quantification tests are obtained after a direct reading on the standard curve and are expressed as Equivalent units per ml (EU/ml), unit equivalent to the international units defined by seroneutralization.

ELISA for equine IFNγ

This test is for quantitative determination of native and recombinant equine IFNγ in solution, cell culture supernatant and serum/plasma samples. The two Abs cross react with native ovine IFNγ and native and recombinant bovine IFNγ. Antibodies are supplied in sterile-filtered (0,2μm) PBS with sodium azide (0,02%). Concentration of equine IFNγ standard range is 0,1-10000 pg/ml and no international standard exists for calibration. The guidelines is described by the MabTech AB.

Results and discussions

In this experiment we tested the safety of an immunological preparation represented by CAV-2 adenoviral vector containing the transgene of interest glycoprotein G rabies virus after intramuscular administration to equine. Thus, we tested the effectiveness of rabies vaccine produced by recombinant DNA technology in domestic equine.

Specific antibodies

We evaluated the rabies seroconversion level in horses that were vaccinated with either inactivated vaccine or with CAV-2 adenoviral vector vaccine containing the rabies virus G glycoprotein (table 1).

At 16 weeks after the rabies immunization, horses react differently depending on the type of vaccine was administered to them. Thus, horses 1-5 received a dose of replicating vaccine vector represented by CAV-G in concentration of 10^8 $TCID_{50}$ /ml, horses 7-11 received dose 2 of commercial inactivated vaccine represented by Nobivac Rabies vaccine (Intervet) and horse 6 served as negative control.

However, it appears that horses vaccinated with CAV-G^+ immune product had a specific seroconversion rate were much higher than those immunized with commercial product Nobivac Rabies. At 16 weeks of taking the two types of rabies vaccines either dosed animals do not develop a specific humoral response level (0.5IU/ml) that would provide protection against wild virus or a challenge test (Fig.1). Dynamics of immune response during research shows that there is no uniformity in the quality of response, which can be attributed to the fact that animals are increasing rent and food in farm systems, they are not animals ”pathogen free”, or are not derived of inbred lines and their age varies from one year to 10 years.

On the other hand we can say that following vaccination with Nobivac Rabies the experimental group of five horses that are aged between one year and seven years has been detected levels of specific antibodies to provide protection against rabies.

It should be noted that none of immunological products confers immunity not too strong, and the manufacturer Intervet mentions that Nobivac Rabies vaccination in horses

was received first time at age a six months old and is necessary a booster vaccination after 21 days.

Table 1. Antibody level in horses after vaccination with CAV-G$^+$ replicative vector

Group	Horse no.	Antibody level at different time			
		W0	W4	W7	W16
CAV-G$^+$	1	<0.125IU/ml	>0.125IU/ml	<0.125IU/ml	<0.125IU/ml
	2	<0.125IU/ml	>0.125IU/ml	<0.125IU/ml	>0.125IU/ml
	3	<0.125IU/ml	>0.125IU/ml	0.5IU/ml	>0.25IU/ml
	4	<0.125IU/ml	>0.25IU/ml	<0.125IU/ml	>0.125IU/ml
	5	<0.125IU/ml	>0.5IU/ml	<0.5IU/ml	<0.125IU/ml
Nobivac Rabies	1	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml
	2	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml
	3	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml
	4	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml
	5	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml
T$^-$	1	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml

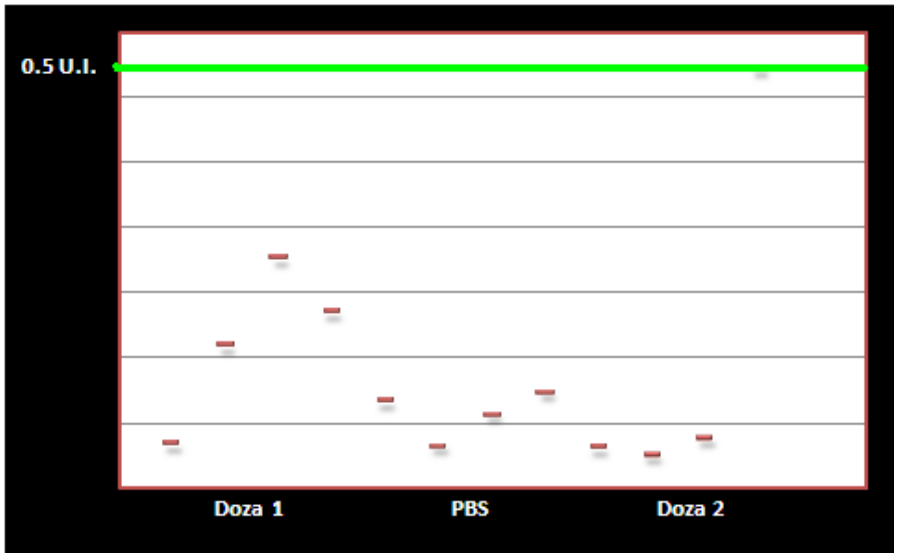

Fig.1. Serum rabies antibody level in week 16 of the experiment

If we compare horses that gave the strongest specific response immune, we observed the response is far more intense in horse vaccinated with CAV-G$^+$ vaccine than the horse vaccinated with Nobivac Rabies (Fig 2). However, the antibody level in horse vaccinated with recombinant vaccine is maintained in a set period of time (week 2 to week 6) and providing rabies seroprotection (at least 0.5IU/ml).

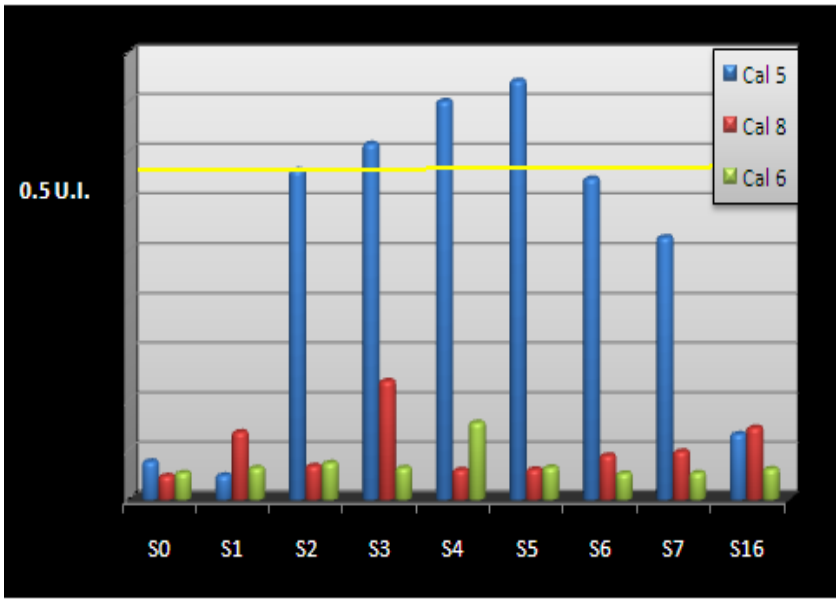

Fig. 2. Dynamics seroconversion to horses with the most effective response

IFNγ assay

To evaluate the activity of cellular immunity effectors we used a quantitative serological method that reveals the level of serum IFNγ in horses vaccinated against rabies using two immunological products.

In group of horse vaccinated with CAV-G vaccine, after dosing serum IFNγ no significant changes are noted, the amount of these cytokines remain at a constant level throughout the study. In group of horses vaccinated with Nobivac Rabies happened the same evolution of serum IFNγ.Following this test it was observed that serum levels of IFNγ is constant during the test it in widely ranging from 1pg/ml to 1000 pg/ml (Fig 3).

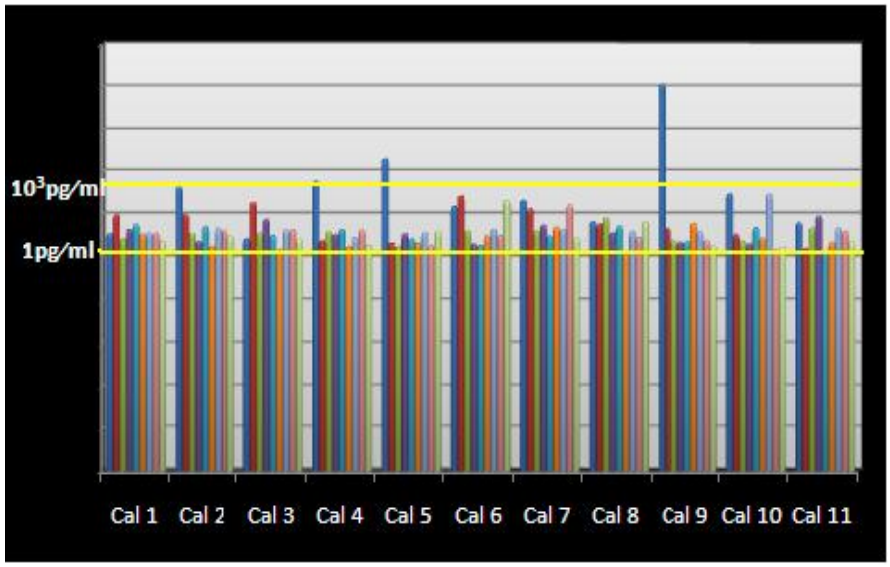

Fig. 3. The equine IFNγlevel detected during the experiment

Conclusions

In this preliminary study we evaluated the possibility of using a CAV-G vector in immunoprophylaxis of rabies in horses. Unfortunately specific humoralresponse was a short one and not provide protection against disease (<0.5IU/ml).

IFNγ levels do not change following inoculation with an inactivated rabies vaccine or a product build by recombinant DNA technology and used as a vector canine adenovirus type 2.

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References

1. Kieny, M.P., Desmettre, P., Soulebot, J.P., et al., (1987) – *Rabies vaccine: traditional and novel approaches*. Prog Vet Microbiol Immunol;3: 73–111.
2. McQuiston, J.H., Yager, P.A., Smith, J.S., et al.,(1999) – *Epidemiologic characteristics of rabies virus variants in dogs and cats in the United States*. J.Am. Vet. Med. Assoc. 2001;218(12):1939–1942.
3. Schneider, L.G., (1995) – *Rabies virus vaccines*. Dev Biol Stand;84:49–54.
4. Wunner, W. H., Briggs, D. J., (2010) – *Rabies in the 21st century*. Plos Neglected tropical disease; vol. 4, Issue 3:1-3.
5. Zhang, F., Liu, Y., Zhang, S.F., et al.,(2006) – *Survey of neutralizing antibody against rabies in dogs in China cities and suburbs*. China J Vet Sci;26(6):634–636.
6. ***www.cdc.gov
7. ***www.oie.int
8. ***www.who.org

PRELIMINARY STUDIES ON TESTING RABIES VACCINE THAT USES REPLICATIVE OR DEFECTIVE FOR REPLICATION CAV-2 VECTORS ON CATS

Remus Gabriel PLEŞCA, Irina Oana TĂNASE , Gheorghe SAVUŢA
USAMV Iasi, Aleea Mihail Sadoveanu nr. 6-8,
remus_plesca@yahoo.com

Abstract
To prevent rabies in domestic animals we need safe and effective vaccines. Replicative vectors expressing the rabies virus glycoprotein, derived from canine adenovirus have been reported to be promising vaccines in various animal models. In this paper we compare the potential of a replicative and a non-replicative vector, both based on canine adenovirus type 2 and expressing the rabies glycoprotein. We used several concentrations of CAV-gp.G vaccine in cats and we evaluated the quality of humoral immune response throughout the study. Cats vaccinated with the highest concentrations of vector , developed a humoral response that they provide protection against rabies (which is strongest after boost vaccination). Also we can see groups of cats vaccinated with high concentrations of anti-CAV 2 antibodies. These results suggest that vectors derived from canine adenovirus 2 could be considered for the development of promising vaccines in cats.

Keywords: CAV-2, vaccine, cats, vector, rabies

Introduction

Rabies is a zoonotic disease that is caused by a virus (12). It is known to be present on all continents except Antarctica and infects domestic and wild animals (20). Rabies occurs in more than 150 countries and territories. Worldwide, more than 55 000 people die of rabies every year and 40% of people who are bitten by suspect rabid animals are children under 15 years of age. Rabies is spread to people through close contact with infected saliva via bites or scratches. Dogs are the source of 99% of human rabies deaths. Most of the deaths occur in the absence of post-exposure prophylaxis. Rabies is nearly always fatal when left untreated. Every year, more than 15 million people worldwide receive a post-exposure preventive regimen to avert the disease – this is estimated to prevent 327 000 rabies deaths annually.

Domestic dog and cat rabies has been controlled by traditional inactivated vaccines. Poor vaccination coverage, sometimes with inferior quality vaccines (9) that fail to maintain persistent levels of neutralizing antibodies suggests that an inexpensive rabies vaccine that elicits long-term protection after a one-time vaccination might facilitate the control of cat rabies in developing countries. Some inactivated rabies vaccines may also potentially have certain risks due to the possibility of an incomplete inactivation of the virus or the inadvertent spread of residual rabies virus pathogenic particles (7) and (14), while some rabies vaccines have been associated with injection site fibrosarcomas in cats (13). These problems led to continued efforts to develop safer rabies vaccines using recombinant subunit proteins (16), recombinant viral vectors (11) and (17) and deoxyribonucleic acid based (DNA) vaccines (18) and (10). Recombinant live vector vaccines possess some advantages over traditional vaccines: they are innocuous (2), at no time is the rabies virus handled, and they induce suitable humoral immune responses. But due to host limit and elements such as potential safety problem for human (3), some recombinant vaccine cannot be used in domestic cats.

Recently, was developed a recombinant vaccine using canine adenovirus as vector, in

which the glycoprotein gene was cloned and expressed. The effectiveness in protection against CVS challenge was demonstrated in dogs (5). Canine adenovirus type-2 does not naturally infect cats, however, when feline kidney cell line were infected, the virus replicated sufficiently. In the present study, we show the posibile application of this recombinant virus for the immunization of cats.

Materials and methods

Vaccines

CAV-G^+ vector was transfected in canine DKcré cell line where caused the appearance of characteristic adenoviruses citopatogen effect as shown in another study (data not available). RecCAV-G^+ purification was done using the CsCl method and titration CAV-2 was made on DKcré cells using formula Reed-Muench and RT-PCR leading to a titer of 2,5 x 10^{10} $TCID_{50}$/ml. Concentration used in this experiment was 10^4 -10^7 TCID50/ml of replicative CAV-G vector and 10^5 – 10^8 TCID50/ml of non-replicative CAV-G vector, in 2 ml dose intramuscular in two separate points.

Nobivac Rabies is an inactivated, adjuvated vaccine against rabies, prepared in cell culture. This vaccine is highly immunogenic and offers complete protection that lasts at least 3 years. Each dose contains ≥ 2 mouse potency I.U. of inactivated rabies virus, strain Pasteur, adjuvated with aluminium phosfate. Every cat was received one dose of 1 ml Nobivac Rabies intramuscular in a single point.

Cats

Was used 20 domestic cats (11 male and 9 females, 1-4 years age, about 1-4 kg) were purchased from Faculty of Veterinary Medicine, Iasi and used in this study. The cats, randomly assigned to 10 experimental groups (two cats per group), were daily exercised, fed comercial cat food (Whiskas) and received tap water and libatum. The cats had previously disinfected externally and internally but they had not been vaccinated against rabies, this is confirmed by specific neutralizing antibody assay of the sera. All cats were housed indoors in confortable cages located in the animal facility at the Faculty of Veterinary Medicine in Iasi, Romania.

Vaccination of cats and bleeding

On day zero were vaccinated 20 cats, which were grouped into 10 groups of two cats per group. Eight groups of cats were vaccinated with CAV-G vector (group 1-4 received CAV-G^+ and groups 5-8 received CAV-G^0) at various concentrations, group nine (cats 17-18) each received a dose of inactivated vaccine Nobivac Rabies produced by Intervet and last group (cats 19-20) was unvaccinated against rabies. The cats received intramuscular injection of 1ml of vaccine in the hind legs. Before inoculation all cats were bled for a pre-immunized control. All cats was bled in seven, 14, 21, 28, 35, 42, 49 and 56 day after the initial vaccination. After 200 days after firs vaccination we did a boost vaccination in groups 1-8 of cats with CAV-G vector (we used the same concentration). All sera were separated and stored at -20°C. After vaccination the cats were monitored and recorded for body temperature daily for seven days and observed for clinical signs.

Rabies neutralizing antibody assay

Rabies virus neutralizing antibody in cat sera were determined using ELISA Platelia Rabies II usum veterinarum and the results were expressed in International Units (UI)/ml of rabies virus neutralizing antibodies (protective titer 0.5 UI/ml) as described by (19). The unknown

sera as well as the calibrated Positive Controls or the Quantification Standards are distributed in the glycoprotein coated wells of the microplates. During incubation of one hour at 37°C, anti-rabies antibodies present in the sample bind to the glycoprotein coated to the microplate wells. After incubation, unbound antibodies and other serum proteins are removed by washings. The conjugate (protein A labelled with peroxidase) is added to the microplate wells. During a second incubation of one hour at 37°C, the labelled protein A binds to the anti-rabies-antibody-antigen complexes attached to the microplate wells. The unbound conjugate is removed by washings. The presence of immune complex is demonstrated by the addition of a solution containing a peroxidase substrate and a chromogen, initiating a color development reaction. After 30 min. incubation at room temperature, the enzymatic reaction is stopped by addition of a solution H2SO4 1N. The optical density reading obtained with a spectrophotometer set at 450 - 620 nm is proportional to the amount of anti-rabies antibodies present in the samples. A standard curve is constructed using the Quantification standards (S1 to S6), obtained by serial dilutions of the R4b calibrated Positive Controls. The optical density values for the unknown samples are compared with the Positive Controls. Sera titres in quantification tests are obtained after a direct reading on the standard curve and are expressed as Equivalent units per ml (EU/ml), unit equivalent to the international units defined by seroneutralization.

Antibodies against canine adenovirus type 2

To verify adenoviral vector canine CAV-2 is present in sera collected from the first eight groups (16 cats) under test, ELISA was used. Sera studied were first subjected to dilutions 1/100, 1/500 and 1/2500. This test is not commercial and was designed only for its use in laboratory research purposes and was elsewhere described (8).

Neutralization test was used as an alternative to laboratory non-commercial ELISA we used. Serum antibody titrations were performed by innoculating 100 µl of sera in serial dilutions (1/5, 1/10, 1/20, 1/40, 1/80, ... $1/10^5$) in a microtiter plate. An equal volume of virus suspension virus suspension containing $100TCID_{50}$ was also added to each serum dilution. The serum-virus mixtures were incubated for 60 minutes at 37°C after which a suspension of DKcré cells (1×10^6cells/ml) in maintenance media was added. Cultures were incubated for 4 days, at which time serum neutralization titer were calculated as the reciprocal of the highest serum dilution that inhibited CPE (4).

Resultats and discussion

In our study we vaccinated cats with CAV-G replicative and non-replicative vector vaccine and we tested its effectiveness at various concentrations. Most effective concentration of recombinant vector was 10^7 $TCID_{50}$/ml for replicating vector (table 1) and 10^8 $TCID_{50}$/ml for defective for replication vector (table 2). At one of cats vaccinated with 10^7 $TCID_{50}$/ml CAV-G vector the rabies specific antibodies appear in the second week after inoculation and reaching the end of the experiment increase permanent to a level 4IU/ml. This cat is protected against rabies rabies (level greater than 0.5IU/ml) in the second week after first inoculation until the end of this experiment. Other groups of cats with was vaccinated with lower concentrations of vaccine vector replicative not develop a specific antibody level to ensure their protection from disease or challenge during eight weeks of this preliminary study. The commercial inactivated vaccine induced a high level neutralizing antibody since two weeks after vaccination (2-4IU/ml) and then a decrease a specific

antibody level in last week of the experiment, but all the injected cats showed an antibody level more than 0.5IU/ml (level of protection).

Table 1. Antibody production in cats after first vaccination with CAV-G replicative vector

Antibodies	Vaccines	Titer	Antibody level at different time (weeks)			
			0	2	4	8
Antibodies neutralizing to rabies	Recombinant ($TCID_{50}$/ml)	10^4	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml
			<0.125IU/ml	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml
		10^5	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml
			<0.125IU/ml	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml
		10^6	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml
			<0.125IU/ml	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml
		10^7	<0.125IU/ml	<0.125IU/ml	0.25 IU/ml	>0.25 IU/ml
			<0.125IU/ml	>0.5IU/ml	>1 IU/ml	>4IU/ml
	Nobivac R (U.I./ml)	2IU/ml	<0.125IU/ml	2 IU/ml	>4 IU/ml	>0.5 IU/ml
			<0.125IU/ml	>4 IU/ml	4 IU/ml	>1 IU/ml
	T°	-	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml
			<0.125IU/ml	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml
ELISA to Adenovirus	Recombinant ($TCID_{50}$/ml)	10^4	1042	1000	783	485
			1366	1396	1476	908
		10^5	1155	1112	1001	1091
			721	812	1079	1656
		10^6	459	954	2871	1656
			212	269	360	1208
		10^7	589	1294	3068	2731
			220	361	687	2281
Neutralizing to Adenovirus	Recombinant ($TCID_{50}$/ml)	10^4	<5	<5	<5	<5
			<5	<5	<5	<5
		10^5	<5	<5	<5	5
			<5	<5	<5	5
		10^6	<5	5	10	40
			<5	<5	5	10
		10^7	<5	10	20	80
			<5	<5	20	20

The antibodies against CAV-2 increased from two weeks after immunization, which is the same with appearance of rabies virus neutralizing antibody (group 4 of cats who received the highest concentration of replicative CAV-G vector). Only last week a cat in group 4 (cat no.7) developed a positive titer neutralization reaction (at least 80).

On the other hand, the most effective concentration of non-replicative CAV-G vector vaccine (10^8 $TCID_{50}$/ml) was administered to group 8 of cats (cats 15 and 16). In cat 16 is a specific humoral response against rabies since at week two (>0.5IU/ml) and the end of this preliminary experiment both cats in group eight developed a humoral response to provide protection against rabies (greater than 0.5 IU/ml). Anti-CAV ELISA test results showed us that cats 15 and 16 (group 8) developed a high rate of seroconversion level since

week two (968-1354) and increase level until week eight (2762-3059). After performing the neutralization test just cats of group eight developed a positive titer (160) in last week of this experiment.

Table 2. Antibody production in cats after first vaccination with CAV-G non-replicative vector

Antibodies	Vaccines	Titer	Antibody level at different time (weeks)			
			0	2	4	8
Antibodies neutralizing to rabies	Recombinant ($TCID_{50}$/ml)	10^5	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml
			<0.125IU/ml	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml
		10^6	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml
			<0.125IU/ml	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml
		10^7	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml
			<0.125IU/ml	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml
		10^8	<0.125IU/ml	<0.125IU/ml	>0.125IU/ml	2IU/ml
			<0.125IU/ml	>0.5IU/ml	>0.25 IU/ml	>0.5IU/ml
	Nobivac R (U.I./ml) T	2IU/ml	<0.125IU/ml	2 IU/ml	>4 IU/ml	>0.5 IU/ml
			<0.125IU/ml	>4 IU/ml	4 IU/ml	>1 IU/ml
		-	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml
			<0.125IU/ml	<0.125IU/ml	<0.125IU/ml	<0.125IU/ml
ELISA to Adenovirus	Recombinant ($TCID_{50}$/ml)	10^5	402	513	654	493
			321	300	280	160
		10^6	155	151	147	124
			116	135	181	113
		10^7	143	162	190	563
			303	312	321	373
		10^8	151	968	1407	2762
			375	1354	2774	3059
Neutralizing to Adenovirus	Recombinant ($TCID_{50}$/ml)	10^5	<5	<5	<5	<5
			<5	<5	<5	<5
		10^6	<5	<5	5	5
			<5	<5	5	5
		10^7	<5	5	5	10
			<5	5	5	40
		10^8	<5	40	40	160
			<5	40	40	160

After 200 days after the first vaccination cats in the first eight groups was revaccinated with the same vector and the same concentration of vaccine vector CAV-G (table 3). Only cat eight of group 4 which has been revaccinated with 10^7TCID_{50}/ml is protected against rabies while in group 8, both cats developed a level of rabies antibodies that ensure protection (>4IU/ml). The results of neutralization test show that cats revaccinated with 10^6 $TCID_{50}$/ml (group 3) developed a positive response (160-320), the same group 4 vaccinated with replicative 10^7TCID_{50}/ml (320->640). The same positive response (at least 80) we obtain in cats vaccinated with non-replicative vector 10^7TCID_{50}/ml (cat 13=80 and cat14=160) and 10^8TCID_{50}/ml (cat 15=640 and cat 16=640).

Table 3. Antibody production in cats after revaccination with CAV-G replicative and non-replicative vector

Antibody	Vaccines	Titre	Antibody level at different time (weeks)	
			0	8
Antibodies neutralizing to rabies	Replicative	10^6	<0.125IU/ml	>0.25IU/ml
			<0.125IU/ml	<0.125IU/ml
		10^7	>0.25IU/ml	>0.25IU/ml
			>4IU/ml	>4IU/ml
	Non-replicative	10^7	<0.125IU/ml	<0.125IU/ml
			<0.125IU/ml	<0.125IU/ml
		10^8	>4IU/ml	>4IU/ml
			>1IU/ml	>4IU/ml
ELISA to Adenovirus	Replicative	10^6	3097	3190
			728	3409
		10^7	2978	3294
			3149	3047
	Non-replicative	10^7	2934	3360
			3284	3312
		10^8	3537	3327
			3141	3047
Neutralizing to Adenovirus	Replicative	10^6	10	160
			10	320
		10^7	5	320
			80	>640
	Non-replicative	10^7	20	80
			<5	160
		10^8	>160	640
			>160	640

If we compare the response obtained after each vaccination in week eight note that the response is more intense after the booster vaccination compared to the first vaccination, indicating the effectiveness of the immune memory cells (Fig. 1).

In the present preliminary study, we examined the suitability of CAV-G, heterologous recombinant virus, for rabies immunization of cats. The results described above demonstrated that the high concentrations of the canine adenovirus type 2 encoding rabies glycoprotein can elicit specific humoral immune response in cats as well as in dogs (6,15).

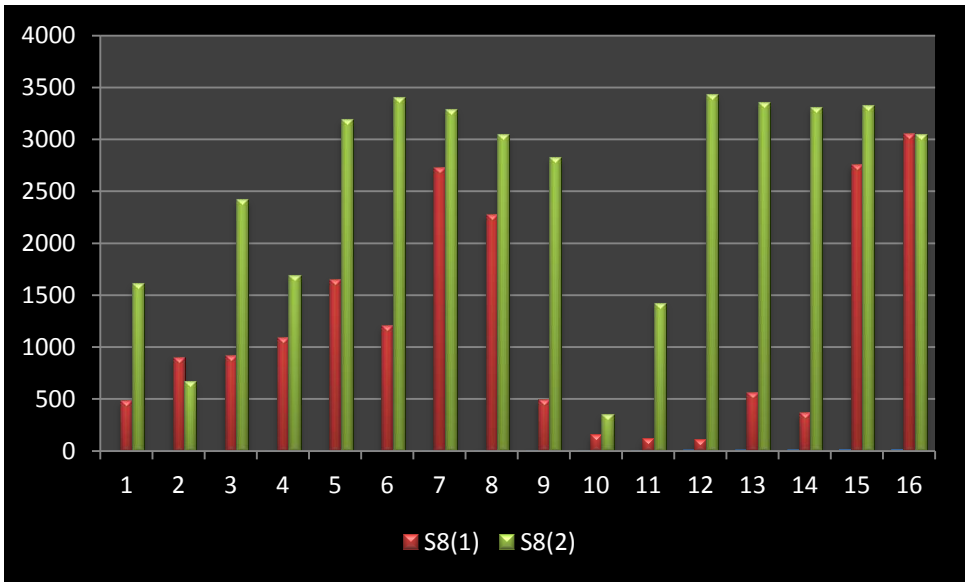

Fig.1. Antibody level of rabies in week eight after both vaccinations in cats

We also studied different concentrations of CAV-2 vector vaccine replicative (four different concentrations) and non-replicative (other four different concentrations) and analyze which of them could be better and more efficient to promote seroconversion. However, the only effective concentration vector for eliciting neutralizing antibodies against rabies virus in cat was 10^7 $TCID_{50}$ /ml for replicative CAV-G and 10^8 $TCID_{50}$ /ml for non-replicative CAV-G.

When we vaccinated cats in groups 1, 2 and 3 with lower concentrations of replicative vector (10^4 $TCID_{50}$ /ml, 10^5 $TCID_{50}$ /ml and 10^6 $TCID_{50}$ /ml), the rabies seroconversion was very weak, and does not confer protection against disease. The same thing happened when we used lower concentrations of CAV-2 replicative vector (group 5-10^5 $TCID_{50}$ /ml, group 6-10^6 $TCID_{50}$ /ml and group 7-10^7 $TCID_{50}$ /ml).

CAV-2 antibody was also proved by ELISA test and neutralization assay of the vaccinated serum sample of cats. Have responded positively to these tests provided only cats vaccinated with the highest concentrations of vector (in both vector categories). We know cats may not naturally be infected with canine adenovirus.

The cats in this trial accepted two time of vaccination, booster vaccination was did after 200 day after first vaccine administration. Data shows that if we compare results from two vaccination in the last week of experiment we observe that the humoral response after rabies booster vaccination is increased. However, develop a response to provide protection against rabies only cats vaccinated with the highest concentrations of recombinant rabies vaccine.

On the other hand the anti-CAV 2 neutralization assay give a positive response after revaccination cats in group 3 (10^6 $TCID_{50}$ /ml) and 4 (10^7 $TCID_{50}$ /ml) of cats immunized with recombinant replicative vector, group 7 (10^7 $TCID_{50}$ /ml) and group 8 (10^8 $TCID_{50}$ /ml) who received a non-replicative vaccine.

Conclusion

Replicative CAV-2 vector containing the rabies virus glycoprotein G determine specific humoral response in cats, that protection can tell only if used in concentrations at least 10^7 $TCID_{50}$ /ml .

Defective for replication CAV-G vector can protect the cats against rabies (highly 0.5IU/ml) if we have a concentration at least 10^8 $TCID_{50}$ /ml .

After booster vaccination we observe a better response in last week after revaccination of cats but are protected just groups immunized with most highly concentrations of recombinant vaccine.

As canine adenovirus type 2 is easy to produce in cell line and a high titer of virus is easy to achieve, to produce a dose of vaccine is much cheaper than other form of vaccine such as DNA vaccine, subunit vaccine and inactivated vaccine. Therefore, a recombinant rabies-canine adenovirus is a promising vaccine candidate in domestic cats.

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References

1. Bouet-Cararo, C., Contreras, V., Fournier, A., Jallet, C., Guibert, J.M., Dubois, E., Thiery, R., Breard, E., Tordo, N., Richardson, J., Schwartz-Cornil, I., Zientara, S., Klonjkowski, B., (2011) – *Canine adenoviruses elicit both humoral and cell-mediated immune response against rabies following immunization of sheep*. Vaccine; 29:1304-1310.
2. Brochier, B., Blancou, J., Thomas, I., et al., (1989) – *Use of recombinant vacciniarabies glycoprotein virus for oral vaccination of wildlife against rabies. Innocuity to several nontarget bait consuming species*. J Wildl Dis;25:540–7.
3. Ferreira, T.B., Alves, P.M., Aunins, J.G., et al., (2005) – *Use of a denoviral vectors as veterinary vaccines*. Gene Ther;12:S73–83.
4. Hamir, A.N., Raju, N., Rupprecht, C.E. (1992) - *Experimental Oral Administration of Canine Adenovirus (Type 2) to Raccoons (Procyon lotori)*. Vet. Pathol.; 29:509-513.
5. Hu, R.L., Liu, Y., Zhang, S.F., Zhang, F., Fooks, A.R., (2007) – *Experimental immunization of cats with a recombinant rabies-canine adenovirus vaccine elicits a long-lasting neutralizing antibody response against rabies*. Vaccine; 25:5301-5307.
6. Hu R.L., Zhang, S.F., Fooks, A.R., et al., (2006) – *Prevention of rabies virus infection in dogs by a recombinant canine adenovirus type-2 encoding the rabies virus glycoprotein*. Microb. Infect;8:1090–7.
7. Kieny, M.P., Desmettre, P., Soulebot, J.P., et al., (1987) – *Rabies vaccine: traditional and novel approaches*. Prog Vet Microbiol Immunol;3: 73–111.
8. Klonjkowski, B., Klein, D., Galea, S., Gavard, F., Monteil, M., Duarte, L., et al., (2009) - *Gagspecific immune enhancement of lentiviral infection after vaccination with an adenoviral vector in an animal model of AIDS*. Vaccine;27(6):928–39.
9. McQuiston, J.H., Yager, P.A., Smith, J.S., et al.,(1999) – *Epidemiologic characteristics of rabies virus variants in dogs and cats in the United States*. J.Am. Vet. Med. Assoc. 2001;218(12):1939–1942.
10. Osorio, J.E., Tomlinson, C.C., Frank, R.S., et al., (1999) – *Immunization of dogs and cats with a DNA vaccine against rabies virus*. Vaccine;17(9–10):1109–16.
11. Paoletti, E., (1996) – *Applications of pox virus vectors to vaccination: an update*. Proc Natl Acad Sci USA;93:11349–53.
12. Perianu, Tudor,(2005) – *Bolile infecţioase ale animalelor*, Viroze, vol. II, Ed. Universitas XXI,

Iaşi.
13. Rudmann, D.G., Van Alstine, W.G., Doddy, F., et al., (1996) – *Pulmonary and mediastinal metastases of a vaccination-site sarcoma in a cat*. Vet Pathol;33:466–9.
14. Schneider, L.G., (1995) – *Rabies virus vaccines*. Dev Biol Stand;84:49–54.
15. Tims, T., Briggs, DJ., Davis, RD., Moore, SM., Xiang, Z., Ertl, HCJ., Fu, ZF., (2000) – *Adult dogs receiving a rabies glycoprotein develop high titers of neutralizing antibodies*. Vaccine; 18:2804-2807.
16. Wunner, W.H., Dietzschold, B., Curtis, P.J., et al., (1983) – *Rabies subunit vaccines*. J Gen Virol;64:1649–56.
17. Xiang, Z.Q., Spitalnik, S., Tran, M., et al., (1994) – *Vaccination with a plasmid vector carrying the rabies virus glycoprotein gene induces protective immunity against rabies virus*. Virology;199:132–40.
18. Xiang, Z.Q., Yang, Y., Wilson, J.M., et al.. (1996) – *A replication defective human adenovirus recombinant serves as a highly efficacious vaccine carrier*. Virology;219:220–7.
19. ***www.oie.int
20. ***www.who.org

MILK MINERAL CONTENT AND HEAVY METAL CONTAMINATION FROM COWS WITH DIFFERENT LEVELS OF MILK PRODUCTION

Elena ROTARU, Liliana TUDOREANU, Gheorghe V. GORAN, Victor CRIVINEANU
Faculty of Veterinary Medicine Bucharest - Interdisciplinary Laboratory for the Study and Modelling of Heavy Metals Accumulation in the Food Chain, 105 Splaiul Independentei, 050097, 5th district, Bucharest, Romania, elenootaru@yahoo.com

Abstract
The objective of this study is to evaluate the concentration of minerals from milk samples from cows with different levels of milk production from an intensive diary farm from the south of Romania situated in an area with temperate continental climate. It was found that minerals concentrations in milk were not influenced by the number of lactations or the total number of day of lactation. Milk daily production per individual did not influence the total concentration of Ca, Cd, Co, Cu, Fe, K, Li, Mg, Mn, Na, Pb, Se, Sr and Zn in the milk samples however individuals with a milk daily productions under 30 litters per day produced milk with smaller Ca and K concentrations than the individuals with an over 30 litter per day production. The average mean minerals concentrations found were in accordance with the Committees of Nutrition (Codex Alimentarius, FAO/OMS) published values.

Key words: minerals, milk, cows

Introduction

Rollin (2002) found that cows from diary farms are suspected to develop mineral deficiencies due selection of individuals with high milk production potential and due to the feed quality which is influenced by the monoculture systems as well as by the soil mineral spoilage, and reduction of mineral supplements added to feed.

Musnier (2008) showed that mineral content identified in blood is an insufficient indicator for the identification of mineral cellular requirement because any physiological abnormality modifies excretion by urine, faeces and milk on one hand and on the other hand the minerals present in blood are not necessarily used by the organism. Moreover urine is not a suitable indicator of the mineral requirements of the organism because in acidosis or liver dysfunction the mineral urinary excretion is increased. The hair mineral concentration is also a poor body mineral status indicator because the cheating has a high Se content, the black hair has high Zn content and the red hair has high Cu content. Therefore the identification of mineral concentration from milk is the best method for the characterization of the mineral profile of the organism (Musnier, 2008).

The objective of this study is to evaluate the concentration of minerals from milk samples from cows with different levels of milk production from a diary farm from the south of Romania situated in an area with temperate continental climate with very hot summers (with low precipitations) and cold winters with irregular blizzards, and temperature increasing.

Materials and methods

A representative group of 15 lactating cows (Holstein) was selected for the survey. The selected animals had a milk production which varied form 15 litters /day to 53.9 litters / day (Table 1). The animals were selected to be representatives for all the lactation stages and total lactations days of the dairy farm.

The survey was conducted between December 2010 and February 2011 therefore the minerals concentrations in milk were influenced by the feed specificity of winter months.

Table 1. Characterization of the animal group selected for the survey

Item No.	Identification No.	Group	Birth date	Lactation No.	No days of lactation	No Litters per day
1	1824	7	24/01/2005	3	83	15
2	59	5	30/01/2007	2	117	16.5
3	1445	5	20/04/2006	2	41	23.2
4	7601	7	27/05/2006	3	200	31.2
5	653	3	25/12/2008	1	87	33.8
6	145	5	1/04/2007	2	56	35.5
7	99	9	28/03/2005	4	121	35.9
8	445	7	13/07/2008	1	245	38.9
9	9079	4	13/07/2004	4	69	40.8
10	3333	5	21/02/2005	4	126	41.3
11	169	5	1/05/2007	2	141	42.2
12	5424	7	17/11/2004	4	60	43.9
13	8877	12	22/10/2004	4	108	47.7
14	9950		20/02/2006	3	126	51.4
15	9851	7	18/01/2005	4	129	53.9

The milk samples of 20 ml were collected manually (fig. 1), after eliminating the first jets, before milking itself which can hinder interpretation of results. Many studies recommended sampling in order to identify the mineral content of total milk obtained from one milking (for livestock milk mixture). Absence of bacteriological determinations allowed a mere sampling but it was followed that the samples were not contaminated with blood (haemolactation). For sampling were used plastic bottles (including lids) to avoid possible contamination of samples by oligominerals contained in rubber or glass.

Milk samples preparation and analysis

For each sample one ml of milk sample was added HNO_3 and H_2O_2 and than the samples was digested by microwave digestion at 190^oC for a total time of 30 minutes. Suitable dilutions were made to over range elements to ensure they fell within the calibration range.

Three standards of 0.01 ppm, 0.1 ppm and 50 ppm were obtained from a multielement standard (MERK) containing 1000 mg/l of Ag, Al, Ba, Ba, Be, Bi, Ca, Cd, Co, Cr, Cu, Fe, Ga, K, Li, Mg, Mn, Na, Ni, Pb, Se, Sr, Tl, Zn.

The instrument was calibrated using a blank and three standards for each element, after inspection a linear fit was applied to all elements. Samples were analyzed in a single sequence. The sample data was measured by interpolation.

Instrument Configuration: Pump rate 50 rpm; Nebulizer Standard concentric; Nebulizer Argon Pressure 0.6 L/min; Spray Chamber Standard cyclonic; Centre tube 2.0 mm.

RF Forward Power 1150 W; Purge Gas Argon; Coolant flow 12 L/min; Auxiliary flow 0.5 L/min; Integration times: High Wavelengths 5 seconds; Low Wavelengths 15 seconds; Analysis mode: Speedy.

Results and discussions

The mean concentration and median concentration for Ca, Cd, Co, Cu, Fe, K, Li, Mg, Mn, Na, Pb, Se, Sr, Zn, are presented in table 2. The samples concentrations of Ag, Al, Ba, Ba, Be, Bi, Cr, Ga, Li, Ni, Tl were below the detection limit of the instrument and below 0.001 (mg/l) in the crude samples.

The statistical analysis of the minerals concentration in milk revealed that several samples had abnormal (outliers) mineral concentrations compared to the mineral values found for the survey group (table 2).

Table 2. Outlier values for some minerals found after statistical analyses of the data

Sample	Element	High value outlier (mg/L)	Small value outlier (mg/L)	No Litters per day	Pathology	Lactation No	No days of lactation
1	Ca	----	601.5	14.0	chronic endometritis	3	83
	K	----	842.0				
	Mg		70.0				
2	Cu	4.55	----	16.5	-	2	117
	Li	1.80	----				
4	Fe	14.90	-----	31.2	-	3	200
5	Fe	10.01	----	33.8	endometritis, vaginitis	1	87
9	Ca	1491.00	-----	40.8	endometritis	4	69
12	Li	1.25	----	43.9	endometritis, antibiotics	4	60
14	Cu	6.60	----	51.5	endometritis	3	126
	Cd	0.15	-----				
15	Mn	0.16	----	53.9	-	4	129

Mean and median concentration for minerals in milk samples are illustrated in table 3.

Table 3. Mean and median concentration for minerals in milk samples compared to the recommended values by the Comittes of Nutrition, S.J. Fomon, (Codex Alimentarius FAO/OMS)

Element	Mean concent. (mg/l)	Median concent. (mg/l)	Std Err Mean (mg/l)	Upper 95% Mean (mg/l)	Lower 95% Mean (mg/l)	Min (mg/l)	Max (mg/l)	FAO/ OMS (mg/l)
Ca	**1058.00**	1074.83	47.61	1157.02	959.01	601.571	1487.582	1250
K	**1449.96**	1563.89	55.48	1565.25	1334.88	673.554	1711.532	1300-1500
Fe	**2.89**	1.62	0.75	4.46	1.33	0.31	14.90	0,5
Cu	**0.77**	0.23	0.34	1.48	0.06	0.11	6.60	600
Li	**0.24**	0.12	0.09	0.43	0.05	0.06	1.80	
Mg	**139.27**	133.30	6.47	152.75	125.80	70.00	227.70	90-240
Mn	**0.05**	0.03	0.01	0.07	0.03	0.00	0.16	10-40
Na	**541.49**	438.40	72.54	692.34	390.63	269.60	1759.50	350-500
Pb	**0.33**	0.31	0.03	0.40	0.26	0.05	0.69	20-80
Cd	**0.06**	0.04	0.017	0.10	0.02	0.01	0.40	1-20
Co	**0.15**	0.14	0.01	0.18	0.11	0.00	0.33	0,4-1,1
Se	**0.43**	0.44	0.04	0.52	0.34	0.00	0.81	
Sr	**0.41**	0.37	0.03	0.47	0.34	0.24	1.00	
Zn	**3.847**	3.84	0.16	4.19	3.50	2.35	5.35	3-5

For the winter period (December to February) an intensive dairy farm system might produce milk with average minerals concentrations as illustrated in table 4.

A statistical analysis of the data by ANOVA was used to identify the influence of lactation number (1, 2, 3, or 4) on the mineral concentrations in milk. No significant difference was found between minerals concentration in milk due to the number of lactation (table 5).

The outlier values obtained for Cu for lactation 3 and 4 increased the mean value of the concentration and also the standard error, therefore the calculation of F ration was influenced, resulting $p > 0.58$. Thus it can be concluded that Cu concentration in milk is not influenced by the lactation number. In order to enhance the statistical significance of the data a larger group of animals is recommended for evaluation with an equal number of individuals for each lactation number.

Table 4. Some values for several chemical elements in milk samples in mg/L (Comittes of Nutrition, S.J. Fomon, FAO/OMS Codex Alimentarius)

Elements	**Milk levels (mg/L)**
Na	350-500
K	1300-1500
Cl	1100-1300
Ca	1250
P	960
S	300
I	47
Fe	0,5
Zn	3-5
Cu	600
Cr	13
Co	0,4-1,1
Mg	90-240
Mn	10-40
Mo	18-120
St	100-200
Ni	30
Cd	1-20
Pb	20-80
As	30-60
B	500-1000

Similar results were obtained for ANOVA analyses for minerals concentration by milked litters per day. The data were analysed by grouping the individuals by four levels of production: 20-30 litters/day; 31-40 litters/day, 40-55 litters/day and less than 25 litters/day groups with milk production.

Due to the small number of individuals in the survey group it is unclear how daily milk production is influencing the calcium and potassium concentration in milk (fig. 2).

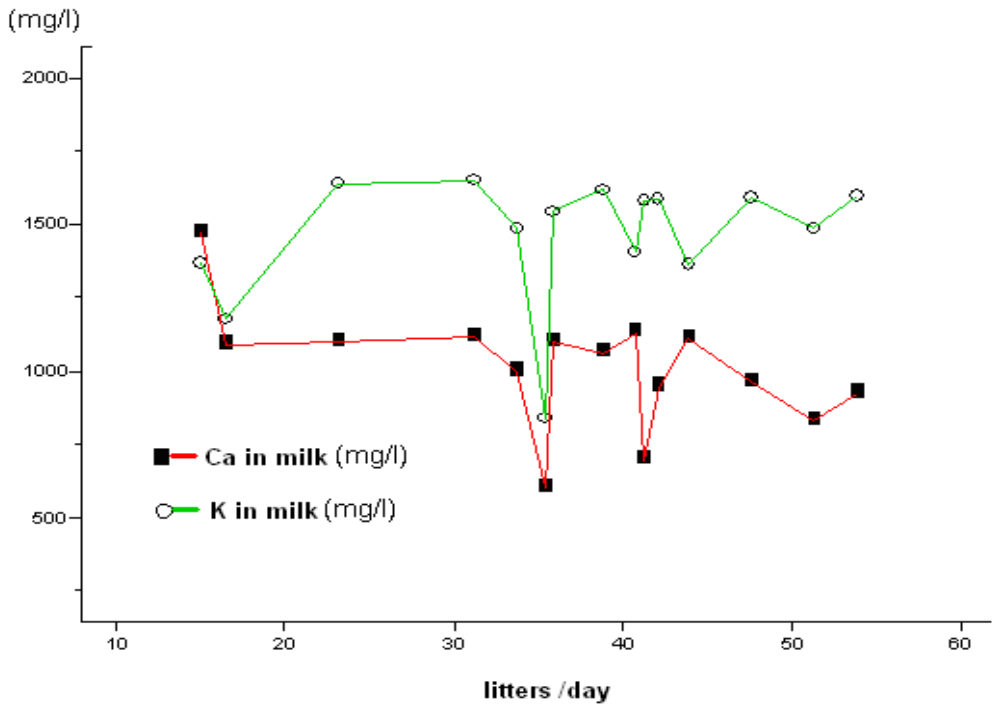

Fig. 2. Variation of calcium and potassium concentration in milk samples by daily milk production for each individual from the survey group

Table 5. ANOVA for mineral concentrations in milk samples by the lactation number

				Mean concentration (mg/l)*			
	Ca	**K**	**Fe**	**Cu**	**Li**	**Mg**	**Mn**
p (prob >F)	>0.65	>0.47	>0.82	>0.58	>0.42	>0.65	>0.86
lactation 1	1031.25 **a**	1554.50 **a**	5.29 **a**	0.21 **a**	0.10**a**	142.22 **a**	0.03 **a**
lactation 2	934.00 **a**	1314.25 **a**	2.47 **a**	0.46 **a**	0.11 **a**	122.02 **a**	0.02 **a**
lactation 3	1140.66 **a**	1503.50 **a**	1.74 **a**	2.35 **a**	0.13 **a**	146.48 **a**	0.03 **a**
lactation 4	988.58 **a**	1516.00 **a**	3.52 **a**	0.92 **a**	0.58 **a**	132.35 **a**	0.04 **a**

				Mean concentration (mg/l)*			
	Na	**Pb**	**Cd**	**Co**	**Se**	**Sr**	**Zn**
p (prob >F)	>0.56	>0.29	>0.51	>0.15	>0.48	>0.78	>0.48
lactation 1	393.82 **a**	0.30 **a**	0.04 **a**	0.14**a**	0.42 **a**	0.35 **a**	3.87 **a**
lactation 2	430.61 **a**	0.39 **a**	0.03 **a**	0.16**a**	0.33**a**	0.35 **a**	4.25 **a**
lactation 3	560.73 **a**	0.48 **a**	0.07 **a**	0.22**a**	0.61**a**	0.37 **a**	3.33 **a**
lactation 4	469.36 **a**	0.27 **a**	0.05 **a**	0.10**a**	0.41**a**	0.41 **a**	3.68 **a**

*Comparisons for all pairs using Tukey-Kramer HSD□□□□□□ 0.05; Levels not connected by same letter are significantly different. Comparison can be made only within the same column.

a) Iron

The mean concentration for Fe in milk samples is much higher as the concentration suggested by FAO/OMS (0,5mg/l - table 4).

Analysis of individual pathological history systematized in Table 4 showed inflammation processes in cows in which milk iron values were highest. Thus in cows with no history of inflammatory pathology values were within normal limits compared to those who were present such pathological events. These were explained by the milk sample content of macrophages activated by inflammatory processes, which accumulates iron without release it in the circulation (Bordessoule, 2006).

Table 6. The Fe concentrations in milk samples and the pathology of the cows from the selected group

Item No	**Lactation No**	**No days of lactation**	**No Litters per day**	**Milk Fe level mg/L**	**Normal Fe level**	**Recent pathological history**
1	3	83	15.0	21.3	Kosikowsky & Mistry, 1997, = 0.5 mg/l	Chronic endometritis
2	2	117	16.5	0.31		not
3	2	41	23.2	4.07		Endometritis
4	3	200	31.2	0.57		not
5	1	87	33.8	10.01		Endometritis, vaginitis
6	2	56	35.5	4.79		Endometritis
7	4	121	35.9	1.49		Pododermatită,

						mastitis
8	1	245	38.9	0.57		Pododermatită
9	4	69	40.8	1.26		Endometritis
10	4	126	41.3	0.68		Not
11	2	141	42.2	0.72		Pododermatită
12	4	60	43.9	3.03		Endometritis, antibiotic
13	4	108	47.7	0.74		Not
14	3	126	51.4	2.52		Endometritis
15	4	129	53.9	5.24		No??

b) Calcium

Calcium is a constant component of the milk that is not affected by its intake, but rather by the hormones "symphony" of its storage and use at the bone level. Analysis of data obtained allowed to conclude that there were registered decreasing levels of calcium in the analyzed samples related to the milk roduction. They were ranging from the lower milk production to the highest one. Otherwise, in cows that had an average milk production of 53.9 L/day the calcium levels registered a lower value compared to those that had an average milk production of 15 L/day. Raported to normal values of 1250 mg/L (FAO / WHO) there were variations from 1471 mg/L in samples from cows that had the lowest milk production to 926 mg/L in the case of samples from cows that had the highest milk production. Feed calcium supplementation should be correlated to the milk production. Predisposition of many cows to hypocalcemia is sometimes difficult to explain. It could be inplicated problems to the target tissue, the response to hormonal stimuli, age-related calcium absorption reduction or the decline of the number of 1.25 dihydroxyvitamin D receptors.

c) Potassium and sodium

Potassium is the most abundant biomineral in milk. In cows, the milk elimination way could reprezent 12% of the total body loss (5). In the case of performance productions, milk elimination levels depends on the feed potassium content and production level. In such situations, potassium elimination can reach 15-40% compared to 12% in a cow with a normal productive quantity (12). Values registered in this study were for the majority of samples in the normal range of 1300 - 1500 mg/L. The lowest value was registered in the cow no. 6, without being able to make a correlation with the milk production. Lee et al (1989) showed that the deficit in magnesium promotes intracellular depletion and urinary excretion of K^+. In addition, hypomagnesemia stimulates the secretion of aldosterone and renin. Corroborating with the low magnesiumlevel in sample no. 6, it could be explained the potassium level in this milk sample. On the other hand several studies highlights the many factors of variation of body potassium levels. Thus, stress is associated with adrenal response and increased excretion of aldosterone. This increases the potassium urinary excretion that represent a major loss for the body. Sodium shows deviations above normal levels (350-500 mg/l) without the posibility of correlation to the mil production per day.

Some studies showed that the K / Na ratio in milk is 3, but it could vary considerably depending on the stage of lactation and the presence of microbial infection of the mammary gland (3). The results obtained in this study did not confirm this statement. The values

obtained in this study could not be differentiated either by an mammary gland infection in cow no. 7 or the different stages of lactation (Table 5).

d) Magnesium

Analysis of the results obtained in this study allowed the conclusion that there were variations of magnesium values in analyzed milk samples; they were within the normal range (90-240mg/L) except for milk sample no. 6. Milk magnesium low values, even if they were within normal limits, may be related to feed used during the stabulation.

Table 7. K / Na ratio values in samples of milk

Sample No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**
K (mg/L)	1347	1182,5	1643	1650,5	1486	842	1544
Na (mg/L)	726	550	350,25	473,6	407,55	297,7	402,4
K/Na	***1,89***	***0,2***	***4,69***	***3,48***	***3,64***	***2,82***	***3,83***

Sample No.	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
K (mg/L)	1623	1407	1584,5	1589,5	1364,5	1595	1486	1601
Na (mg/L)	380,1	617	341,65	524,5	692	269,65	482,6	493,5
K/Na	***4,26***	***2,28***	***4,63***	***3,03***	***1,97***	***5,91***	***3,07***	***3,24***

e) Zinc

Savu (1999) and Goran (2009) reported the romanian legislative tolerance for zinc that should not exceed 5 mg/L milk (normal 3-5 mg/L). In the samples analyzed zinc levels exceeded this value only in sample no 6. Variations of the other values can not be correlated with the daily milk amount. Loss of zinc by milk elimination way in cows was reported by Ghergariu as a particular situation. In cows mammary gland has homeostatic value for zinc. In case of deficiency, zinc elimination in milk reduces considerably while in the case of zinc exces its milk elimination increases to an intake level equivalent to 600 ppm. After that zinc milk elimination caps as the mammary gland has the capacity to discriminate against large concentrations in the interest of protecting the newborn ().

f) Strontium

Savu (1999) indicated the presence of this alkaline earth element in the soil and plants and points out that the total amount of ingested strontium pass in cow's milk a proportion that depends on the individual production milk amount. Variations in the strontium levels in milk samples analyzed did not correlate to the amount of daily milk production. The increased strontium level registered in cow no. 1 was surprising in the context of the other results.

Milk is the main product of animal origin in which strontium is concentrated due to chemical similarity to calcium. In the body, strontium follows calcium metabolism and is

found in different organs and body fluids. Also, it is excreted in urine and milk. In all stages of lactation strontium competes with calcium, and absorption and milk transfer of strontium is therefore inversely proportional to the feed calcium content. In addition, milk calcium (hence strontium) is found mostly in the form of caseinate, corelated to the amount of protein ingested by the animal. Strontium (as calcium) bind casein and precipitate it, and increase the risk for its transition in cheese.

Conclusions

1. ANOVA analyses revealed that the concentrations of Ca, Cd, Co, Cu, Fe, K, Li, Mg, Mn, Na, Pb, Se, Sr, Zn are not influenced by the lactation number or the daily milk production of individuals. However for physiological and pathological reasons it is recommended to consider a larger group of animals for this kind of surveys.
2. An organism that had as genetic and physiological priority milk secretion in quantities far greater than the requirements of the species could register milk composition deficiencies. This could demonstrate the priority mobilization of minerals (homeorhesis) to the mammary gland in conditions of productive performance in lactating cow.
3. One milk sample had abnormal high Zn values exceeding the maximum admissible values in milk for human consumption. Furtherer investigation is needed to elucidate this finding .
4. Strontium presence was detected and further investigation is required in order to identify its provenience which we suspect to be from feed and water as well. The larger strontium concentration was identified for the same sample as the one having the largest calcium concentration which confirms that strontium is using the same pathways
5. The milk total concentrations of Ca, Cd, Co, Cu, Fe, K, Li, Mg, Mn, Na, Pb, Se, Sr, Zn, is not influenced by daily milk production. However all minerals concentrations were reduced in the milk sample from the individual with less than 20 litter of milk per day production, which might be explained by the feeding programme which differs according for each caw according to its daily milk production.

Bibliography

1. Ghergariu S. - Oligominerale şi oligomineraloze, Ed. Academiei, Bucureşti, 1980.
2. Goran G.V. - Toxicologia produselor alimentare, Ed. Printech, Bucureşti, 2009, 165-177.
3. Guenguen L., Rombauts P., 1961, Dosage du sodium, du potassium, du calcium et du magnésium par spectrophotométrie de flamme dans les aliments, le lait et les excreta Ann. Biol. anim. Bioch. Biophys. Volume 1, Number 1, 1961, 80 - 97.
4. Kadzere C.T., Murphy M.R., Silanikove N., Maltz E., 2002, Heat stress in lactating dairy cows: a review. Livestock Production Science. Vol. 77: 59-91.
6. Lee Russel Mc Dowell. Minerals in Human and Animals Nutrition, Academic Press, 1989, 98-114.
7. Musnier L. – Bovins – Bien evaluer les carences en oligo-elements, La Depeche veterinaire, No. 976, 16 fevrier 2008.
8. Rollin F. – Mise en evidence des carences en oligo-elements dans les exploatations bovines , Congresso de Ciencias veterinarias – Proceeding of the Veterinary Sciences Congress, 2002, SPVC Oeiras, 10-12 out, pp 95-106.
9. Sattler N., Fecteau G., Girard C., Couture Y., Tremblay A. 2001. Evaluation des équilibres potassiques chez la vache laitière et étude des variations journalières et selon le stade de production. Can Vet J. 42; 107-115.

10. Savu C. – Poluarea mediului şi prezenţa substanţelor toxice în alimente, ed. Semne, 1999, Bucureşti, 26-28, 83-116.
11. Schneider P.L., Beede D.K., Wilcox C.J. 1986. Responses of Lactating Cows to Dietary Sodium Source and Quantity and Potassium Quantity During Heat Stress. J. Dairy Sci. 69,1: 99-110.
12. Valarcher J.F., Schelcher F., Fourcras G., Espinasse J., 1995, Equlibre hydroionique: mecanismes regulateurs et patologie. Point Vet., 27 (numero special), 697-704.
13. Willard M.D., 1989, Disorders of Potasium Homeostasis. Veterinary Clinics of North America: Small Animal Practice, Vol. 19, 2: 241-259.

FINE AND IMMUNOHISTOCHEMICAL STRUCTURE OF THE PANCREAS IN OSTRICH (*STRUTHIO CAMELUS L*)

H.F ATTIA*, I.M.A EL-ZOGHBY
Histology & Cytology Dept. Faculty of Veterinary Medicine. Benha University
*Department of medical laboratories and Medical biotechnology.
Faculty of applied medical sciences, Taif university
Corresponding author. Drhossam222@hotmail.com

Abstract
Thirteen samples of the ostrich pancreas were used to show the histological, histochemical, immunohistochemical and fine structure of the pancreas. H&E, Gomori method and Crossman's trichrome; PAS and alcian blue stain were used for histological and histochemical demonstration respectively. Immunohistochemical detection of alpha, beta and delta cells was denoted by cytoplasmic reaction. It was covered by thick CT capsule that lined by mesothelial cells. The pancreatic acini consisted of pyramidal cells with basophilic nuclei and prominent nucleolus and large, coarse granules (Zymogen granules) in the apical part of the cytoplasm. Numerous islets of langerhans were distributed between the pancreatic acini. It consisted mainly of three types of cells, Alpha cells (A cells) which were numerous, columnar in shape with oval nuclei and uniform granules. Beta cells (B cells) which were polygonal in shape with small basely situated nuclei .It characterized by large granules of different shapes that, were surrounded by haloes. Delta cells (D cells), which were triangular in shape and characterized by dark cytoplasm and large uniform granules. The islets cells were located also as dispersed cells between the pancreatic acini. Positive immunohistochemical reaction appeared in the cytoplasm of alpha, beta and delta cells.

Key words. Pancreas-ostrich-immunohistochemistry-fine structure

Introduction

The ostrich are used principally for the production of meat of high nutritive value**,** and of low cholesterol level (Anon, 1998). Furthermore ostrich are used for the production of hide and feather (Horbanczuk, Sales, Cleeda, Konecka, zinab and Kawaka 1998). The avian and the ostrich pancreas are located on the right side of the abdominal cavity in all birds. It is tightly bound by mesentery and blood vessels positioned between the descending and the ascending duodenal loops (Sturkie, 1986). It is composed of two main lobes, dorsal and ventral, which extend from the apex of the duodenal loop to the point where the pancreatic ducts enter the distal duodenum. Another smaller lobe, extending from the head of the pancreas towards the spleen has been termed as the splenic lobe (Bezuidenhout, 1986).

Numerous papers were found to be dealing with the histological structure of the pancreas (Watanabe et al., 1975 ;Guha and Ghosha, 1978; Ruffier . Simon and Ridcau 1998 and Nurhayat et al., 2004) of geese (Nagasao et al., 2003), of pigeon (Mihail and Cracium 1982) and of duck, (Peter et al., 1970 ;Gomez and Garcia,1974 and Do Prado et al.,1989) . In spite of this, papers were discussed the fine histological structure of the pancreas in ostrich were rare (Slornelli, Ricciardi, Miragliotta, Coli and Giannessi, 2006 and Ben Bacha et al., 2007).

The current study, therefore aimed primary to investigate the histological, histochemical, immunohistochemical and fine structure of the ostrich's pancreas with special reference to the cellular contents of both exocrine and endocrine parts.

Materials and methods

Thirteen specimens of the different lobes of the pancreas were collected and examined for free of any diseases and abnormalities. They collected from ostrich abattoir in Cairo. The specimens immediately immersed in the fixatives. Many fixatives were used; Susa. Bourn's and 10 % buffered neutral formalin fluids. The specimens were dehydrated, cleared and embedded in paraffin. Sections (4-5 u) were cut and stained with Hematoxyline & Eosin for general characters; Crossmon's trichrome for identification of collagen fibers ; Silver impregnation technique for identification of reticular fibers and combination of PAS and alcian blue for identification of both acid and neutral mucopolysacharides. These methods after Bancroft, Cook, Stirling and Turner (1994).

The specimens were prepared for staining with immunohistochemical stains using specific antiserum for (the endocrine islets of langerhans cells), in institute of cancer, Cairo University.

The endogenous peroxidase and non-specific binding sites for antibodies were suppressed by treating sections with 0.5% hydrogen peroxide for 30 min and 10% normal rabbit serum for 10 min at room temperature , respectively. Furthermore, sections were processed for standard immunohistochemical techniques using the avidin-biotin-complex (ABC) method (Hsu, Raine and Fanger, 1981). We used peptide specific antibodies isolated from mammalian species for a number of reasons. First ,there was very high amino acid sequence homology between the mammalian-derived hormones used in our study and their bird counterparts. Second, previous studies demonstrated that mammalian-derived antibodies such as rabbit anti-somatostatin and rabbit anti-glucagon , as used in our investigation, cross reacted with their bird counterparts (Polak,., Pearse, Adams and Garand, 1974). Negative controls were carried out by incubating sections with phosphate-buffered saline (PBS) instead of the primary antiserum. Positive controls were also conducted with tissue sections from the gastrointestinal tract of rabbits known to contain the hormones studied. The sections were incubated in primary antisera in PBS-containing bovine serum albumin) 2.5 %and Triton X-100 (0.2%) for 1 h at room temperature. Subsequently, the binding of primary antisera was detected using rabbit anti-mouse antisera and Strept ABC. Finally, the chromogen protocol was used to reveal the distribution of bound peroxidase (Ku et al.,2000).

For TEM a very small pieces of Ixlxl mm were fixed in 2.5% glutaraldehyde in IM phosphate buffer (pH. 7.3) for 24 hours then post fixed in cold IM phosphate buffered 1% osmium tetroxide (pll. 7.3) for 3 hours, rinsed in phosphate buffer for 30 minutes then dehydrated (Hayat,1986). Semi-thin sections were stain by Toluidine blue. Ultra thin sections were obtained and mounted on copper grids then stained with uranyl acetate and lead citrate (Reynolds, 1965 and Hayat, 1986). For the TEM in college of science using Sumy Electron Optics SEO at 25 Kv.

Results

The pancreas of the ostrich was found to be a typical secretory gland housing islets of hormones secreting cells. The former appeared in the form of a compound acinar gland whose

adenomeres were packed with cuts of the draining duct system to form lobes. The pancreas was covered by a thick capsule of connective tissue mainly consisted of collagen (Fig.1) and reticular fibers. This capsule was covered by flat mesothelial cells (Fig.1a). From the capsule, thin incomplete and ill developed CT septa penetrated the architecture of the pancreas dividing it into numerous pancreatic lobules (Fig.2). These lobules appeared incomplete or small circumscribed lobules surrounded by thick layer of CT. Arterioles, venuoles and ducts were located in the course of the CT septa.

The parenchyma of the pancreas was consisted of exocrine part and endocrine part. The exocrine part was consisted of the secretory acini and the duct system.

The secretory acini were consisted of 4-5 large pyramidal cells oriented around narrow lumen with basal, basophilic and vesicular nuclei with prominent one or more nucleolus. The cytoplasm was acidophilic and appeared crowded with coarse granules in the apex of the cells (Fig.3). The basal part of the cells cytoplasm was appeared basophilic due to accumulation of the rough endoplasmic reticulum. The fine structure of the acini cells was pyramidal to columnar in shape with basely situated oval, euchromatic nuclei with prominent nucleolus. (Fig.4). The apical cytoplasm was studded with numerous secretory granules (zymogen granules), which appeared large, spherical and electron dense in homogenecity. Numerous mitochondria well developed Golgi apparatus and rough endoplasmic reticulum. Some granules were located adjacent to the zymogen granules, small in size with less dense matrix (Fig.5). The acini were surrounded by fine reticular fibers separating it from the surrounding parenchyma (Fig.6).

The duct system started within the adenomeres by cellular ductules called centro-acinar duct which were located in the center of the pancreatic acini. It was lined by flat epithelial cells with pale acidophilic cytoplasm and flatten darkly stained nuclei (Fig. 3). The fine structure of these cells was appeared flat with large centrally located nucleus with condensed chromatin on the periphery and prominent nucleolus. The cytoplasm was appeared as undifferentiated and non granular (Fig. 4).

The centro-acinar cells drained into small ducts between the pancreatic acini. Intercalated duct was located between the pancreatic tissues and appeared as small duct lined by low cuboidal epithelium with strong acidophilic cytoplasm and central basophilic nuclei (Fig.7). Within the pancreatic lobules, another larger duct called intralobular duct, which was surrounded by little amount of collagen fibers and lined by an epithelium varying from cuboidal to columnar cells (Fig.8 &9). In the CT septa that partially separated the lobules, interlobular ducts could be demonstrated which represented the larger duct that drained the pancreatic tissues. It was lined with simple columnar epithelium with basely situated, oval vesicular nuclei and acidophilic cytoplasm. (Fig. 10 and 2)

The endocrine part (Islets of langerhans)

It was appeared as circumscribed areas between the pancreatic acini either single (Fig.11), or multiple structure (Fig.12 and 15). It was widely distributed between the pancreatic

tissues lobes especially in the center of the pancreas. The cellular contents made two types of the islets; alpha islets which were consisted mainly of alpha cells and few delta cells. Beta islets which were consisted mainly of beta cells and alpha and delta cells, on the basis of immunohistochemical reactions and electro microscopy .

Alpha cells, which appeared columnar to pyramidal cells with large basophilic nuclei (Fig. 11 and 12). It was characterized by lightly stained cytoplasm. These cells together forming alpha islets or distributed singly with the other cells of the beta islets or between the acini cells. It reacted positively with the glucogon antisera (Fig.13). The cytoplasm was slightly electron dense and crowded with large electron dense secretory granules (Fig.17 and 17a).Their size ranging from 500-550nm in diameter. They were numerous and appeared spherical to oval in shape. Some of these granules showed ill distinct space between the limitative membrane and their contents. Mitochondria were numerous. Numerous RFR and few free ribosomes.

Delta cells appeared oval to triangular cells with centrally basophilic located nuclei and acidophilic cytoplasm were located in both alpha and beta islets. It was few in number and represented the delta cells (Fig.11). These cells gave positive immunohistochemical reaction with the antisera of somatostatin. (Fig.16). It was numerous in the simple or multiple islets and appeared triangular or irregular in shape with centrally located ovoid nuclei with prominent one or more nucleolus. The cytoplasm was appeared dark electron dense and studded with numerous round shape secretory granules; some of these granules were appeared immature with slightly electron dense. The limiting membrane of the secretory granules was tightened over their contents (Fig.19 and 17). Numerous mitochondria, RER and free ribosomes. The size of the granules ranging from to 300-500nm.

Beta cells which appeared large ovoid cells with large vesicular, dark basophilic nuclei with prominent nucleolus were mainly located in the beta islets and some times between the acini cells. (Fig.12). These cells gave positive immunohistochemical reaction with the antisera of insulin (Fig.14).

It was ovoid or polygonal in shape with small basely situated nuclei with numerous condensed chromatins. The cytoplasm showed medium electron dense (Fig. 18). The cytoplasmic granules showed different shapes and size, 180-260nm. It might be round in shape and surrounded by clear haloes between the limitative membrane and their contents or rode shape and other secretory granules were showed plemorphism.

The histochemical reaction showed moderate PAS positive reaction in the cells of the acini and CT fibers of the parenchyma (Fig.20). While it was strong PAS positive reaction in the mesothelial cells of the capsule and the zymogens granules in the apex of the cytoplasm of the acini cells. Faint alcianophilic reaction in the CT fibers of the pancreatic parenchyma. Negative PAS and alcian blue reaction the cells of the islets of langerhans.

Discussion

The pancreas was covered by thick capsule of connective tissue mainly consisted of collagen and reticular fibers. This capsule was covered by flat mesothelial cells. This finding

was augmented in all mammalian and poultry species (Fitzgerald ,1969 and Baumel et al.,1993). The avian pancreas was mainly composed of exocrine glands Those glands consisted of tall columnar epithelial tissues which had acidophilic zymogen granules on their apical surface (Feher and Fancsi 1971). Moreover, small centro-acinar cells without granules were observed in the central lumen of the acinus (Scheuerer 1988 and Gulmez, 1998).

Pancreatic ducts from the acinus to the point where it empties its contents into the duodenum are arranged in following order; centro-acinar duct, intercalated ducts (the smallest in size), intralobular ducts and interlobular ducts (Fitzgerald ,1969 , Baumel , 1993 and Nurhayat 2003).

Intercalated duct was located between the pancreatic tissues and appeared as small duct lined by simple cuboidal epithelium with strong acidophilic cytoplasm and basophilic nuclei (Nagasao et al., 2003 and Slornelli et al.,2006). On contrast Mutoh et al., 1999 in chicken pancreatic intercalated duct gave changeable description of the duct as it lined by quite inconspicuous cells that seem to appear as stellate cells.

The acini cells cytoplasmic granules were stained positively with PAS but not with alcian blue. The fuchsinophilic and alcianophilic reactivity of the granules was found to be a characterstic feature of the serous secreting adenomeres (Abdel-Malek and Kandil, 1979).

The islets were distributed in the pancreatic tissues and consisted of three types of cells (A, B and D) which were distributed in the contour of the islets as described in the Indian chicken (Guha and Ghosh , 1978, Rawdon,1998 and Schwarz et al.,1983). The islets were found to be two types ; the large alpha islets, consisting mostly of alpha and delta cells and the beta islets, containing beta, delta and sometimes alpha cells. The delta cells were abundant in the pancreatic islets. The A cells called light cells while B cells called dark cells (Hellman and Hellerstrom,1960).

The islets were appeared as large or small which was randomly distributed in the architecture of the pancreatic tissue in all lobes as that of the (Guha and Ghosh , 1978) in birds . While Miakami and Ono, 1962 in chicken and Smith 1974 in Japanese quail reported that the islets distribution were mainly localized to the splenic lobe.

The distribution of the islets cells in the pigeon was similar to that of the ostrich except there were additional cells called IV and acinar D cells (Mihail and Cracium,1982).

The islets cells were spread between the acinar cells in ostrich pancreas. As ,the endocrine parts of the duck pancreas were scattered singly or in small groups of islets of various shapes and sizes in the intersititium of the exocrine part (Nurhayat et al.,2004).

D cells were numerous cells between the pancreatic islets and their abundance was very interesting and it might have an important role in bird physiology. It secrete pancreatic hormone which has insulin like activity (Power, 1967). While Epple, 1968 emphasized the possible lipid mobilizing role of the delta cells.

The fine structure of alpha cells was large columnar in shape with oval nuclei with prominent nucleoli. The cytoplasm was slightly electron dense and crowded with large electron dense secretory granules. Beta cells were ovoid or polygonal in shape with small basely situated

nuclei with numerous condensed chromatins. The cytoplasmic granules showed different shapes and size, round in shape and surrounded by clear haloes between the limitative membrane and their contents or rode shape and other secretory granules were showed plemorphism. Delta cells appeared triangular or irregular in shape with centrally located ovoid nuclei with prominent one or more nucleolus. The cytoplasm was appeared dark electron dense and studded with numerous round shape secretory granules. These results were in agreement with Laurent, et al., 1988, in islets of langerhans of ducks.

The immunohistochemical structure of the islets cells was positive to antisera of insulin, as in chicken (Manfred et al., 1995) and antisera of somatostatin, as in chicken (Takayangi et al., 1996) and anti sera of glucogon as in quail and duck respectively (Miakami et al., 1985 and Lucini et al., 1996).

References

1. Abdel-Malek,Ratiba,G and Kandil,M.H.A (1979): The lingual glands of some animal species. Egypt Vet. Med .J. 27(27)349-357.
2. Anon,A(1998): Policy for grading ostrich skins.Ostrimark SA Co-op. Alexandria, South Africa.
3. Ben Pacha, Abir ; Youssef G; Sofine B; Habib M and Hafedh M (2007): Ostrich pancreatic phosholipase A2 purification and biochemical characterization . J. of. Chromatography B , vol 857, (1): 108-114.
4. Bancroft, J.D; Cook,H.C; Stirling, R.W and Turner,D.R(1994): "Manual of histological techniques and their diagnostic application'.2nd Ed.,Churchill Livingstone, Edinburgh, London, New York.
5. Bezuidenhout AJ. (1986): The topography of the thoraco-abdominal viscera in the ostrich (Struthio camelus). Onderstepoort J Vet Res. 1986 Jun;53(2):111-7.
6. Baumel JJ. (1993): Handbook of Avian Anatomy: Nomina Anatomica Avium. 2nd Ed. Cambridge, MA, USA: Nuttall Ornithological Club.
7. Do Prado ML; Campos MN; Ricciardi Cruz AR(1989) : Distribution of A, B, and mixed pancreatic islets in two bird species (Anas plalyrhincus Gallus gallus, Linne, 1758)—a morphomctric study.Gegenbaurs Morphol Jahrb. 1989; 135(2):379-84.
8. Epple, A(1968): Comparative studies on the pancreatic islets .Endocr.Japan15.107-122.
   Fitzgerald TC. (1969): The coturnix quail, anatomy and histology. Iowa, USA: Iowa State Univ Press.
9. Feher G, Fancsi T.(1971): Vergleichende morphologie der bauchspeicheldrüse von hausvögeln. Acta Vet Acad Sci Hung 1971; 21:141-64.
10. Gomez A . J and Garcia H .O(1974): Scanning electron microscopy of the islets of Langerhans in Ducks and Chickens. Acta Diabetol Lat. 11(3): 225-239.
11. Guha, B and A Ghosh (1978): A cytomorphological study of the endocrine pancreas of some Indian birds. Gen Comp Endocrinol. 1978 Jan ;34 (l):38-44 340340.
12. Gulmez ,N (1998): Gross anatomy of the pancreatic lobes and ducts in six breeds of domestic ducks and six species of wild ducks in China. Anat Hislol Embryol. Dec;27(6):413-7
13. Hayat, M. A. (1986):- Basic techniques for transmission electron microscopy. 1st Ed.Academic Press, Inc. Florida.
14. Hellman, Bo and Hellerstrom C (1960): The islets of langcrhans in ducks and chicken with special reference to the argyrophil reaction. Cell &Tissue Rcse.52(2):278-290.
15. Horbanczuk,J; Sale,J;Celeda, T; Konecka, A; Zinaba, G and Kawaka, P (1998): Cholesterol content and fatty acid composition of ostrich meat as influenced by subspecies. Meat Sci., (50): 385-388.
16. Hsu, S.M., Raine, L., Fanger, H.(1981): Use of Avidin-Biotin-Peroxidase Complex (ABC) in Immunoperoxidase Techniques: A comparison between ABC and Unlabeled Antibody (PAP) Procedures. J .

17. Ku SK, Lee JH, Lee HS.(2000): An immunohistochemical study of the insulin-, glucagon- and somatostatin-immunoreactive cells in the developing pancreas of the chicken embryo. Tissue Cell., 32(1):58-65.
18. Laurent, F; Hindelang , C ; Strosser , M.T and Mialhe P (1988): The ultrastructure of A,B and D pancreatic cells in normal and in diabetic ducks. Biol Struct.Morph; (1):34-41.
19. Lucini C, Castaldo L, Lai O.(1996)An immunohistochemical study of the endocrine pancreas of ducks. Eur J Histochem. 1996;40(1):45-52.
20. Manfred R; Ivan B; Richarn B; Jurgen Z and Caroline M (1995): Immunohistochemical localization of the insulin like growth factor IandII in the endocrine pancreas of birds, reptiles and amphibian. General and comparative endocrinology,vol(100)(3):385-396.
21. Miakami S; Taniguchi K and Ishikawa T (1985): Immunocytochemical localization of the pancreatic islets cells in the japaneese quail, coutrnix coutrnix japonica. Nippon Juigaku Zasshi, Jun 47(3), 1.357-369.
22. Miakami,S and Ono.K (1962): Glucagon deficiency induced by extirpation of the alpha islets of the fowl pancres. Endocr.7,464-473.
23. Mihail N, Cracium C (1982): An ultrastructural description of the cell types in the endocrine pancreas of the pigeon. Anat Anz 152(3): 229-237.
24. Mutoh, K, V. Vakuri, II and Taniguchi, Kb (1999): Intercalated duct cells in chicken pancreatic islet with special reference to the Alloxan administration. Dept. Vet. Anatomy. J. Vet Med Sci. 61(5):493-496.
25. Nagasao J, Sugiyama D, Yoshioka K, Amasaki H, An T, Yue Z, Mutoh
26. Nurhayat Gulmez (2003): Are Glands Present in Goose Pancreatic Ducts'? Alight Microscope Study. TOP. J. Pancreas (Online) 2003; 4(3): 125-128.
27. Nagasao J, Sugiyama D, Yoshioka K, Amasaki H, An T, Yue Z, Mutoh K. :(2003): Morphological relationship between intercalated duct and pancreatic islet in streptozolocin and/or camostat mesilate administrations in the chicken. Anat Histol Embryol. 32(2):89-93.
28. Nurhayat Gulmez (2003): Are Glands Present in Goose Pancreatic Ducts? Alight Microscope Study. JOP. J. Pancreas (Online) 2003; 4(3):125-128.
29. Nurhayat G. Lmez*, Hakan Kocamiþ, Bahin Aslan, M.Mtaz Nazli (2004): Immunohistochemical Distribution of Cells Containing Insulin, Glucagon and Somatostatin in the Goose (Anser anser) Pancreas. Turk J Vet Anim Sci 28 (2004) 403-407.
30. Peter S, Himmelmann B and Kern HF (1970): Ultrastructure of avian islands of Langerhans (chicken, duck, pigeon). Verh Anat Ges .64:105-114.
31. Polak, J.M., Pearse, A.G.E., Adams, C., Garand, J.C(1974):
32. Immunohistochemical and Ultrastructural Studies on the Endocrine Polypeptide (APUD) Cells of the Avian Gastrointestinal Tract. Experientia, 1974; 30: 564-567 .
33. Power, L (1967): Insulin like activity of a third islet cell hormone .Lancet 1, 1138-1140.
34. Rawdon, B.B (1998): Morphogenesis and differention of the avian endocrine pancreas with particular reference to expermintal studies on the chick embryo.Microsc.Res.Tech.43.292-305.
35. Reynolds, E. S. (1965): The use of lead citrate at high ph as an electron opaque in electron microscopy. J. Cell 26: 208-215.
36. Ruffier , L ; J Simon , N Rideau (1998): Isolation of functional glucagon islets of Langerhans from the chicken pancreas. Gen Comp Endocrinol. 1998 Nov ;112 (2): 153-62 .
37. Scheuerer M.(1988): Histologische, Histochemische und Ultrastrukturelle Untersuchungen am Pankreas der Japanischen Wachtel. Inaugural-Dissertation. München.
38. Schwarz R Ali A M and El-Bab MR (1983): A histomorphological study on the endocrine portion of the pancreas of the pancreas in swan and fowl . Z Mikrosk Anat Forsch; 97(4): 556-564.
39. Slornelli. M. R. ; M. P. Ricciardi, V. Miragliotta, A. Coli, E. Giannessi (2006): Morpho-structural Study of the Pancreas and Pancreatic Duct in Ostrich (Slruthio camelus L.). Acta Vet. Brno 2006, 75: 157-160.
40. Smith,P.H (1974): Pancreatic islets of coturnix quail. Alight and electron microscopic study with special reference to the islet organ of the splenic lobe. Anat Rec.178, 567-586.
41. Sturkie PD. (1986): Avian Physiology. 4th Ed. New York, NY, USA: Springer Verlag,
42. Takayangi M; Y okada; K Kita; J Naito and T Watanabe (1996): Somatostatin-14 and somatostatin-28 in chicken pancreatic islets D cells. Tissue and cell, 28 (4):495-500.

43. Watanabe T, Ki Paik Y and Yasuda M (1975): Fine structure of the pancreatic islets in domestic fowl with special reference to the cell type and secretion. Arch Histol Jpn. 38(4): 259-247.

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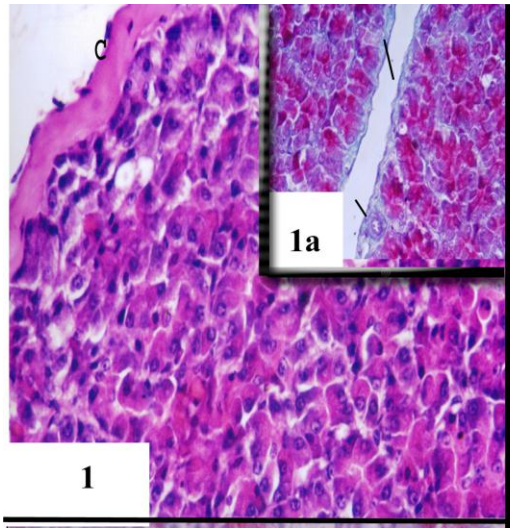
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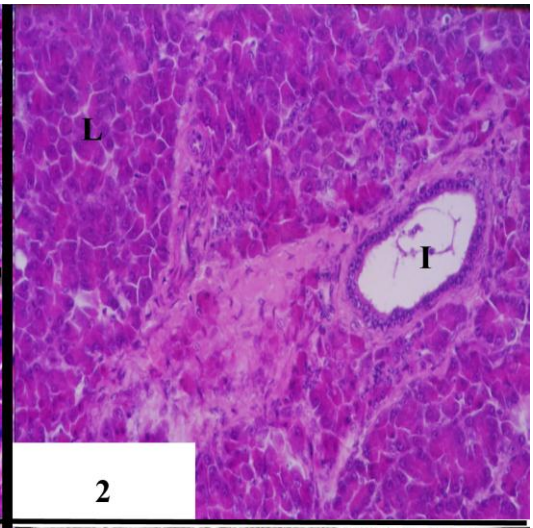
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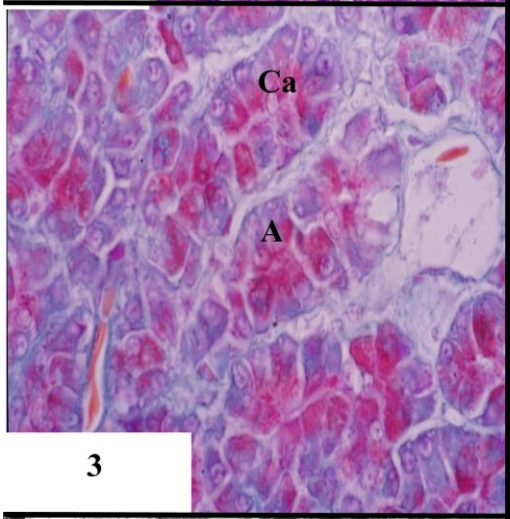
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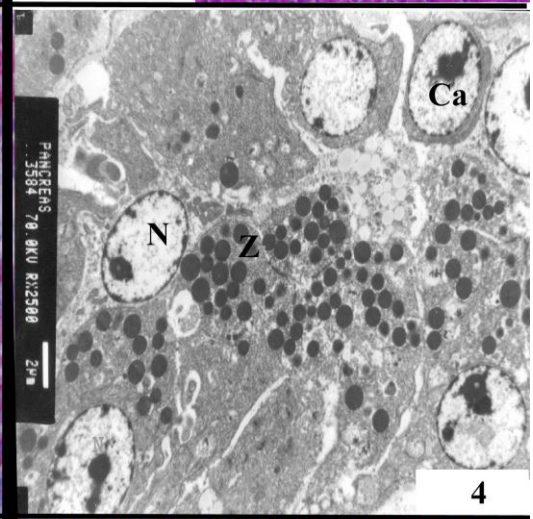
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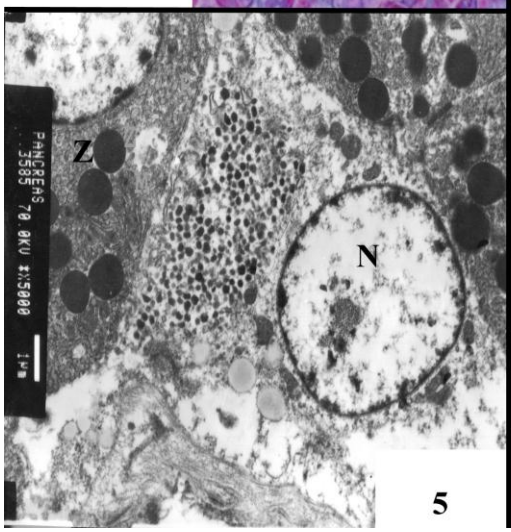
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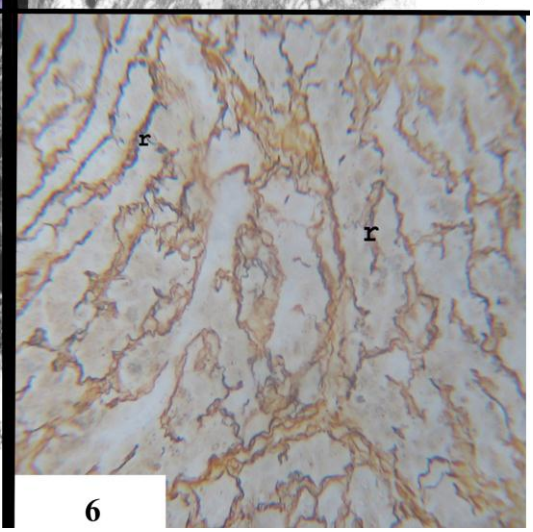
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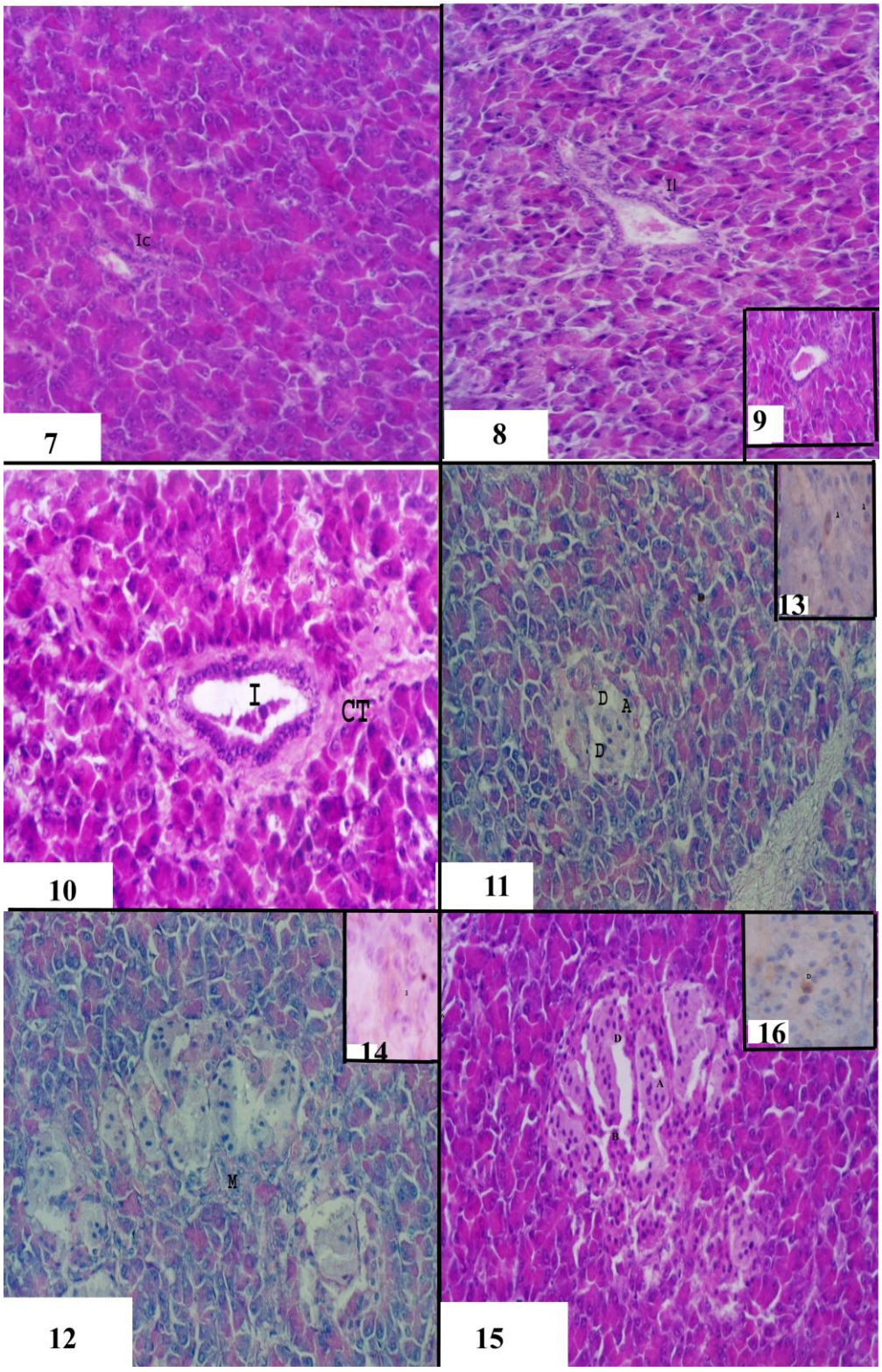
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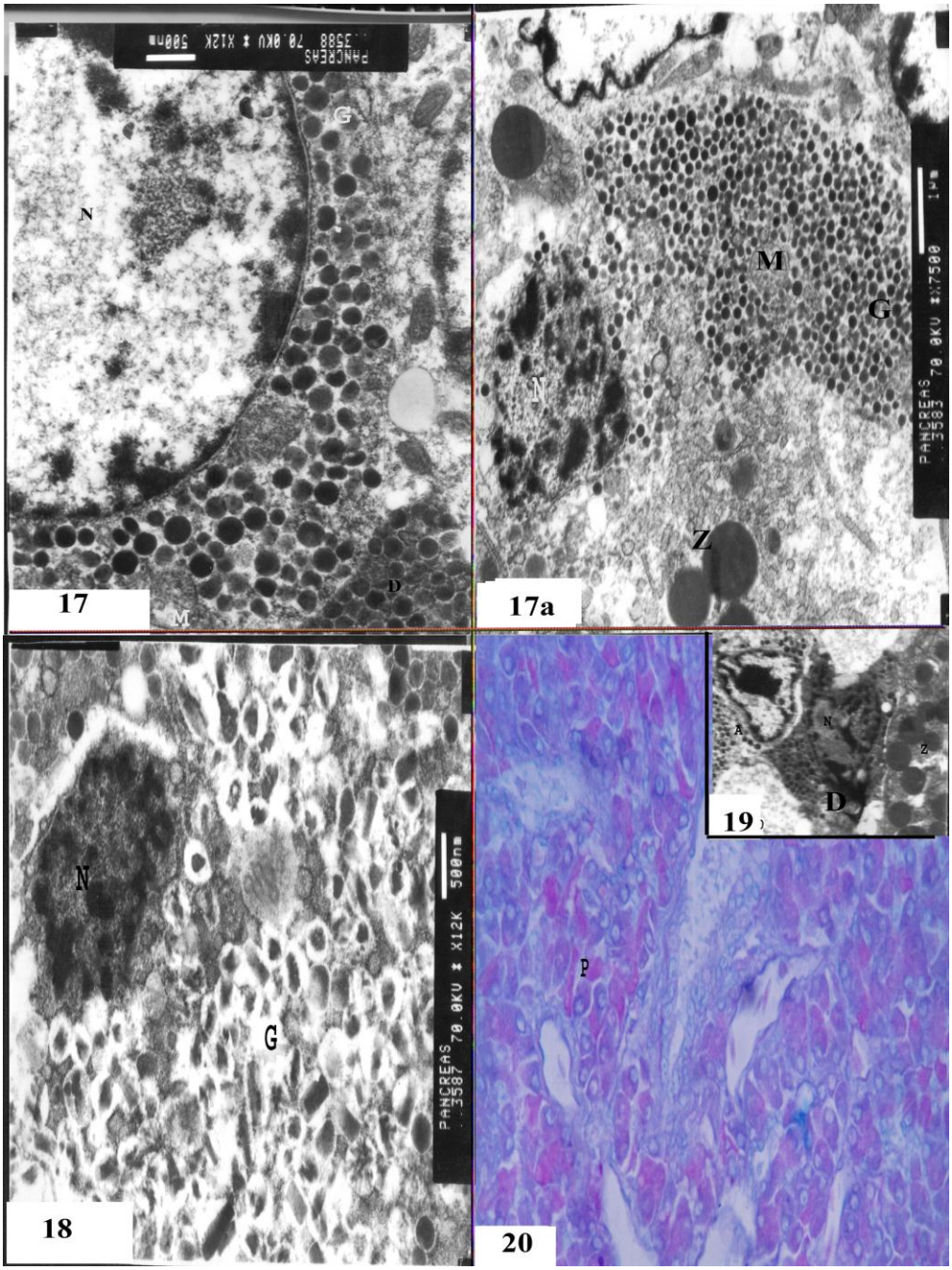
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PHARMACEUTICAL VIGILANCE FOLLOWING THE REPIRATORY DISEASES TREATMENT IN CATTLE

Ramona MARIUŢA, Luminița Diana HRIȚCU, Gh. SOLCAN
University of Agricultural Sciences and Veterinary Medicine Iasi, Faculty of Veterinary Medicine, M. Sadoveanu Alley 8, Iasi, ROMANIA, Email gsolcan@uaiasi.ro.

Abstract
A pharmaceutical vigilance program following the respiratory diseases treatment were implemented in a dairy cow and a beef farm, on 214 clinical cases.The pathogens involved in the occurrence of pneumopathies were represented by Pseudomonas aeruginosa (36%), Streptococcus zooepidemicus (22%), Pasteurella hemolytica (20%), Mycoplasma bovis (10)%, Pneumocistis carinii (6%), Aachanobacter pyogenes (4%) and Fusobacterium necrophorum (1%). From the 214 cases of respiratory diseaseas treated, 2,8% presented side effects and 1,86 % presented adverse reactions. The side effects were: edema at the inoculation place, intense itching at the inoculation place, abatement, inappetence, mucous membranes congestion, epiphora, sub-fever condition, persistent thirst, muscular trembling, tabes, frequent urination, slight anafilactic reaction; the adverse reaction were apathy, muscular trembling, convulsions, violent anafilactic reactions, collapse, dyspnea, lungs edema, diarrhea, lack of urination, tabes and death. The calves between 0-6 months old are the most vulnerable to side effects and adverse reactions due to medicine administration errors, incorrect weight evaluation or due to the body's failure to get used to the given medicine.
Keywords: cattle, respiratory diseases, treatment, adverse reactions

The pharmaceutical vigilance refers to all the activities performed for the detection, assessment, validation and prevention of adverse reactions or of other medicine-related problems. The final objective of the pharmaceutical vigilance activity consists in improving the pacient safety and assisstance, by promoting a reasonable medicine usage. The medicines continuous monitoring in the pharmaceutical vigilance system allows the identification of those medicines causing undesired adverse reactions and their control. This is mainly done by means of the suspected adverse reactions notification programme. When the safety profile of a medicine/medicines of its class indicates it, for instance, when an abnormal frequency of a known adverse reaction is detected or when unexpected serious adverse reactions are detected, a signal or an alert is generated. Normally, it takes several localised or dispersed notifications to generate a signal, depending on the described event gravity and on the available information quality.

Materials and methods

The study was performed within two farms, one of dairy cows and another for beef cattle. At a first stage, the animals, the exploitation and micro-climate conditions were analysed.

Then, a study was performed concerning the occurrence of respiratory diseases on age categories: adult cows, heifers and calves, meanwhile establishing treatment protocols with several types of antibiotics. This was done to allow a bench marking concerning the effectiveness of the antibiotics used, the occurrence of side effects or adverse reactions to the antibiotics used and to establish which is the most recommended antibiotics for each disease.

The diagnostic was established by the corroboration of clinical morpho-pathological and para-clinical data associated with a minute epizootic survey. For both the confirmation and the choice of a safer and more effective therapeutical protocol it was necessary to perform some laboratory parasitological, virusological , bacterial or mycological exams, from necropsic material, excretions and secretions in 78 cases.

We also tried to achieve as many aims of the pharmaceutical vigilance activity. Thus, we took into consideration all the antibiotics utilised for the respiratory diseases of the animals.

Results and discussions

Regarding the proportion of the etiopathogenic agents involved in respiratory affections, the results from Fig. 1 were found.

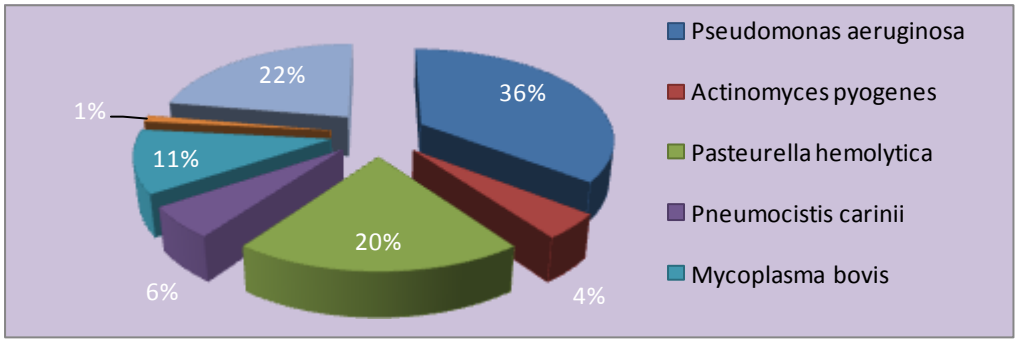


Fig. 1. Pathogenic agents involved in respiratory diseases of cattle

Based on the results of the antibiograms, the therapy with anti-microbian substances was performed, in function of the symptomatology and of the antibiotics sensitivity of the involved pathogenic agent, on all the sick animals. The antibiotics used in respiratory diseases in cattle and number of cases treated with each of it are presented in Fig. 2.

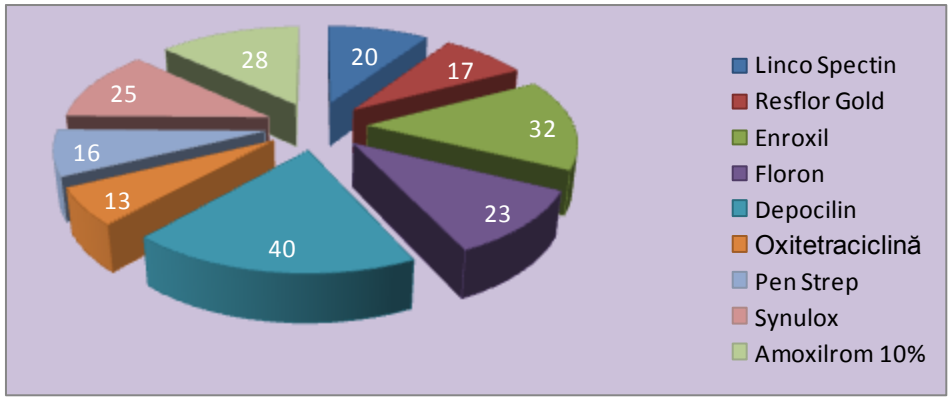


Fig. 2. The antibiotics used in respiratory diseases in cattle (nr. of cases)

One may note that on the first position, in terms of usage on animals diagnosed with respiratory diseases is the antibiotic Depocillin used on 18,69% of the cases, followed by Enroxil 10% used on 14,95% of the cases of pneumopathies. Third position is held by Amoxilrom 10% given to 13,08% of the cases, followed by Synulox given to 11,68%, then Floron injectable used in 10,74% of cases, Linco Spectin in 9,34%, Resflor Gold injectable solution in 7,94% and Penstrep in 7,47%. The last position is held by Oxitetraciclin given only to 6,07% of the cases.

There were considered all the antibiotics used for respiratory diseases of the animals *between* 2007 – 2011, thus resulting a table for the monitoring of the secondary and adverse reactions (Table 1).

Table 1. The occurrence of secondary or adverse reactions following the therapy with antibiotics on the animals with respiratory diseases

No.	*Medication used*	*Pneumopathies*	*Side effects*	*Adverse Reactions*
1	***Enroxil 10%***	*32*	*1*	*1*
2	***Penstrep***	*16*	*1*	*-*
3	***Amoxilrom 10%***	*28*	*1*	*-*
4.	***Linco Spectin***	*20*	*-*	*-*
5	***Resflor Gold sol inj.***	*17*	*1*	*1*
6	***Floron sol inj.***	*23*	*2*	*-*
7	***Depocillin***	*40*	*-*	*1*
8	***Oxitetraciclin (Engemycin10%)***	*13*	*-*	*1*
9	***Synulox***	*25*	*-*	*-*
T O T A L		***214***	*6*	*4*

Analyzing the data from table. 2, one may note that from 214 cattle treated with antibiotics between 2007 – 2011, 6 (2,8%) presented side effects and 4 (1,86%) presented adverse reactions. It must be mentioned that side effects were signaled at majority of antibiotics used.

Main symptoms of side effects and adverse reactions encountered after antibiotherapy are presented in Table 2.

Concerning the identification of animal sub-groups presenting a particular risk for the occurrence of adverse reactions, we must mention that the following were taken into consideration: dosis, age, sex and related affections.

Following a careful observations and a minute anamnesis in the case of animals presenting side effects or adverse reactions following the antibiotics therapy, it was noted that the reactions were individual, the antibiotics being used according to the prescription. The animal sex was not important for the occurrence of side effects or adverse reactions.

Concerning the related affections, it was noted that side effects occurred at the animals with a poor condition and presenting symptoms both from the digestive and

respiratory area, and the adverse reactions occurred particularly at the animals with an advanced disease degree, (depression, hyperthermia, prolonged decubitus), or with a concurent pathology digestive and respiratory. Such kind of symptoms are also described by many other authors (1, 2, 3, 9, 11).

We also tried to establish the age category most vulnerable to side effects and adverse reactions (Table 3).

Table 2. Symptoms of side effects and adverse reactions to antibiotics therapy of respiratory diseases in cattle

Antibiotics used	No of cases	Side effects	No of cases	Adverse Reactions
Enroxil 10%	1	Moderate edema at the inoculation place	1	Inflammatory edema at the inoculation place, muscular trembling
Penstrep	1	Edema at the inoculation place, intense itching at the inoculation place, sub-fever condition	-	-
Amoxilrom 10%	1	Edema at the inoculation place, muscular trembling	-	-
Linco Spectin	-	-	-	-
Resflor Gold sol inj.	1	-	1	Respiratory disorders, cramps, anafilactic shock, death.
Floron sol 300mg/ml	2	Abatement, inappetence, muscular trembling, tabes	-	-
Depocilin	-	-	1	Inflammatory edema at the inoculation place, anafilactic shock
Oxitetraciclin (Engemycin10%)	-	-	1	Tabes, convulsions, death
Synulox	-	-	-	-
TOTAL	6		4	

Analyzing the data from table3, one may find that most cases of side effects or adverse reactions occurred at the calves between 0 – 6 months, respectively 6 cases (60%), followed by heifers with 2 cases (20%), calves above 6 months, and adult bovins, 1 case each (10%). So most often the side effects or adverse reactions occur on the calves of 0 – 6 months which presented the highest ratio of 60%. These could be due to medicine administration errors, to incorrect weight evaluation or due to the body's failure to get used to the given medicine.

Table 3. Age category of animals presenting side effects or adverse reactions to antibiotics

	No of cases	Adult bovins	Heifers	Calves ≥ 6 months	Calves 0 – 6 months
Enroxil 10%	3	1			2
Penstrep	1		1		
Amoxilrom 10%	1				1
Linco Spectin	-				
Resflor Gold sol inj.	2			1	1
Floron sol.inj. 300mg/ml	2		1		1
Oxitetraciclin (Engemycin10%)	1				1
Synulox	-				
TOTAL	10	1	2	1	6

Conclusions

1. The pathogens most frequently involved in the occurrence of respiratory diseases in 214 cattle were: *Pseudomonas aeruginosa* (in 36% of the cases), *Streptococcus zooepidemicus* (22%), *Pasteurella hemolytica* (20%), *Mycoplasma bovis* (10%), *Pneumocistis carinii* (6%), *Actinomyces piogenes* (4%) and *Fusobacterium necrophorum* (1%).
2. From 214 cases treated 2,8% revealed side effects and 1,86 % presented adverse reactions. The side effects were: edema at the inoculation place, intense itching at the inoculation place, abatement, inappetence, mucous membranes congestion, epiphora, sub-fever condition, persistent thirst, muscular trembling, tabes, frequent urination, slight anafilactic reaction; the adverse reaction were apathy, muscular trembling, convulsions, violent anafilactic reactions, collapse, dyspnea, lungs edema, diarrhea, lack of urination, tabes and death.
3. The calves between 0-6 months old are the most vulnerable to side effects and adverse reactions due to medicine administration errors, incorrect weight evaluation or due to the body's failure to get used to the given medicine.

Bibliography

1. Bârză, H., May, I., Ghergariu, S., Hagiu, N., 1981 - Patologie şi clinică medicală veterinară. Ed.did.şi pedag., Bucureşti, Ed. Ştiinţa, Chişinău.
2. Belu, H.C., Şuţeanu, E., 2000 – Efecte adverse ale unor antibiotice. Rev. Rom. de Med.Vet. nr.3 (Supliment), p.177.

3. Booth, N.H., Mc.Donald, L.E., 1988 – Veterinary Pharmacology and Therapeutics. 6th ed. Ames: Iowa State University Press. 1227 pp.
4. Carp-Cărare, M., Timofte Dorina, 2002 - Imunologie şi imunopatologie, Casa de editură Venus, Iaşi.
5. Goodman, D., Gilman, A., 1990 – The pharmacological bovis of therapeutics. Mac Millan, New York.
6. Licperta, E., 1980 – Farmacologie veterinară. Ed. Ceres, Bucureşti.
7. M.V.M., 1993 - The Merck veterinary manual. Merck and Co., Rahway, New York.
8. Răpuntean, Gh., Răpuntean, S., 1999 – Bacteriologie specială veterinară. Ed. Agronomică, Cluj Napoca.
9. Solcan Gh., Boghian V., Rollin F. – 2005 – Patologie şi clinică medicală veterinară vol I – ed "Ion Ionescu de la Brad Iaşi"
10. Stătescu, V., Savopol, E., 1999 – Incompatibilităţi medicamentoase. Ed. Medicală, Bucureşti.
11. Upson, D.W., 1988 – Handbook of Clinical Veterinary Pharmacology. 3rd ed. Manhattan: Upson Enterprises, 729 p.

PHARMACEUTICAL VIGILANCE FOLLOWING THE ENTERITIS TREATMENT IN A DAIRY COW AND A BEEF FARM

Ramona MARIUŢA, Luminița Diana HRIȚCU, Gh. SOLCAN
University of Agricultural Sciences and Veterinary Medicine Iasi, Faculty of Veterinary Medicine, M. Sadoveanu Alley 8, Iasi, ROMANIA, Email gsolcan@uaiasi.ro.

Abstract
A pharmaceutical vigilance program following the enteritis treatment were implemented in a dairy cow and a beef farm, on 369 clinical cases. The pathogen agents most frequently involved in the occurrence of enterites were: Escherichia coli, Pseudomonas aeruginosa, Salmonella enteritidis, Salmonella typhimurium, Rotavirus, Coronavirus and Eimeria bovis. The antibiograms showed an increased sensitivity to 7 antibiotics, which were included in the therapeutical protocol within the antibiotics therapy: Enroxil 10%, Penstrep, Colisin, Sulfadiarom, Betamox LA, Amoxilrom 10% and Gentamicin. Monitoring the side effects and the adverse reactions it was noted that out of 369 clinical cases treated with antibiotics, only 1,35% displayed side effects and 0,81% presented adverse reactions. The most vulnerable to side effects or adverse reactions were calves between 0 – 6 months.

Keywords: enteritis, calves, treatment, adverse reactions

Introduction

The objective of the pharmaceutical vigilance activity consists of improving the pacient safety and assisstance, by promoting a reasonable medicine usage.
The aims of the pharmaceutical vigilance activity are:

- Identification and cuantification of adverse reactions previously not-recognised;
- Monitoring the frequency of known adverse reactions;
- Identification of animal sub-groups presenting a specific risk for the occurrence of adverse reactions (risk which may be related to the given dosis, sex, age or associated conditions);
- Continuous monitoring of the medicine safety throughout its lifespan, to make sure that related risks and benefits remain acceptable. This also involves the monitoring of the medicine safety in case new therapeutical indications are approved;
- Comparison of the medicine safety profile against the safety profiles of other compounds of the same therapeutical class;
- Detection of prescription and administration errors;
- Identification of new pharmaceutical and toxicological features and of fundamental mechanisms of adverse reactions/events;
- Detection of significant interactions between medicines, interactions that may be detected only after a mass usage;
- Analysis and dissemination of information needed for a correct prescription and for the regulation of medicine circulation;
- Assessment and communication of the risk-benefit ratio for all the medicines existing on the market;

- Control (based on the significant arguments) of the ”fake-positive” adverse reactions which may occur among the professionals in the health field or in the media.

The medicines continuous monitoring in the pharmaceutical vigilance system allows the identification of those medicines causing undesired adverse reactions and their control. This is mainly done by means of the suspected adverse reactions notification programme. When the safety profile of a medicind e/medicines of its class indicates it, for instance, when an abnormal frequency of a known adverse reaction is detected or when unexpected serious adverse reactions are detected, a signal or an alert is generated. These signals or alerts may come of any source.

Normally, it takes several localised or dispersed notifications to generate a signal, depending on the described event gravity and on the available information quality.

Materials and methods

The study was performed within two farms, one of dairy cows and another for beef cattle. At a first stage, the animals, the exploitation and micro-climate conditions were analysed.

Then, a study was performed concerning the occurrence of digestive affections on age categories: adult cows, heifers and calves, meanwhile establishing treatment protocols with several types of antibiotics. This was done to allow a bench marking concerning the effectiveness of the utilised antibiotics, the occurrence of side effects or adverse reactions to the antibiotics used and to establish which is the most recommended antibiotics for each disease.

The diagnostic was established by the corroboration of clinical morpho-pathological and para-clinical data associated with a minute epizootic survey. For both the confirmation and the choice of a safer and more effective therapeutical protocol it was necessary to perform some laboratory parasitological, virusological, bacterial or mycological exams, from necropsic material, excretions and secretions in 82 cases.

We also tried to achieve as many aims of the pharmaceutical vigilance activity. Thus, we took into consideration all the antibiotics utilised for the digestive affections of the animals.

Results and discussions

Regarding the percentage of the enteropathogenic agents involved in enteral affections, the results from Fig. 1 were found:

Following the laboratory analyses, it was established that the most frequently involved are bacteria, namely *Escherichia coli* at 31% of the cases, followed by *Pseudomonas aeruginosa* diagnosed on 23% of the cases. Third most frequent was *Salmonella enteritidis* with the same ration as the parasite *Eimeria bovis*, respectively 12%, followed by Coronavirus in 9% of the cases, *Salmonella typhimurium* in 7% of the cases and on the last position was *Rotavirus i*n 6% of the cases.

Following the determination of the involved pathogenic agents, where possible we also performed antibiograms, in order to make a efficient therapeutical decision.

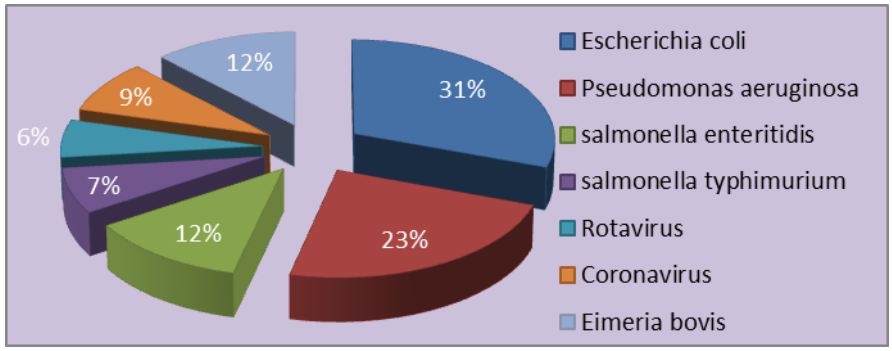


Fig. 1. Involvement of pathogenic agents in digestive affections of cattle

Thus it was noted that in function of the cases and pathogenic agents, the antibiograms provided results showed in Table 1.

Studying the antibiogram results, therapy with antimicrobian substances was performed, in function of the symptomatology and of the involved pathogenic agent sensitivity to antibiotics, for all the sick animals.

Table 1. The results of the antibiograms of the pathogenic agents involved digestive diseses of the calves

Pathogenic Agents	**Sensitivity testing to anti-infectious substances of the pathogenic bacteria using the difusimetric method**
Escherichia coli	***Sensitive to*** - amoxicilin, colistin sulphate, norfloxacin, enrofloxacin, gentamicin,trimetroprim
Pseudomonas aeruginosa	***Sensitive to*** - ceftazidime, polimixine B, ***moderately sensitive to*** - oxacilin, lincomicin, florfenicol, gentamicin and penicilin G;
Salmonella enteritidis	***Sensitive to*** - amoxicilin , ampicilin, enrofloxacin,
Salmonella typhimurium	***Sensitive to*** - amoxicilin, ampicilin, enrofloxacin
Eimeria bovis	***Sensitive to*** - sulfadiarom and amprolium

There were considered all the antibiotics used for digestive affections of the animals from the farms between 2007 – 2011, thus resulting a table for the monitoring of the

secondary and adverse reactions (Table 2). Analysing the data from table. 2, one may note that from 369 animals treated with antibiotics between 2007 – 2011, 5 (1,35%) presented side effects and 3 (0,81%) presented adverse reactions. It must be mentioned that side effects were also signaled at many antibiotics.

Concerning the identification of animal sub-groups presenting a particular risk for the occurrence of adverse reactions, we must mention that the following were taken into consideration: given dosis, age, sex and related affections.

Following a careful observations and a minute anamnesis in the case of animals presenting side effects or adverse reactions following the antibiotics therapy, it was noted that the reactions were individual. The sex was not related with the occurrence of side effects or adverse reactions because these occurred both at females and at males almost equally. Concerning the related affections, it was noted that side effects occurred at the animals with a poor condition and presenting symptoms both from the digestive area and the respiratory system, and the adverse reactions occurred in particular at the animals with an advanced disease degree, in a poor condition (depression, hyperthermia, prolonged decubitus), or with a diverse pathology associated with the specific degestive or respiratory signs. At the same time, 2 of the 3 cases presenting adverse reactions have died.

Table 2. The occurrence of secondary or adverse reactions following the therapy with antibiotics on the cattle

No. Pos	*Medication used*	*Enteritis (nr of cases)*	*Side effects*	*Adverse Reactions*
1	***Betamox LA***	*46*	*1*	
2	**Sulfadiarom**	*50*	*1*	-
3	***Enroxil 10%***	*70*	*1*	*1*
4	***Gentamicin***	*41*	*1*	*1*
5	***Penstrep***	*69*	*1*	-
6	***Colisin***	*50*	-	*1*
7	***Amoxilrom 10%***	*43*		
T O T A L		*369*	*5*	*3*

Main symptoms of side effects and adverse reactions encountered after antibiotherapy are presented in Table 3. Such kind of symptoms are also described by many other authors (1, 2, 3, 11).

We have tried to establish the most vulnerable age to side effects and adverse reactions (Table 4). In terms of ratios, one may note that the calves of 0 – 6 months represent 55,5 % from the total of 9 cases presenting side effects and adverse reactions, followed by the calves above 6 months and heifers with 22,2% each, and on the last position there is the category of adult cows with only 11,1% of total cases.

The conclusion is that most often the side effects or adverse reactions occur on the calves of 0 – 6 months. These could also present the above-mentioned reactions more

frequently both due to medicine administration errors, to incorrect weight evaluation or due to the body's failure to get used to the given medicine.

Table 3. Types of side effects and adverse reactions signaled at the performance of antibiotics therapy on the animals

Antibiotics used	No of cases	Side effects	No of cases	Adverse Reactions
Betamox LA	1	Mucous membranes congestion, Epiphora, tabes, slight anafilactic reaction	-	-
Sulfadiarom	1	Edema at the inoculation place, on extended area, sub-fever condition, Persistent thirst	-	-
Enroxil 10%	1	Moderate Edema at the inoculation place, deviation, short term inappetence	1	Inflammatory Edema at the inoculation place, muscular trembling
Gentamicin	1	Persistent thirst, frequent urination, inappetence, uncertain walking, deviation	1	Diarrhea, convulsions, anafilactic reactions, lack of urination, death
Penstrep	1	Edema at the inoculation place, intense itching at the inoculation place, sub-fever condition	-	-
Colisin	-	-	1	Anafilactic shock, dyspnea, lungs edema
Amoxilrom 10%	-	-	-	-
Total	5		3	

Table 4. Identification of age sub-groups of animals presenting a specific risk for the occurrence of side effects or adverse reactions

	No of cases	Adult bovins	Heifers	Calves ≥ 6 months	0 – 6 months
Betamox LA	1				1
Sulfadiarom	1				1
Enroxil 10%	2	1			1
Depocillin	1		1		
Gentamicin	2	-	1	1	
Penstrep	1				1
Colisin	1			1	
Amoxilrom 10%					
TOTAL	9	1	2	2	5

Conclusions

1. The pathogen agents most frequently involved in the occurrence of bovine enterites were: *Escherichia coli, Pseudomonas aeruginosa, Salmonella enteritidis, Salmonella typhimurium, Rotavirus, Coronavirus and Eimeria bovis.*
2. The antibiograms showed an increased sensitivity to 7 antibiotics, which were included in the therapeutical protocol: Enroxil 10%, Penstrep, Colisin, Sulfadiarom, Betamox LA, Amoxilrom 10% and Gentamicin.
3. Monitoring the side effects and the adverse reactions it was noted that out of the total 369 cases subject to antibiotics therapy, only 1,35% displayed side effects and 0,81% presented adverse reactions.
4. The most vulnerable for side effects or adverse reactions occurrence were calves between 0 – 6 months.

Bibliography

1. Adameşteanu, I., 1974 – Iatropatiile în medicina veterinară. Ed. Ceres, Bucureşti.
2. Belu, H.C., Şuţeanu, E., 2000 – Efecte adverse ale unor antibiotice. Rev. Rom. de Med.Vet. nr.3 (Supliment), p.177.
3. Booth, N.H., Mc.Donald, L.E., 1988 – Veterinary Pharmacology and Therapeutics. 6th ed. Ames: Iowa State University Press. 1227 pp.
4. Carp-Cărare, M., Timofte Dorina, 2002 - Imunologie şi imunopatologie, Casa de editură Venus, Iaşi.
5. Decun, M., 1986 - Infecţiile colibacilare la animale. Ed.Ceres, Bucureşti.
6. Goodman, D., Gilman, A., 1990 – The pharmacological basis of therapeutics. Mac Millan, New York.
7. Licperta, E., 1980 – Farmacologie veterinară. Ed. Ceres, Bucureşti.
8. M.V.M., 1993 - The Merck veterinary manual. Merck and Co., Rahway, New York.
9. Răpuntean, Gh., Răpuntean, S., 1999 – Bacteriologie specială veterinară. Ed. Agronomică,C-N.
10. Solcan Gh., Boghian V., Rollin F. – 2005 – Patologie şi clinică medicală veterinară vol I – ed "Ion Ionescu de la Brad Iaşi".
11. Stătescu, V., Savopol, E., 1999 – Incompatibilităţi medicamentoase. Ed. Medicală, Bucureşti.
12. Upson, D.W., 1988 – Handbook of Clinical Veterinary Pharmacology. 3rd ed. Manhattan: Upson Enterprises, 729 p.

DATA UPON THE SUCCESSION OF THE BICAZ RESERVOIR ICHTHYOFAUNA

Ramona SORIC, Liviu MIRON
lmiron@uaiasi.ro
Faculty of Veterinary Medicine "Ion Ionescu de la Brad" University

Abstract
Bicaz lake was made in the 60^{ies}. At the lake formation the fish population comprised 25 species. As time passes by certain changes of the ichtyofauna structure took place, so in the period 1970 – 2000 the number of fish species is decreasing to19 species and nowadays there are 15. In the last decade changes occurred, in the qualitative composition the of the Bicaz reservoir ichthyofauna. In 4 years of periodically seasonal capture study (2008-2012) we have identified 15 fish species, by net fishing in 6 work stations situated in Potoci bay, around three trout farms. The aim of this study was to analyze and to establish the structure of the Bicaz reservoir ichthyofauna in order to know the probabilities of transmission of some diseases from the wild fishes to the ones in captivity or the reverse case. Compared to the previous decade we report the the presence of Hucho hucho L., Phoxinus phoxinus L. , and starry sturgeon - Acipenser stellatus L., the last one probably escaped from the floating cage breading system.
Key words: Bicaz, ichthyofauna, succession

The Izvoru-Muntelui Bicaz reservoir, which is the fifth largest in Europe, is used for aquaculture in parallel with other common uses. This is the reason why since 1960 when the lake was made, countless studies have been realized regarding the evolution of the newly created ecosystem. The most comprehensive studies can be found in the monographs focusing on limnophysics and limnobiology of this ecosystem, by the efforts of over 25 specialists leaded by Miron *et al*. 1983 and Miron *et al*. 2010.

Also, in order to know the probabilities of transmission of some diseases from the wild fishes to the ones in captivity, or for the purpose of understanding some aspects from parasites life cycle, regular fishing aimed to capture ichthyofauna from Bicaz lake. This allowed establishing a succession of the fish populations from the beginning till present by using the published data by Miron *et al*., 1983, Battes *et al*., 1983, Miron & Simionescu, 1994, and Miron *et al*., 2010.

Material and Methods

The collection of the biological material was made by net fishing, using (Ø 1,6 cm; 1,8 cm; 2,0 cm; 2,2 cm; 2,4 cm; 3,0 cm) in 6 stations along the three existing trout farms in Potoci bay. The nets were submerged for 12-24 ours usually at night and after their removal the fish species identification was made according to the determination key.

Results and discussions

An important aspect of this paper in order to reveal the ichthyo-faunistic succession of the lake is given by quarterly fishing with 'nets' of various dimensions in the area of salmonid farms in order to characterize the ichthyofauna of Bicaz lake in terms of parasites and parasites transfers. This way, the multiannual dynamic of the quality composition of the ichthyofauna has revealed for this study stage, the existence among the fish captured of specimens resulted from aquaculture. In the ichtyo-faunistic succession, 25 species of 5 families (*Salmonidae*, *Thymallidae*, *Cyprinidae*, *Cobitidae*, *Percidae*) and later *Acipenseridae*

in 1966, have been included (Table 1). These included 19 local species, four introduced by acclimatization and two observed near Bistriţa River as stationary water species (*Alburnus alburnus* L. and *Gobio gobio obtusirostris Val*); the rest are rheofil species, found in the effluent streams habitat. Along the time, the number of fish species has decreased, from 25 in the first decade to 19 in the second decade and 15 in the forth. We have encountered in 2012 only 15 fish species present in the area of Potoci bay from the Bicaz reservoir.

We report the presence of *Hucho hucho* L., an endemic salmonid of the Carpathic-danubian area, that was missing in the period 1997-2009. Since 1983, the cyprinid *Phoxinus phoxinus* L, was not identified by other authors and this description in Potoci bay reveals the importance of the reef effect of salmonid farm.

The submerged caged favours the development of pelagic fishes through the additional source of nutrients provided by periphyton attached to cage nets. Pelagic fish contact with fish in floating cages in search for food, favours interspecific transmission of parasite: For example the protozoan *Ichthyophthirius multifiliis* L., the only species of the genus, have no host specificity and can be infective for many others fish species. The periphitic bioderma constitutes a major reservoir of food for fish, including the zooplankton organisms and species to which are belonging the parasite species that grow here, being protected by submerged vegetal cover.

The starry sturgeon Acipenser stellatus L.,, was for the first time captured in the Bicaz reservoir, probably escaped from the farm.

Table 1. Bicaz reservoir ichthyofauna succession

Familia şi specia	***1960 Miron I.***	***1970 Battes et al.***	***1983 Miron I.***	***1997 Miron L.***	***2009 ACVAPUR***	***2012***
Fam. Salmonidae						
Salmo fario L.	+	+	(+)	+	+	+
Salmo trutta lacustris L.	+	+	+	+	+	+
Salvelinus fontinalis Mitchill	+	(+)	(+)	-	-	-
Rhabdofario mykiss Walbaum	-	+	+	+	+	+
Hucho hucho L.	+	+	+	+	-	+
Coregonus lavaretus mareoides L.	+	(+)	(+)	-	-	-
Coregonus albula ladogensis Pra.	+	-	-	-	-	-
Fam. *Thymallidae*						
Thymallus thymallus L.	+	-	-	-	-	-
Fam. *Cyprinidae*						
Rutilus carpathorossicus L.	+	+	+	+	+	+
Squalius cephalus L.	+	+	+	+	+	+
Phoxinus phoxinus L.	+	+	+	-	-	+
Scardinius erythrophthalmus L.	+	-	-	-	-	-
Alburnus alburnus L.	+	+	+	+	+	+

Alburnoides bipunctatus Bloch	+	(+)	(+)	-	-	-
Blicca bjoerknav L	+	-	-	-	-	-
Abramis brama L.	-	+	+	+	+	+
Chondrostoma nasus L.	+	+	+	+	+	+
Gobio gobio obtusirostris Val	+	+	+	(+)	+	+
Gobio uranoscopus frici V	+	-	-	-	-	-
Barbus barbus L.	+	+	+	(+)	+	-
Moioagă *Barbus meridionalis peteny* Haeckel	+	+	+	-	-	-
Carasius carasius L.	+	+	+	(+)	+	+
Fam. *Cobitidae*						
Grindel *Noemacheilus barbatulus L.*	+	-	-	-	-	-
Sabanejewia balacanica Karaman	+	+	+	-	+	+
Cobitis taenia taenia	+	+	-	+	-	-
Fam. *Percidae*						-
Perca fluviatilis L.	-	-	-	+	+	+
Fam. Acipenseridae						
Acipenser ruthenus L.	-	+	-	-	+	+
Acipenser stellatus L.	-	-	-	-	-	+

The oligotrophic structure of the lake, allows the development only to the low waist predatory fish species, with insufficient trophic resources for development. Although to the subsequent breeding periods of specie, offspring appear in relatively numerous quantities, it disappears during development, being captured by predators, so that large catches are rare. In figures 1and 2 we observe that the perch *Perca fluviatilis* represents 63,46% with a total capture, of 396, but with a medium weigh of 100g/fish.

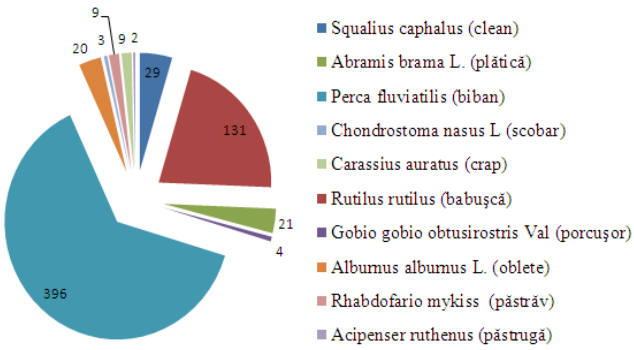


Fig. 1 Potoci bay ichthyophauna structure

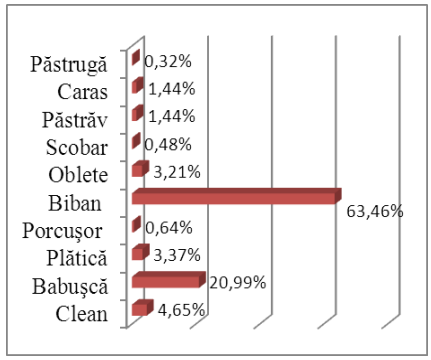


Fig. 2 Percentage distribution of the number of fish caught in 2008-2012

Conclusions

1. The reef effect of salmonid farm favours the development of the pelagic fish, through additional source of nutrients provided by periphyton.
2. The oligotrophic structure of the lake, allows the development only to the low waist predatory fish species, with insufficient trophic resources for development
3. Compared to the previous decade, we have identified the existence of 15 species of fish in Bicaz reservoir, indicating the presence of *Hucho hucho L.*, *Phoxinus phoxinusL.*, and starry sturgeon *Acipenser stellatus* L., the las one probably escaped from directed rearing systems.
4. The attraction of pelagic fish around floating cages according the reef effect for searching feed, favours interspecific transmission of the parasites: for example *Ichthyophthirius multifiliis* F., the only species of the genus, with low host specificity may infect many species of fish, the trout also being in this list.

References

1. Battes K., 1971 - *Caracteristici ale lacului de acumulare Bicaz în cursul anului 1970*. Lucr. Staţ. "Stejarul", Limnol., 357-398
2. Miron I., *et al*., 1983 – *Lacul de acumulare Izvoru Muntelui – Bicaz*, Editura academiei Republicii Socialiste România, Bucureşti, p. 224
3. Miron I, Miron L., 1993 – *The system of lacustrin aquaculture in Romania* - Lacurile de acumulare din Romania – tipologie, valorificare, protecţie vol. 1, p. 38-41
4. Miron Liviu, Cătălin Simionescu – 1994, Cercetări privind compoziţia ihtiofaunei din lacul de acumulare Bicaz, Lacurile de acumulare din România – tipologie, valorificare, protecţie, pag. 83-91, 1994
5. Miron Liviu, 1999 – P*arazitofauna Salmonidelor crescute intensiv în lacurile de acumulare montane şi în unele păstrăvării clasice din România, Lacurile de acumulare din România*, Vol 2, p. 43-48
6. Miron I., *et al*. 2010 – *Succesiunea ecologică (Râul Bistriţa – Lacul de acumulare Bicaz). Monografie limnologică II,* Ed. Universităţii Alexandru Ioan Cuza, Iaşi

ERRATIC PARASITISM WITH *LIGULA INTESTINALIS* L. PLEROCERCOIDS ON PERCH *PERCA FLUVIATILIS* L. CASE STUDY

Manuela MIRON[1], Ramona SORIC[2]
[1]UAIC Iasi– [2]USAMV Iasi
mironmanuela@yahoo.com; ramonas@uaiasi.ro

Abstract
Parasitological studies of fish from Bicaz reservoir was made especially from the last decade, on the cultured trout bred in floating cages. In this oligotrophic reservoir, the dominant species from the 15 actual free living fish populations is so far perch Perca fluviatilis L.(Perciformes, Percidae). From 586 fish captured, 396 were perch (62,9%). The weight range (g) of captured perch from the lake varied between 5,9 -1600 g weight, the fish length range (cm) being 6-38 cm, the dominant average being 10-18 cm. The aim of this case study was to signalize an erratic infestation of perch with plerocercoids from Ligula intestinalis L., a cestode usually found on bleak Alburnus alburnus L. After the attack and ingestion of the infested bleak, four plerocercoids with the average length of 7 cm, invaded the gastric cavity of the digestive tract, an abnormal localization of parasite. The necropsy of the perch revealed that the distension is secondary to invasive parasites, determined a gastric reflux and the plerocercoids appeared back in the lumen of the esophagus and in the oral cavity of the perch when it was captured. Also, the presence of a large number of little stones in the gastric cavity suggested the harmfulness of invasive parasite and the pain provoked on perch.

Key words: perch, *Ligula intestinalis*, Bicaz reservoir

Introduction

Bicaz man-made lake is an huge reservoir (3000 ha surface area, 32 km length, 2 km large and 92 m deep).The oligotrophic structure of the lake, allows the development only to the low waist predatory fish species, with insufficient trophic resources for development[3].

Although to the subsequent breeding periods of species, offspring appear in relatively numerous quantities, it disappears during development, being captured by predators, so that large catches are rare.

The reef effect of salmonid farm favours the development of the pelagic fish, through additional source of nutrients provided by periphyton.

The analysis of the thermal properties of the water from Bicaz reservoir, in different work-stations around the salmonid farms and in all seasons of the year, presented a thermal stratification of the water column, from the surface to the bottom of the lake.

The surface horizons, favours the development of bacteria, protozoa, helminth, arthropods and parasitic fungus groups.

The periphyton bioderma constitutes a major reservoir of food for fish, including the zooplankton organisms and species to which are belonging the parasite species that grow here, being protected by submerged vegetal cover.

Most of perch species are migrating along their life, but not in large distances. They rest in fish population in the same location. So, each population of perch in different parts along the lake should have the same parasites, and the host-parasite interaction is probably

constant. For spawning, females of perch are using also artificial spawning substrates, and the cages from salmonicolous farms existing in Potoci gulf and in other 3 locations nearby this zone, assuring an reef effect for this behavior of perch female.

The contact of fish from pelagial with floating nurseries in search of food, favours interspecific porting of the parasites. Also, the nutritional behavior favor to enlarge the parasites founded as endohelminthic communities of the perch.

Material and methods

Fish were examined between 2010-2012. The study examined a total of 596 fishes, representing 15 species. Parasitological research included skin, fins and all internal digestive tract, swim bladder, kidney, gall bladder, and heart. The diagnostic of most captured fish was established according the para-clinical examination, associated with environmental factors measurement, anamnetical and necropsy examination respectively. The parasites were identified according to keys by Gusev (1985), Bauer (1987), Bruno (2006)[2].The fish were caught by different size nets.

Results and discussions

In the next decades from the lake formation (1961), there are described 19 species of fish caught in Lake Bicaz (Battes et al., 1983); as time goes on, the fish drop to 15 species respectively in the 4 th decade [4], maintained at 15 species in the 5th decade (Miron L. and R. Soric, 2012, unpublished data). *Alburnus alburnus* L., represent a good food resource for piscivorous fish like perch, representing 20 % from all species captured from the lake (fig. 1).

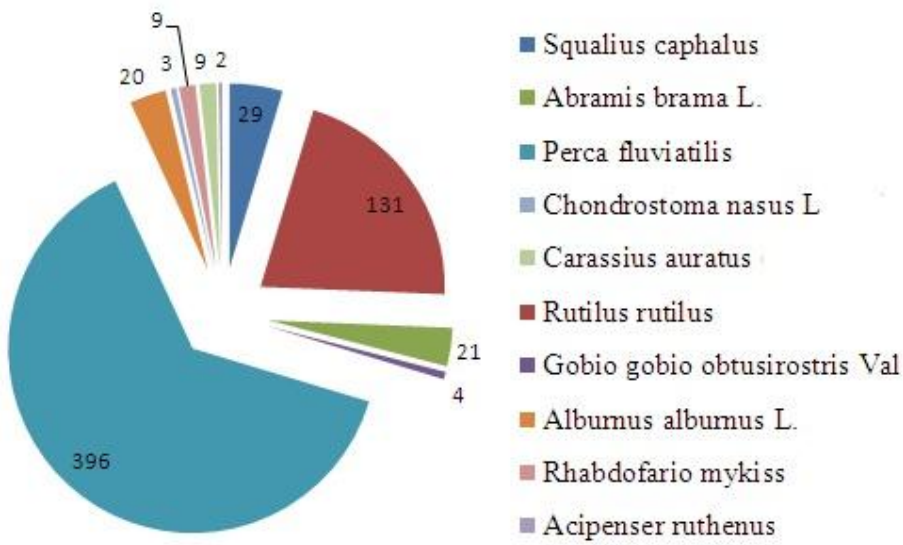
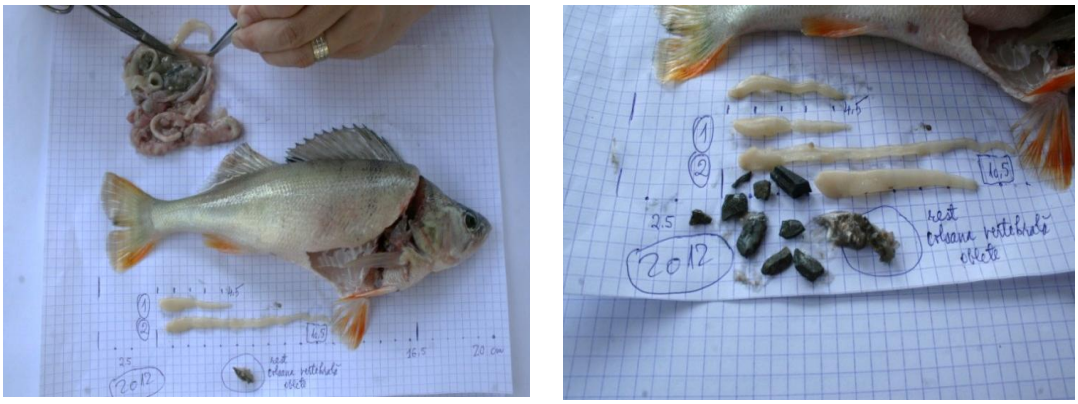


Fig. 1. The fish capture from Bicaz reservoir in 2012

From 586 fish captured, 396 were perch (62,9%) (fig.2). The weight range (g) of captured perch from the lake varied between 5,9 -1600 g weight, the fish length range (cm) being 6-38 cm, the dominant average being 10-18 cm.

An interesting component of erratic parasitism was found by us in the summer of 2012 to capture a perch of 262 g digestive tract. Upon opening the stomach we found the presence of 4 larvae of tapeworms living plerocercoids of *Ligula intestinalis* L. Their origin was due to voracious eating habits of perch, in this case swallowing a bleak cestode larvae infected with contaminated predator.

The aim of this case study was to signalize an erratic infestation of perch with plerocercoids from *Ligula intestinalis* L., a cestode usually found on bleak *Alburnus alburnus* L.(fig.2a,b).



a b

Fig.2. a,b. Plerocercoid larvae în the stomach of a perch

After the attack and ingestion of the infested bleak, four plerocercoids with the average length of 7 cm, invaded the gastric cavity of the digestive tract, an abnormal localization of parasite. The necropsy of the perch revealed that the distension is secondary to invasive parasites, determined a gastric reflux and the plerocercoids appeared back in the lumen of the esophagus and in the oral cavity of the perch when it was captured. Also, the presence of a large number of little stones in the gastric cavity suggested the harmfulness of invasive parasite and the pain provoked on perch.

Conclusions

1. Oligotrophic nature of the lake led to the development of those species of fish that could use biomass resources that phyto-and zoo-plankton such as cyprinids *Alburnus alburnus* L. with a high adaptability of this species in Lake Bicaz
2. Part of structure of the nekton from the lake, the planktonic fish respectively, migrate in pelagial zone using perifitic deposits created by floating farm, acting as an artificial reef.

3. Competition induced by other species for the ecosystem would be the effect of the predatorism of the perch on bleak, and the consequent erratic parasitism with plerocercoids of *Ligula intestinalis* L.
4. Do fish feel pain ? We suppose yes, only according infested perch with living plerocercoids who determined pain and a desperate behavior, eating pieces of little stones from the benthic substrate of the lake.

References

1. Battes K., 1971 - *Caracteristici ale lacului de acumulare Bicaz în cursul anului 1970*. Lucr. Staţ. "Stejarul", Limnol., 357-398
2. Bruno D. W., Nowak B., Elliott D.G., 2006 – *Guide to the indentification of fish protozoan and metazoan parasites in staind tissue section*, Diseases of Aquatic organism, vol. 70, p 1-36
3. Miron I., şi colab., 1983 – *Lacul de acumulare Izvoru Muntelui – Bicaz*, Editura academiei Republicii Socialiste România, Bucureşti, p. 224
4. Miron Liviu, Cătălin Simionescu – 1994, Cercetări privind compoziţia ihtiofaunei din lacul de acumulare Bicaz, Lacurile de acumulare din România – tipologie, valorificare, protecţie, pag. 83-91, 1994

HAEMATOLOGICAL, BIOCHEMICAL AND MICROBIOLOGICAL STUDIES AT PIGEONS TREATED WITH A PRODUCT BASED ON METRONIDAZOLE, OXYTETRACYCLINE, FURAZOLIDONE AND BISMUTH SUB-NITRATE

Nicodin Iosif FIŢ, Flore CHIRILĂ, George NADĂŞ, Sorin RĂPUNTEAN, Laurenţ OGNEAN, Sebastian TRĂNCĂ, Cosmina CUC (BOUARI)
University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Faculty of Veterinary Medicine, 3-5 Mănăştur Street, 400372, Romania, e-mail: nfit@usamvcluj.ro

Abstract
The study aimed to reveal the changes in erythrocytes and leukocytes and biochemical parameters in healthy pigeons treated orally with a mixture of metronidazole, oxytetracycline, furazolidone and bismuth sub-nitrate.n the same time we studied the changes in intestinal microbial flora in case of therapy with the four antimicrobial components. For the experiment we used three groups of birds that were treated with different composition of metronidazole (10 mg), oxytetracycline hydrochloride (6 mg), furazolidone (2mg) and bismuth sub-nitrate (2 mg). The first group was treated for 5 consecutive days with 1 g of this product, the second group was treated with double dose and group 3 was the control group. Before and after 5 days of treatment was collected hematological and biochemical samples for analyses. We also isolated after the treatment from these groups 22 strains of Staphylococcus spp., 14 of Streptococcus spp., 7 of Bacillus cereus, 3 of Clostridium perfringens, 14 of Escherichia coli and 4 of Proteus spp. that were tested for antibiotic resistance. The results of erythrocyte parameters showed a slight increase in the beginning of the experiment, not significant in the group with double dose, especially hemoglobin, mean corpuscular volume and mean erythrocyte hemoglobin and after treatment values were similar to those of control group. Mean leukocyte parameters were not varied significantly between the second collection and to the control group. Biochemical profile of the treated pigeons varied with insignificant average values between the two collections. After 5 days of treatment it was found some slightly higher parameters: total protein, globulin, aspartate aminotransferase and creatine phosphokinase. It was also found in oxytetracycline resistance of only 9.09% of strains of staphylococci and streptococci strains of 7.14%. The strains of Bacillus cereus and Clostridium perfringens were not resistant. In Gram negative bacteria situation was different, E. coli resistance was observed for 7.57% of the strains and Proteus spp for 25%. Results obtained from tests with furazolidone showed that for this substance Gram negative bacteria were more sensitive, with 16.66% resistant strains of E. coli and 0% for Proteus spp. The results demonstrates that this product does not significantly alter leucocytes, lymphocytes, and biochemical parameters on pigeons and in the same time have a good efficacy at most pathogens at pigeons.

Keywords: oxytetracycline, furazolidone, antimicrobial resistance, bacteria from pigeons

Introduction

Microbial resistance to tetracyclines is installed gradually especially after repeated and prolonged treatments. This resistance is due to antibiotic exposure and is plasmid mediated. The emergence of resistance occurs due to active transport system deficiency or decrease in membrane permeability, which makes the antibiotic not realize active concentrations in bacterial cells. Mechanism of resistance of microorganisms to nitrofurans seems to be influenced by the activity of genes that coordinate H nitroreductase (RdxA) and H flavin oxidoreductase (FrxA).

This paper aimed to highlight changes in erythrocyte, leucocyte and biochemical parameters in healthy pigeons orally treated with a mixture of metronidazole, oxytetracycline,

furazolidone and bismuth sub-nitrate powder and susceptibility test of bacteria isolated from treated birds.

Materials and methods

The experiment took place from March to November 2011 in the Hospital of the Faculty of Veterinary Medicine and Laboratory of Microbiology and Physiology.

Animals

Testing was conducted on three groups of clinically healthy pigeons aged from 1 to 3 years administrated with a powder having the following composition: Metronidazole 10mg, Oxytetracycline hydrochloride 6 mg, Furazolidone 2 mg, Bismuth sub-nitrate 2 mg.

The groups were divided as follows:

 Group 1 (n = 10) – the animals were administrated with recommended preventive dose for 5 days (1g/kg food per day);

 Group 2 (n = 10) - the animals were administrated with recommended curative dose for 5 days (2g/kg food per day);

 Group 3 (n = 10) – animals that were not treated but housed under the same conditions;

The animals were examined (including standard clinical examination and laboratory screening) before the experiment during the 7 days before the beginning of the study; Study period included a strict timetable, differentiated for days and hours.

Experimental design

It consisted of blood collection on day zero for haematological and biochemical exams and then once a day for three days censecutively to the administration in doses described above. Daily clinical examinations were made also supervising food and water intake. Final examination (post-therapy) was performed at 10-24 hours after the last administration of products and consisted of repeated clinical, hematological and biochemical examinations. All groups were clinically examined for 10 days after the final administration. Hematological parameters investigated were: hematocrit, hemoglobin, erythrocytes, MCV, MCH, MCHC, white blood cells (WBC), neutrophils, monocytes, eosinophils, basophils, lymphocytes. Biochemical tests aimed AST - aspartate aminotransferase, alkaline phosphatase ALP, CK - creatine phosphokinase, CA - calcium, Phos - phosphorus, TP - total protein, gamma-glumatil-transferase GGT, BUN, blood urea nitrogen, WHITE - albumin, GLOB - globulin, MG-magnesium. Were chosen as most relevant to observe any organ metabolism disorders or general disturbances of metabolism. For interpretation of results were taken as reference haematological and biochemical data presented by Reece, 1996 and Ghergariu et al., 2000.

At the end of the experiment from the groups treated with erythromycin were collected fecal samples. Using morphological, cultural and biochemical examinations, bacterial strains were isolated and tested regarding the resistance to oxytetracycline and furazolidone, according to procedures described by Quinn et al, and CLSI.

Results and discussions

The results of haematological and biochemical exams in pigeons treated with metronidazole, oxytetracycline hydrochloride, furazolidone, bismuth sub-nitrate powder as preventive and curative-dose compared with untreated control group are presented as mean values and standard deviations in Tables 1, 2 and 3.

Table 1. Mean red cell parameters recorded in preclinical testing of the product based on Metronidazole, Oxytetracycline, furazolidone, bismuth sub-nitrate on adult pigeons.

Group nr.		*1*	*2*	*3*
		Average + standard deviation	*Average + standard deviation*	*Average + standard deviation*
Before treatment				
Hct(%)		*42,78±3,47*	*47,3±5,23*	46,25±3,29
Hb(g/dl)		*8,14±2.29*	*14,12±3,02*	9,26±0,92
RBC nr. (T/l)		*3,42±0,49*	*3,14±0,07*	3,35±0,65
RBC indices	MEV/(fl)	*125,87±14,34*	*150,57±15,32*	141,18±26,78
	MEH(pg)	*25,35±6,89*	*45,01±9,88*	29,25±5,51
	MCHC(g/dl)	*21,74±7,67*	*30,62±9,63*	22,32±6,05
After tretment				
Hct(%)		*40,50±13,82*	*43,35±3,56*	40,79±13,98
Hb(g/dl)		*8,36±3,45*	*14,75±3,68*	8,85±2,94
Nr.Eritrocite(T/l)		*2,70±0,85*	*3,48±0,48*	3,05±0,94
RBC indices	MEV/(fl)	*138,64±49,52*	*126,12±16,55*	126,13±41,52
	MEH(pg)	*28,22±11,1*	*41,06±11,77*	27,52±9,52
	MCHC(g/dl)	*18,84±6,07*	*34,35±10,07*	20,45±6,17

Synthesis of WBC count revealed a good potential of both nonspecific and specific defense, whose level was not affected by treatment variables, tested on the target species. Proportions of leukocyte subpopulations showed high levels of tolerance to preventive and curative doses of the product without sensitization or adverse effects on leucopoiesis and leukocyte functions in general.

Table 2. Mean leukocyte parameters recorded in preclinical testing of the product that contains Metronidazole, Oxytetracycline, furazolidone and bismuth sub-nitrate on adult pigeons

Group nr.		*A 1*	*A 2*	*A 3*
		Average + standard deviation	*Average + standard deviation*	*Average + standard deviation*
Before treatment				
Leukocytes (G/l)		*16,9±0,89*	*19,52±6,35*	*14,2±2,66*
WBC count (%)	Heterophils	*42±3,24*	*36,8±1,3*	*34±4,12*
	Eosinophils	*5,2±1,79*	*1,4±1,52*	*7,6±2,88*
	Basophils	*0±0*	*00*	*0±0*
	Limphocytes	*37±0,9*	*51,4±6,11*	*38,4±0,89*
	Monocytes	*15,2±3,9*	*10,4±5,59*	*20±3,46*

After treatment				
Leucocite (G/l)		*17,6±4,72*	*16,78±0,83*	*14,8±2,97*
WBC count (%)	Heterophils	*37,2±5,36*	*35,4±3,65*	*31,6±1,67*
	Eosinophils	*1,6±1,52*	*1±0,71*	*1,8±0,84*
	Basophils	*0±0*	*0±0*	*0,8±0,74*
	Limphocytes	*44,2±5,93*	*46,6±7,13*	*47,2±1,79*
	Monocytes	*17±9,54*	*14,6±3,65*	*18,6±2,41*

Biochemical analysis revealed significant differences between groups of animals of the same species, outlining the characteristics of a metabolic protein profile, carbohydrate, mineral and enzyme corresponding to good health and maintenance level continued throughout the observation period pre-and post-treatment, revealing along the lack of dysmetabolic effects, the lack of preventive and curative dose toxicity.

Table 3. Mean biochemical parameters recorded in preclinical testing of product that contains Metronidazole, Oxytetracycline, furazolidone and bismuth sub-nitrate on adult pigeons

Group nr.		*A 1*	*A 2*	*A 3*
		Average + standard deviation	*Average + standard deviation*	*Average + standard deviation*
Before treatment				
TP	(g/dl)	3,34±0,36	3,4±0,25	3,3±0,44
ALB	(g/dl)	2,8±0,12	1,74±0,24	2,8±0,12
GLOB	(g/dl)	0,64±0,21	0,78±0,16	0,54±0,32
GLU	(mg/dl)	299,2±28,23	214,4±7,96	299,2±28,23
AST	(U/l)	83,2±28,34	196,2±12,21	83,2±28,34
CK	(U/l)	380,8±210,75	913,4±59,56	380,8±210,75
UA	(mg/dl)	4,02±2,31	5,9±0,83	3,22±0,62
CA	(mg/dl)	10,6±1,01	8,62±0,65	10,6±1,01
PHOS	(mg/dl)	3,16±1,04	6,32±0,55	2,96±1,03
NA^+	(mmol/l)	160±3,67	157,6±5,64	159,4±3,05
K^+	(mmol/l)	3,96±1,02	4,48±0,31	4,34±1,75
After treatment				
TP	(g/dl)	3,44±0,38	3,46±0,15	3,36±0,42
ALB	(g/dl)	2,44±0,44	1,78±0,29	2,26±0,46
GLOB	(g/dl)	0,96±0,21	0,96±0,11	1,12±0,26
GLU	(mg/dl)	273,2±25,45	216,6±15,66	267,2±54,66
AST	(U/l)	91,2±22,64	190,4±20,12	124,6±38,06
CK	(U/l)	707,8±206,99	948,4±62,72	933,2±82,14
UA	(mg/dl)	4,68±1,3	6,1±0,64	4,52±1,01
CA	(mg/dl)	9,48±0,34	8,24±1,5	9,58±0,51
PHOS	(mg/dl)	5,52±1,18	6,58±0,55	4,6±0,86
NA^+	(mmol/l)	157,2±5,72	156,6±3,44	156,6±6,58
K^+	(mmol/l)	4,62±0,85	4,56±0,22	4,72±0,86

Resistance of microorganisms to treatment with vitamin Oxifuran pointed out that the phenomenon of resistance of microorganisms to oxytetracycline and furazolidone is not a major problem yet.

In support of these statements are indicative of the issues that are presented in Table 4.

Following susceptibility testing of Gram-positive and Gram-negative bacteria to oxytetracycline, we observed that *Staphylococcus* was resistant in case of 9.09% strains and sensitivie for 77.27% of tested strains. For streptococci from 14 strains, 7.14% were resistant and 66.6 susceptible to oxytetracycline. A similar situation was observed for *Bacillus cereus* where there were no strains resistant to oxytetracycline. Testing of anaerobic strains of *Clostridium perfringens* species also showed no resistance to oxytetracycline. A different situation was observed in *E. coli* strains where from 14 isolates, 78.57% were resistant, while for *Proteus* and half of them were resistant (Table 4).

Table 4. Resistance to oxytetracycline of the bacterial strains tested

Tested strains	Total strains (nr.)	Resistant (%)	Moderate sensitive (%)	Sensitive (%)
Staphylococcus spp.	22	9,09	13,63	77,27
Streptococcus spp.	14	7,14	21,42	66.66
Bacillus cereus	7	0	28.57	71.42
Clostridium perfringens	3	0	33.33	66.66
Escherichia coli	14	78.57	7.14	14.28
Proteus spp.	4	25	25	50

Testing of strains resistant to furazolidone showed that strains of *Salmonella spp* and *Proteus spp* studied have not developed resistance to this antibiotic, most of the strains being sensitive and only a small number moderate sensitive. Strains of *Staphylococcus spp, Streptococcus spp* and *Escherichia coli* tested were resistant depending on the specie. Thus in *Staphylococcus spp*., a percentage of 18.75% of strains were resistant, for *Streptococcus* in percentage of 28.57% and for E. coli 16.66 of strains were resistant to furazolidone (Table 5).

Table 5. Resistance to furazolidone of the bacterial strains tested

Tested strains	Total strains (nr.)	Resistant (%)	Moderate sensitive (%)	Sensitive (%)
Staphylococcus spp.	16	18.75	25	56.25
Streptococcus spp.	7	28.57	14.29	57.14
Escherichia coli	12	16.66	25	58.33
Salmonella spp.	4	0	25	75
Proteus spp.	3	0	33.33	66.66

Making a comparison of data obtained by us on oxytetracycline and furazolidone resistance of pathogens isolated from animals, with other data obtained by other researchers in the field on pathogens isolated from chickens in different areas, we apreciate that the levels of resistance are still low. In this context we mention some results obtained by some

researchers in the country that surveyed resistance strains from birds. In a large study of oxytetracycline resistance of bacteria of avian origin, Tudor (2008) noted that the testing of 448 strains of *E. coli* obtained a sensitivity of 1% of the strains tested, most of them being resistant (86%). Similar data were observed in this study, bacteria being in percentage of 78.57% resistant to oxytetracycline. Studies on *Staphylococcus strains* obtained by the same author in our country shows an increase in bacterial resistance to oxytetracycline up to 66% of the strains tested. In our studies, staphylococcal strains showed a level of resistance of only 9.09%. Other studies by Gharaibeh et al. in 2010 on 67 strains of *C. perfringens* isolates from chickens that have been tested in several types of antibiotics (tylosin, amoxicillin, ampicillin, penicillin, florfenicol, danofloxacin, enrofloxacin, chlortetraciclină, doxycycline, and oxytetracycline) shows that from all tested antibiotics, only amoxicillin and oxytetracycline had the MIC_{50} of 0.5 mg/ml, so you can recommend oxytetracycline in anaerobic infections in birds and carnivores. For *Clostridium perfringens* strains similar data were obtained, with no resistant strains to treatment with oxytetracycline.

Antibiotic test data on germs like *Salmonella enterica* to furazolidone are presented by Andor et al., 2008 where it is found that of 106 strains tested, 86% of them were resistant, 5.6% were moderate sensitive and only 4.7% were sensitive to treatment with furazolidone.

Conclusions

In the overall analysis of our results compared to the literature data shows that resistance to oxytetracycline and furazolidone is still reduced in most strains studied by us and that it varies from one bacterial species to another depending on the geographic area studied. However we can say that for infections involving frequently resistant species this product can be recommended mostly because has three antibiotics with broad antimicrobial spectrum, including some protozoa.

Bibliography

1 Andor C., E. Avram, C. Ceică, Cosmina Cuc, Gh. Răpuntean (2008) STUDY REGARDING RESPONSE TO ANTIMICROBIAL AGENTS OF *SALMONELLA ENTERICA* STRAINS, ISOLATED FROM VARIOUS ECOLOGICAL AREAS. Bulletin UASVM, Veterinary Medicine 65(1).
2 Brady, M.S. & Katz, S.E. (1992) *In vitro* effect of multiple antibiotic/antimicrobial residues on the selection of resistance in bacteria. *J. Assoc. Anal. Chem. Int.,* 75, 738-742.
3 Brady, M.S., Strobel, R.J. & Katz, S.E. (1988) *In vitro* analytical system for determining the ability of antibiotics at residue levels to select for resistant bacteria. *J. Assoc. Anal. Chem.,* 71, 295-298.
4 Gharaibeh S., Al Rifai R., Al-Majali A. (2010) Molecular typing and microbial susceptibility of Clostridium perfringens from broiler chickens.
5 Ghergariu S. Pop A., Laszlo K., Marina Spânu (2000). Manual de laborator clinic veterinar. Editura All Educaţional.
6 Reece W.O. *Functional anatomy and physiology of domestic animals*, Lippincot, 2005.
7 Tudor Cătălin (2008) - Evoluţia antibiorezistenţei la principalele antibiotice folosite în avicultura românească. Raport SC Romvac Company SA.

HAEMATOLOGICAL, BIOCHEMICAL AND MICROBIOLOGICAL STUDIES ON SHEEP TREATED WITH 10% ERYTHROMYCIN SOLUTION

Nicodim Iosif FIŢ, Flore CHIRILĂ, George Cosmin NADĂŞ, Laurenţ OGNEAN, Sebastian TRÂNCĂ, Cosmina CUC (BOUARI)

University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Faculty of Veterinary Medicine, 3-5 Mănăştur Street, 400372, Romania, e-mail: nfit@usamvcluj.ro

Abstract

This paper is aiming to highlight changes in erythrocyte, lymphocytes and biochemical parameters on Tigaie and crossbred sheep breed, clinically healthy, treated with 10% erythromycin solution intramuscularly. We also followed the changes in intestinal microbial flora to treatment with erythromycin. For the experiment we used three groups of sheep that were treated with different doses of erythromycin. The first group was treated with 2 ml intramuscularly per 50 kg per day, group two was treated with 4 ml intramuscularly per 50 kg per day, and group three was treated with saline sterile solution. Administrations were made daily for three consecutive days. In the first day and in day 3 of treatment, blood samples were collected for hematological (12 parameters) and biochemical (10 parameters) analysis. After the treatment of these groups, 28 strains of Staphylococcus spp., 10 of Streptococcus spp., 5 of Bacillus cereus, 4 of Arcanobacterium spp. and 8 of Pasteurella spp were isolated, strains that were tested for resistance to erythromycin. The results of erythrocyte parameters showed a significant increase ($p < 0.05$) of hematocrit in the group who received double dose of erythromycin for 3 days. Average leukocyte parameters increased slightly during treatment but had no significant values between the two blood collections and the control group. Biochemical profile in treated sheep varied, with average value significantly ($p < 0.05$) only in aspartate aminotransferase and alkaline phosphatase between the two collections. Other erythrocyte values, leukocyte and biochemical parameters were insignificant ($p \geq 0.05$). Erythromycin resistance was observed in only 7.14% of staphylococci and 12.5% in Pasteurella spp. strains. The streptococci, Bacillus cereus and Arcanobacterium spp. were not resistant. Results obtained from tests with erythromycin shows that this product may modify the hematological and biochemical parameters but not with clinical expression. Because the results were good in antimicrobial studies on Gram positive bacteria, we recommend its use in diseases caused by this type of bacteria.

Keywords: erythromycin, antimicrobial resistance, bacteria from sheep

Introduction

Microbial resistance to macrolides gradually installs especially after repeated and prolonged treatments. This resistance is due to antibiotic exposure and is plasmid mediated. The emergence of resistance occurs due to the deficiency in active transport system or decrease of membrane permeability, which makes the antibiotic concentrations in bacterial cells to be reduced (Corpet and World, 1987; Lebek and Egger, 1983). Plasmids can also carry genes responsible for resistance to other antibiotics - aminoglycosides, chloramphenicol, sulfonamides. Plasmids can be transferred to other bacteria by transduction or conjugation (Brady et al, 1988; Brady and Katz, 1992; Brady et al, 1993)

Antibiotic administration to animals represents the last option for treatment since milk and meat production suffers and secondly because sometimes inappropriate administration may even cause disease (Pintea et al, 1982; Rollins et al, 1975; Reece , 1996).

This paper is aiming to highlight changes in erythrocyte, lymphocytes and biochemical parameters on Tigaie and crossbred sheep breed, clinically healthy, treated with 10% erythromycin solution intramuscularly. We also followed the changes in intestinal microbial flora to treatment with erythromycin.

Materials and methods

The experiment took place from March to November 2011 in the Hospital of the Faculty of Veterinary Medicine and Laboratory of Microbiology and Physiology.

Animals

Testing was conducted on three groups of clinically healthy Tigaie breed sheep aged between 1 and 4 years. The groups were divided as follows:

► Group 1 (n = 10) - sheep that received curative recommended daily dose consecutively for 3 days (2 ml intramuscularly each 50 kg per day);

► Group 2 (n = 10) - sheep received double dose daily consecutively for 3 days (4 ml intramuscularly each 50 kg per day);

► Group 3 (n = 10) - sheep that received physiological solution 2 ml intramuscularly and were housed in the same conditions;

The animals were examined (including standard clinical examination and laboratory screening) before the experiment during the 7 days before the beginning of the study; Study period included a strict timetable, differentiated for days and hours.

Study design

It consisted of blood collection on day zero for haematological and biochemical exams and then once a day for three days censecutively to the administration in doses described above. Daily clinical examinations were made also supervising food and water intake. Final examination (post-therapy) was performed at 10-24 hours after the last administration of products and consisted of repeated clinical, hematological and biochemical examinations. All groups were clinically examined for 10 days after the final administration. Hematological parameters investigated were: hematocrit, hemoglobin, erythrocytes, MCV, MCH, MCHC, white blood cells (WBC), neutrophils, monocytes, eosinophils, basophils, lymphocytes. Biochemical tests aimed AST - aspartate aminotransferase, alkaline phosphatase ALP, CK - creatine phosphokinase, CA - calcium, Phos - phosphorus, TP - total protein, gamma-glumatil-transferase GGT, BUN, blood urea nitrogen, WHITE - albumin, GLOB - globulin, MG-magnesium. Were chosen as most relevant to observe any organ metabolism disorders or general disturbances of metabolism. For interpretation of results were taken as reference haematological and biochemical data presented by Campbell 2004, Ghergariu et al., 2000, and Gylstorff, 1983.

At the end of the experiment from the groups treated with erythromycin swabs with nasal discharge were collected. Using bacteriology techniques according to the procedures described by Quinn et al, 1994, were isolated 14 strains of *Staphylococcus intermedius*, 16 of *Staphylococcus spp.*, 10 of *Streptococcus spp*, 5 of *Bacillus cereus*, 6 of Listeria spp., 4 of *Arcanobacterium pyogenes* and 8 of *Pasteurella multocida*, which were tested in terms of resistance to erythromycin by disc diffusion method according to CLSI.

Results and discussions

The results of haematological and biochemical tests on sheep treated with 10% solution of erythromycin administrated in normal and double-dose compared with controls treated with physiological solution are presented as mean values and standard deviations in Tables 1, 2 and 3.

Table 1. Mean red cell parameters recorded in preclinical testing of 10% erythromycin solution on sheep, preventive dose (group 1) curative dose (group 2) and untreated control (group 3)

Group		*1*	*2*	*3*
		Average + standard deviation	*Average + standard deviation*	*Average + standard deviation*
Before treatment				
Hct(%)		36.2±7.6	*30.47±4.97*	*36.79±2.76*
Hb(g/dl)		10.6±1.52	*12.81±1.48*	*12.47±1.65*
Nr. erythrocyte (T/l)		11.6±1.82	*11.69±1.25*	*6.93±1.48*
Red blood cell indices	MCV /(fl)	33.6±3.65	*32.8±4.55*	*36.2±6.22*
	MCH (pg)	10.8±1.3	*11.41±1.29*	*11.08±1.71*
	MCHC g/dl)	32.02±2.12	*32.59±1.01*	*32.12±1.22*
After treatment				
Hct(%)		39.8±5.36	*37.34±5.81*	*37.01±1.51*
Hb(g/dl)		12.8±1.3	*13.1±2.16*	*12.76±1.57*
Nr. erythrocyte (T/l)		13.2±1.64	*12.98±1.63*	*7.18±1.07*
Red blood cell indices	MCV /(fl)	36.6±3.36	*36.4±3.78*	*38±4.85*
	MCH (pg)	11±1.58	*10.42±1.71*	*11.62±1.58*
	MCHC (g/dl)	32.94±1.31	*30.17±4.87*	*31.77±1.21*

Table 2. Mean WBC parameters recorded in preclinical testing of 10% erythromycin solution on sheep, preventive dose (group 1) curative dose (group 2) and untreated control (group 3)

Group		*1*	*2*	*3*
		Average + standard deviation	*Average + standard deviation*	*Average + standard deviation*
Before treatment				
Leukocytes (G/l)		7.2±2.81	9.64±2.04	9.88±2.11
Blood count (%)	Neutrophils	39±9.35	37.2±11.69	51.6±7.77
	Eosinophils	5.2±2.28	3.6±2.7	6.8±3.77
	Basophils	1±1.22	0.4±0.55	0.8±0.45
	Lymphocytes	50.8±13.26	55±9.14	30.8±6.26
	Monocytes	4±1.58	3.8±1.92	10±2

After treatment				
Leukocytes (G/l)		8.72±2.1	10.38±1.33	9.33±1.75
Blood count (%)	Neutrophils	39.6±10.21	37±4.64	53.6±5.37
	Eosinophils	5.8±2.59	4.4±1.14	6.6±2.3
	Basophils	0.8±0.84	1±1	0.6±0.55
	Lymphocytes	50.4±10.21	54.4±7.09	26.4±3.36
	Monocytes	3.4±1.52	3.2±1.64	12.8±2.28

The results of erythrocyte parameters showed a significant increase ($p < 0.05$) of hematocrit in the group who received double dose of erythromycin for 3 days. Average leukocyte parameters increased slightly during treatment but had no significant values between the two blood collections and the control group.

Table 3. Mean biochemical parameters recorded in preclinical testing of 10% erythromycin solution on sheep, preventive dose (group 1) curative dose (group 2) and untreated control (group 3).

Group		*1*	*2*	*3*
		Average + standard deviation	*Average + standard deviation*	*Average + standard deviation*
Before treatment				
ALB	(g/dl)	42.8±1.48	42.6±1.52	40.82±4.4
ALP	(U/l)	273.2±81.14	306.4±44	103.2±14.97
AST	(U/l)	333.8±88.74	249±83.74	87±10.2
CA	(mg/dl)	10.64±1.21	11±1.37	11.22±1.79
TP	(g/dl)	6.12±0.46	6.16±0.28	7.28±1.83
GLOB	(g/dl)	8.06±0.59	8.08±0.68	7.68±0.58
BUN	(mg/dl)	32.8±11.45	34.4±7.67	28.8±5.36
CK	(U/l)	17±2.92	16±2.92	19.8±2.77
PHOS	(mg/dl)	5.26±0.09	5.28±0.04	5.28±0.8
MG	(mg/dl)	1.88±0.16	1.92±0.15	2±0.16
After treatment				
ALB	(g/dl)	43.6±1.52	42.2±0.84	40.82±1.94
ALP	(U/l)	299.2±48.49	331.2±34.27	110.4±16.74
AST	(U/l)	437.4±61.46	316.4±61.92	75.4±17.44
CA	(mg/dl)	11.56±0.8	11.72±0.96	10.86±1.01
TP	(g/dl)	6.22±0.75	6.1±0.27	6.58±0.59
GLOB	(g/dl)	8.3±0.62	8.06±0.59	8.28±1.41
BUN	(mg/dl)	38±6.52	33.8±10.43	29.4±5.41

CK	(U/l)	15.4±4.34	17.4±2.61	19.6±2.41
PHOS	(mg/dl)	5.28±0.05	5.27±0.05	5.3±0.38
MG	(mg/dl)	2.14±0.27	1.9±0.17	1.98±0.11

AST - aspartate aminotransferase, alkaline phosphatase ALP, CK - creatine phosphokinase; CA - Calcium, Phos - phosphorus, TP - total protein, GGT, gamma-glumatil transferase; BUN, blood urea nitrogen, WHITE - albumin, GLOB - globulin, MG-magnesium

Biochemical profile in treated sheep varied, with average value significantly ($p < 0.05$) only in aspartate aminotransferase and alkaline phosphatase between the two collections. Other erythrocyte values, leukocyte and biochemical parameters were insignificant ($p \geq 0.05$). Erythromycin resistance was observed in only 7.14% of staphylococci and 12.5% in *Pasteurella spp*. strains. The streptococci, *Listeria spp.*, *Bacillus cereus* and *Arcanobacterium spp*. were not resistant.

Resistance test of microorganisms to treatment with 10%erythromycin revealed that the phenomenon of resistance of microorganisms to erythromycin had no marked trend on microbial agents involved in primary and secondary infections in chickens and pigeons produced by pathogens sensitive to this antibiotic.

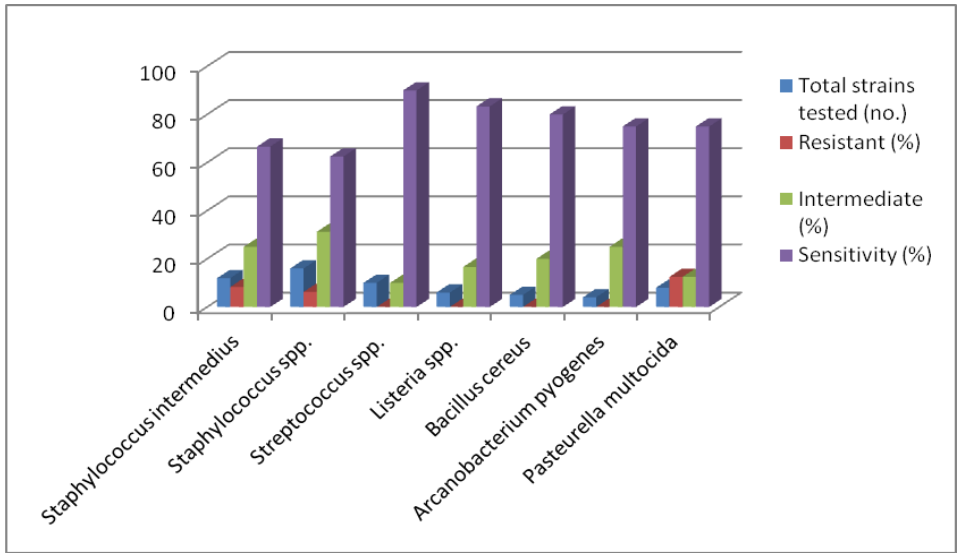


Fig. 1. Sensitivity to erythromycin of bacterial strains isolated from sheep treated with erythromycin

In support of these statements are indicative the aspects presented in Chart 1, which presents data obtained from tests of resistance to erythromycin of some bacterial strains isolated from sheep. *Staphylococcus intermedius* presented a resistance of 8.33% and a sensitivity of 66.66% in case of tested strains. Of the 16 strains of *Staphylococcus spp.* a rate of 62.5% were susceptible to erythromycin, 31.25% were moderate sensitive and 6.25% were

resistant. In case of streptococci, from 10 strains tested 0% were classified as resistant and 90% as sensitive to erythromycin. None of *Listeria monocytogenes* strains was resistant but 16.6% were moderate sensitive and 83.33% were sensitive to erythromycin. A similar situation was observed for *Bacillus cereus* where there were no strains resistant to erythromycin. In case of *Arcanobacterium pyogenes* a number of 75% of the strains were sensitive, 25% moderate sensitive and none was resistant. *Pasteurella multocida* strains showed resistance for in 12.5% and a sensitivity of 75%.

Making an overall assessment of the results, erythromycin resistance can be observed from Chart 1 that the 7 types of bacteria tested, only three reported resistant strains - *Staphylococcus intermedius, Staphylococcus spp.*, and *Pasteurella multocida* and four species (*Streptococcus spp., Listeria spp., Bacillus cereus, Arcanobacterium pyogenes*) no resistant strains were found. Resistance variation was between 6.25% and 8.33% for staphylococci (for Gram positive) and 12.5% in Gram-negative bacteria. The sensitivity of the tested strains was between 62.5% and 90%. The percentage of moderate sensitivie strains ranged between 10 and 31.25%.

In this context we mention some results obtained by other researchers that surveyed resistance in the country, so a study on 89 strains of *Staphylococcus aureus* isolated from northeastern Romania in 2006 were tested for resistance to erythromycin by MIC determination. There was a resistance to this product at a rate of 19%, being slightly higher thanthe value of this study (Dobreanu et al., 2006).

Conclusions

Results obtained from tests with erythromycin shows that this product may modify the hematological and biochemical parameters but not with clinical expression. Because the results were good in antimicrobial studies on Gram positive bacteria, we recommend its use in diseases caused by this type of bacteria.

Bibliography

1. Brady, M.S. & Katz, S.E. (1992) *In vitro* effect of multiple antibiotic/antimicrobial residues on the selection of resistance in bacteria. *J. Assoc. Anal. Chem. Int.,* 75, 738-742.
2. Brady, M.S., Strobel, R.J. & Katz, S.E. (1988) *In vitro* analytical system for determining the ability of antibiotics at residue levels to select for resistant bacteria. *J. Assoc. Anal. Chem.,* 71, 295-298.
3. Brady, M.S., White, N. & Katz, S.E. (1993) Resistance development potential of antimicrobial residue levels designated as 'safe levels'. *J. Food Protect.,* 56, 229-233.
4. Campbell T. W. (2004) Hematology of Lower Vertebrates.
5. Corpet, D. Lumeau, S. (1987). Effect of low levels of antimicrobials on drug resistant populations of intestinal bacteria in gnotobiotic mice. In Fedesa report: Rational view of antimicrobial residues. An assessment of human safety. Abstracts of a seminar held in Zurich, March 24.
6. Dorneanu Olivia, , Miftode Egidia,, Vremeră Teodora, E Năstase, Filip Olga, V Luca-(2006) Prevalence And Characteristics Of Staphylococus Aureus Isolated From Infections In Northeast Romania. Journal of Preventive Medicine; 14 (3-4): 66-70
7. Ghergariu S. Pop A., Laszlo K., Marina Spânu (2000). Manual de laborator clinic veterinar. Editura All Educaţional.
8. Gylstorff I: Handbuch der Geflügelphysiologie, 1983; Wallach JD, Boever WJ: Diseases of Exoic Animals.
9. Lebek, G. Egger, R. (1983). The effect of low levels of antibiotics on the selection of resistance in intestinal bacteria in vitro observations. Cited in Fedesa report: Rational view of antimicrobial residues. An assessment of human safety. Abstract of a seminar held in Zurich, March 24.
10. Pintea V., Codruţ M., Manta D.A.M Salageanu G., (1982) - Fiziologie Medical-Veterinară; Ed. Didactică şi pedagogică, Bucureşti

11. Reece O. W. (1996). Physiology of domestic animals.Blackwell Publishing
12. Rollins, L.D., Gaines, S.A., Pocurull, D.W., & Mesear, H.D. (1975). Animal model for determining the no effect level of an antimicrobial drug on drug resistance in the lactose fermenting enteric flora. *Antimicrob. Agents Chemother.,* 7, 661-665.
13. Quinn P.J., Carter M.E., Markey B., Carter G.R., (1994) Clinical veterinary Micribiology. Edit. Wolfe
14. Clinical and Laboratory Standards Institute (CLSI), *Performance standards for antimicrobial disk susceptibility*, Approved Standard tenth edition M02-A10, Clinical and Laboratory Standards Institute, Wayne, Pa, USA, 8th edition,2009.

ISOLATION OF ADIPOSE-DERIVED MESENCHYMAL STEM CELLS FROM MURINE ADIPOSE TISSUE

Cristina ILEA, Ioan Ş. GROZA, Mihai CENARIU, Laura CĂTANĂ, Hussam ARYAN, Emoke PALL
University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca
Adress: 3-5 Calea Mănăştur street, postal code 400372, Cluj-Napoca
E-Mail: criss_vet@yahoo.com

Abstract
Regenerative therapy based on the use and manipulation of mesenchymal stem cells (MSCs)is a novel treatment modality that could be applied in different types of pathologies which require tissue remodelin. Several potential sources for obtaining MSCs are bone marrow, umbilical cord blood and peripheral blood. While the first source requires an invasive procedure and its efficiency depends of donor's age, the umbilical cord blood can be harvested only at birth and the third source, the peripheral blood, provides o low number of MSCs that are hard to expand in culture, recent studies showed that adipose-derived stem cells (ADSCs) are another valuable source of MSCs. These pluripotent cells with similar properties to bone marrow-derived mesenchymal stem cells are easier to obtain, using less invasive methods, but with the same stemness potential. The aim of our study was the evaluation of two different methods for isolation of ADSCs, followed by cultivation and morphological characterization of obtained cell populations.
Materials and methods. Mouse adipose tissue samples were acquired from abdominal and periinginal fat from 12-14 weeks old CD1 male mice that were killed by cervical dislocation. The obtained samples, according to Protocol I were digested with 0.075% collagenase type II, for 15-20 minutes at room temperature. According to Protocol II, the enzimatic digestion with collagenase type II was completed by mechanic dissociation with a Magnetic Stirrer with *Hot Plate for 30 minutes at 250 rpm at 37°C. Isolated cells were cultivated in supplemented IMDM medium, followed by morphological characterization.*
Results and discussions. Analyzing the proliferative rate and morphological characteristics of adherent isolated cell populations, we concluded that by using enzymatic digestion combined with magnetic dissociation we managed to obtain a stabile population of adipose derived mesenchymal stem cell, the proportion of fibroblast-like cells being 90%, comparative to enzymatic digestion were we obtain a percentage of 20% of fibroblast-like cells, 80% being round-shaped cells, characteristic of adipose progenitor cell.
Keywords: adipose mesenchymal stem cells, enzymatic digestion, mechanic dissociation

Introduction

Regenerative therapy, based on the use and manipulation of mesenchymal stem cells (MSCs) is a novel treatment modality that could be applied in different types of pathologies which require tissue remodeling (1). Several potential sources for obtaining MSCs are bone marrow, umbilical cord blood and peripheral blood. While the first source requires an invasive procedure and its efficiency depends of donor's age, the umbilical cord blood can be harvested only at birth, and the third source, the peripheral blood, provides o low number of MSCs that are hard to expand in culture (2,3). Recent studies showed that adipose-derived stem cells (ADSCs) are another valuable source of MSCs. These pluripotent cells with similar properties to bone marrow-derived mesenchymal stem cells are easier to obtain, using less invasive methods, but with the same stemness potential (4).

The aim of our study was the evaluation of two different methods for isolation of ADSCs, followed by *in vitro* cultivation of isolated ADSCs and morphological characterization of obtained cell populations.

Materials and methods

Mesenchymal stem cells were isolated from 12-14 weeks old CD1 mice after they were killed by cervical dislocation. Mouse adipose tissue dissected from abdominal and subcutaneous inguinal fat were cut in fine pieces. To isolate ADSCs we used two different protocols:

Protocol I: Adipose tissue samples were washed with phosphate-buffered saline solution (PBS, Sigma) in a proportion of 1:1. Enzymatic digestion was performed using Collagenase type I (3mg/ml) (Sigma-Aldrich) and 2% BSA (Sigma) in PBS, for 20 minutes, at room temperature. Collagenase was removed by dilution with α-MEM medium (Gibco) supplemented with 10% fetal calf serum (FCS, Gibco) and centrifuged at 1200 rpm for 10 minutes. In this way, most of the matured adipocytes remained in the supernatant. The ring containing adipose cells and supernatant were discarded and the cell pellet was filtered through a 40µm filter mesh (Milipore) to remove debris. Obtained stromal cell precipitate was resuspended in culture medium represented by IMDM supplemented with 10% FCS, 1% antibiotic-antimicotic, 1% glutamine, 1% non-essential amino acids (NEA) and 5% horse serum. After 24 h, the nonadherent cells were removed by washing with PBS, the culture medium being changed every 48 h. After 70% confluence, was performed the first passage.

Protocol II: The small pieces of adipose tissue were digested with 0.2% collagenase type I (Sigma-Aldrich) diluted in 4-5 ml PBS (Sigma). The enzymatic digestion was completed by mechanic dissociation using a magnetic stirrer with hot plate, at 37°C, for 30 minutes 250 rpm. After shaking, the lower fraction was aspirated and suspended in a sterile tube (the superior fraction contained mature adipocytes). Collagenase was inactivated by FCS in a proportion of 1:1, followed by centrifugation (1200 rpm, 10 minutes). Supernatant was discarded, and after filtration of cell pellet with a sterile mesh (Milipore), the stromal cell precipitate was resuspended in culture medium represented by Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12) (Sigma) supplemented with 10% fetal calf serum (FCS, Gibco), 5% horse serum (Sigma) and 1% MycoZap (Mycoplasma Elimination Reagent, Lonza) in 60 mm culture dishes at 37°C, 5% CO_2 and 90% humidity.

Non-adherent cells were removed after 4 days, and the culture medium was changed every 4 days. The first passage was performed done when cell population reached a confluence of 80-90 %.

Results and disscutions

The mouse is a suitable experimental model system to study the cell biology and biochemical characteristics of MSCs (5). These cells are considered to be an ideal source of stem cells for regenerative medicine. They can be purified and expanded maintaining their multipotent capacity. Even though many investigators have attempted to establish appropriate methods to isolate and maintain mouse MSCs, their cultivation being more difficult than human MSCs (6). To optimize MSC cultivation, we used two different methods of isolation and cultivation.

To obtain mouse ADSCs culture, we isolated mesenchymal stem cells using 2 different protocols (enzymatic digestion, versus enzymatic digestion completed by mechanic dissociation).

Using Protocol I, the initial culture (p0), at day 2 of cultivation contained a heterogeneous cell population, composed by round-shaped, polygonal-shaped and enlarged-shaped cells and a low number of fibroblast-like cells (fig.1). Due to an increased growth

rate, isolated cells were disposed in cell agglomerations, composed by 8 to 10 cells. All these morphological characteristics, in association with the asymmetric stem cell division indicated by the culture heterogeneity, proves that in the pre-existent culture, the cells were represented by committed adipose mesenchymal stem cells/progenitor cells (APCs).

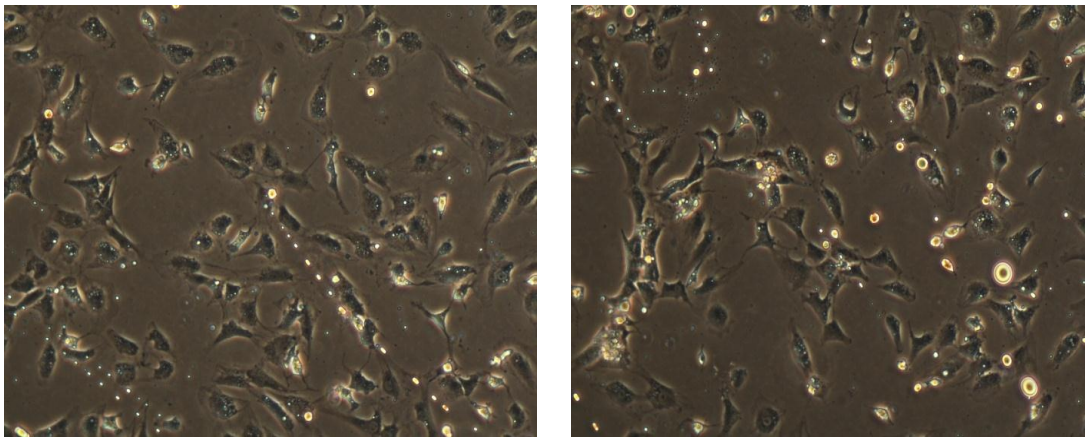


Fig. 1 – APCs in culture (protocol I) in day 2 (original)

Because of the highly increased doubling number of APCs, in the day 4 of cultivation, the confluence reached a percentage of 80-90% (fig.2). To avoid contact inhibition, we realized the first passage (p1). The culture medium was changed every day. Due to the highly increased proliferative rate, p2 and p3 were done after 2 days each. During all three passages, the cultures presented a heterogeneous population of cells (round shaped cells, enlarged shaped cells, polygonal cells) similar to the primary culture, the percentage of fibroblast-like cells being 20%. At p3, we observed a spontaneous adipogenic differentiation, which confirm that isolated cells were adipose progenitor cells (APCs).

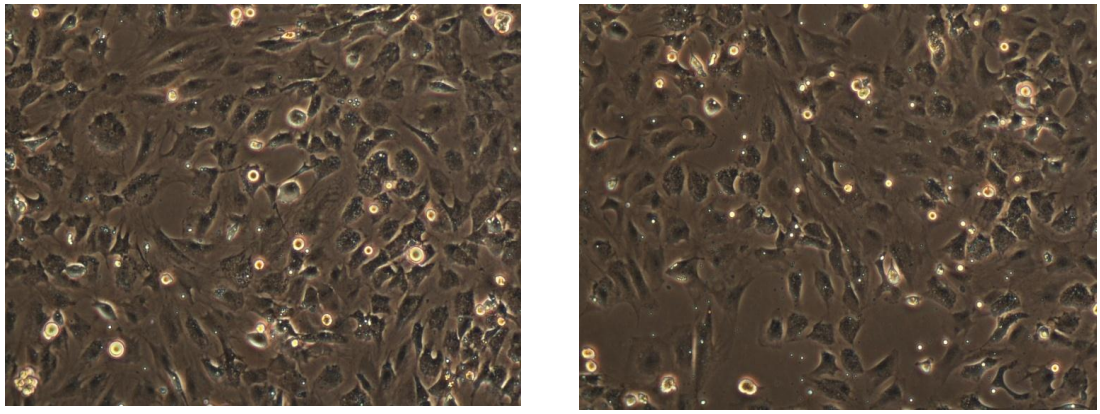


Fig. 2 – APCs in culture (protocol I) in day 4 (original)

In the initial culture (p'0) obtained using protocol II, the adherent cells were observed starting from the day 2 of culture and the nonadherent cells were removed only in the day 4 of culture. Compared to the p0 obtained using protocol I, here, the cultures presented a

morphologically homogeneous population of fibroblast-like spindle cells and a low number of star-shaped cells, characteristics of mesenchymal stem cells (fig.3). In the day 8 of culture, the MSCs were disposed in colonies formed by 10-15 cells (fig.4).

Recent studies showed that the cellular pellet collected at the bottom of the centrifuged adipose tissue samples showed the highest concentration of ADSCs (7). There is evidence that the ability of adipose precursor cells to grow and differentiate varies among fat depots and changes with age (8).

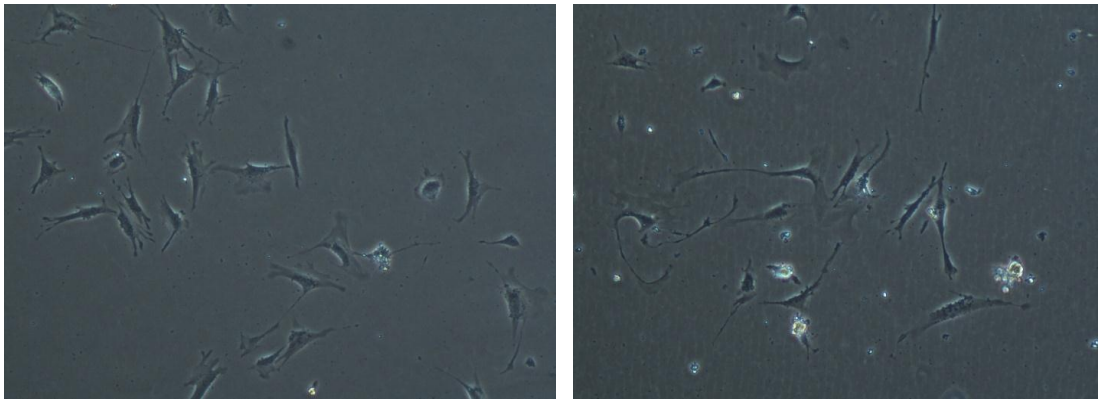


Fig. 3 – ADSCs in culture (protocol II) in day 4 (original)

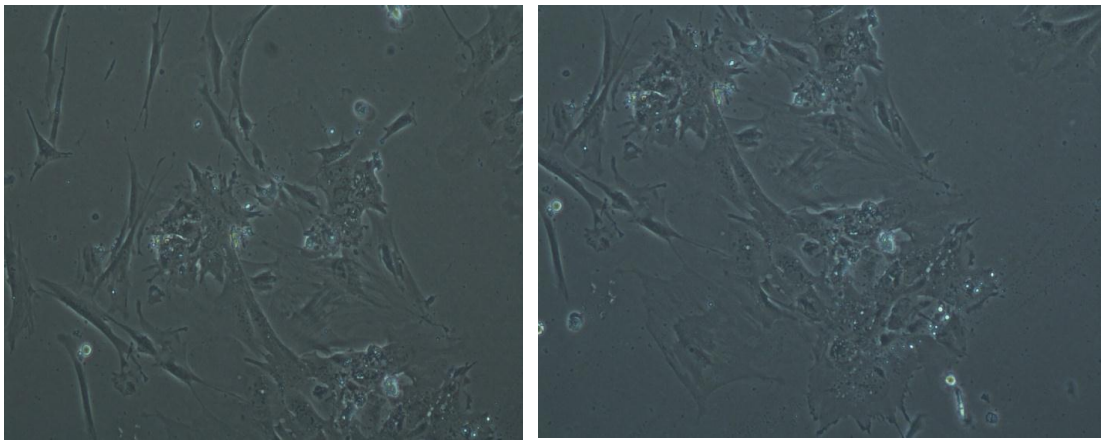


Fig.4 – ADSCs in culture (protocol II) in day 8 (original)

A 80-90% confluence was reached in the day 16 of culture, reason why the first passage (p'1) was done so late. Passages p'2 and p'3 were done every 2 weeks each. During all three passages, the homogeneity of cell populations was maintained, 80% of the cells being fibroblast-like spindle cells and the rest of 20% being polygonal-shaped and star-shaped cells. The symmetric division of cultured cells demonstrates the presence of adipose derived mesenchymal stem cells (ADSCs).

As the recent studies show, the precipitated lipoaspirate fraction after centrifugation contains endothelial cells, pericytes, tissue fibroblasts, and some other cell types (9) adherent

to plastic, of all these cells only MSC seem to find necessary conditions for active growth. After several passages the cultures are free from "ballast" cell types and become almost homogeneous (10). These properties show that lipoaspirate is superior to the bone marrow by the yield of stromal cell (11), due to peculiarities of blood supply to the adipose tissue and high density of capillaries.

All these properties make adipose tissue an important source of MSCs and an attractive source for clinical autografting. Compared with other tissues, adipose tissue contains a high volume of MSCs that can be easily expanded in vitro differentiating into various types of cells (12, 13).

Conclusions

Using protocol I, at day 2 of cultivation, the initial culture (p0) contained a heterogeneous cell population composed by round-shaped, polygonal-shaped and enlarged-shaped cells and a low number of fibroblast-like cells, the confluence of 80-90% being reached in day 4. The same characteristics were maintained at p1, p2 and p3, the confluence being reached at day 2. The morphological characteristics, in association with the asymmetric stem cell division indicate that in the pre-existent culture, the cells were represented by committed adipose mesenchymal stem cells/progenitor cells (APCs).

In the primary culture (p'0) obtained using protocol II, the adherent cells were observed starting from the second day of culture, characterized by a morphologically homogeneous population of fibroblast-like spindle cells and a low number of star-shaped cells. The first passage (p'1) was done in the day 16 of culture, when the confluence reached at 80-90% and p'2 and p'3 were done every 2 weeks each. During all three passages, the homogeneity of cell populations was maintained, this symmetric division demonstrating the presence of adipose derived mesenchymal stem cells (ADSCs).

Analyzing the proliferative rate and morphological characteristics of adherent isolated cell populations, we concluded that by using enzymatic digestion combined with mechanic dissociation we can obtain a stabile population of ADSCs. The proportion of fibroblast-like cells was 90%, comparative to enzymatic digestion were we obtain a percentage of 20% fibroblast-like cells and 80% being round-shaped cells, characteristic of APCs.

References

1. Ippokratis Pountos, Peter V. Giannoudis, 2005, Biology of mesenchymal stem cells, Injury, Int. J. Care Injured, 36S, S8—S12 ;
2. Erices A, Conget P, Minguell J., 2000, Mesenchymal progenitor cells in human umbilical cord blood. Br J Haematol.,109:p. 235-242;
3. Ralf Huss, 2000, Isolation of Primary and Immortalized $CD34^-$ Hematopoietic and Mesenchymal Stem Cells from Various Sources, STEM CELLS, p.18:1-9;
4. Zuk PA, Zhu M, Mizuno H, et al., 2001, Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng.;7:211e26;
5. Hisha H, Nishino T, Kawamura M, et al., 1995, Successful bone marrow transplantation by bone grafts in chimeric-resistant combination.Exp Hematol 23:347;
6. Nakagami H, Morishita R, Maeda K, et al., 2006, Adipose tissuederived stromal cells as a novel option for regenerative cell therapy.J Atheroscler Thromb 13:77;

7. Conde´-Green A, Gontijo de Amorim NF, Pitanguy I., 2009, Influence of decantation, washing and centrifugation on adipocyte andmesenchymal stem cell content of aspirated adipose tissue:a comparative study. J Plast Reconstr Aesthet Surg;
8. Schipper BM, Marra KG, Zhang W, et al., 2008, Regional anatomic and age effects on cell function of human adipose-derived stem cells. Ann Plast Surg;60:538e44;
9. S. Hung, C. Cheng, C. Pan, et al., 2002, Ibid., 20, 522-529;
10. H. Wang, S. Hung, S. Peng, et al., 2004, Stem Cells, 22, 1330-1337;
11. P. Bianco and P. G. Robey, J., 2000, Clin. Invest., 105, 1663-1668;
12. Yane˜z R, Lamana ML, Garcia-Castro J, et al., 2006, Adipose tissue-derived mesenchymal stem cells have in vivo immunosuppressive properties applicable for the control of the graft-versushost disease. Stem Cells 24:2582;
13. Yamamoto N, Akamatsu H, Hasegawa S, et al, 2007, Isolation of multipotent stem cells from mouse adipose tissue. J Dermatol Sci48:43.

COMPARISON OF SCIENTIFIC RESEARCH PERFORMANCES IN ROMANIAN VETERINARY MEDICINE FACULTIES

Liviu Miron[1], Carmen Anton[2], Silviu-Mihail Tiţă[3]
[1]Faculty of Veterinary Medicine, Ioan Ionescu de la Brad University Iaşi
[2]National Inventics Institute
[3]Al.I.Cuza University Iaşi/Romania
silviutita@yahoo.com

Abstract:
Measuring the scientific performance at the level of higher education is, globally, the task assumed by numerous organizations who are trying to develop different rankings concerning the ability of the Universities to offer degree programs or careers in scientific research. In Romanian, criteria for research evaluation performances are similar with international criteria and the main indicator are: ISI articles, citations of ISI articles, books, articles publish in international data bases (Web of Science, Scopus etc), article publish in national review ranking B+ or B, international or national conferences and for innovation Romania uses international patents (USPTO, EPO, JPO), national patents (OSIM).The aim of this study is to elaborate a comparative analysis between the performances of scientific research for the main four veterinary medicine faculties: Timisoara, Bucuresti, Cluj-Napoca, Iasi.

Keywords: *research, patents, performances, indicators, veterinary medicine.*

INTRODUCTION

Higher education is a principal component of the development and prosperity of a country. The processes of globalization are exerting a substantial influence on the system of higher education and promoting an increase in the competition between universities of different countries for the most talented youth and highly qualified teaching staff (Markusova, Tsygankova, Krylova 2009**).**

Performance in veterinary medicine for the general public probably means the discovery of animal parasites or specific drugs. Veterinary medicine enjoys the status of a ‘‘learned profession’’ within the health sciences. Two terms in that statement, ‘‘learned profession’’ and ‘‘science,’’ heavily depend on research. (Buss, 2005). Publication achieved research results or patenting different instruments used in research are factors to be taken into account when we evaluate the performance of scientific research.

Assessments of quality and productivity of academic research programs become more and more important in gaining financial support, in hiring and promoting research staff, and in building academic reputation (Groot, Garcia-Valderrama, 2006). In addition, the main purpose of performance measurement is to identify the role of the reviewed university or faculty to the progress of the scientific community to which it belongs.

Results of scientific research are often evaluated by two methods: peer-review or bibliometrics. "Peer-review is a functional evaluation system, but its performance would increase if they were removed the following disadvantages: bias, prejudice, abuse, failure to identify errors, fraud and errors (Kundzewicz, Koutsoyiannis 2005). Bibliometrics is the application of mathematical and statistical methods to books and other media (Pritchard 1969).

According to the standards, the second method depends largely on the first, in that first articles published in journals that are indexed in suitable to be cited databases, are evaluated by the editors of these publications. Later, if it exceed the evaluators criteria and standards, one can measure some bibliometric indicators such as the number of citations, number of items, h-index, g-index, number of patents etc.

Both have been criticized and both have pros and cons (Abramo et al. 2008). Computerized publication and citation counting has been an important tool for comparisons at various levels (e.g., countries, institutions or individual scientists), judgment for merit and evaluation of research policy and management (Gauffriau et al. 2008; Larsen 2008). In turn, universities use such data for promotions and appointments as well as for encouragement measures (Baskurt , 2010).

Compared to other areas in which scientific work matters more, in veterinary medicine disseminating knowledge through patents has a high importance. Patents are one of the most prevalent measures of innovation, and for good reason: they are easily accessible in electronic form; by definition, they are linked to inventiveness; they are classified by category and sub-categories; they identify individuals and organizations; and they contain a trace of what knowledge they build upon through the citation of prior art (Nelson, 2009).

RESEARCH METODOLOGY

The research started with the idea to make a comparative analysis of the scientific results of the veterinary medicine faculties, beneficial to the key persons in these faculties in order to identify methods by which performance can be improved. Faculties are considered the main provision of education and research in this area, as members of the University of Agricultural Sciences and Veterinary Medicine in Bucharest, Cluj, Iasi and Timisoara.

The used data are collected for the analyzed faculties from three different sources: reports on universities ranking in Romania for each field of faculty separately, Institute of Scientific Information (ISI Philadelphia USA) - Web of Science and SCOPUS database. In terms of the followed period, that was between the years 2005-2012, with various small corrections. These corrections were necessary because some departments have no article during 2005-2007 in WoS and Scopus international databases and the ranking reports do not covered the years 2005 and 2011, 2012.

Key Indicators for assessing the used scientific research are those internationally relevant: the number of ISI papers, number of scientific articles indexed in ISI, scientific articles indexed in BDI, full time research / teaching personal and allocated financial resources. The last two indicators allowed me to calculate two other important indicators of labor productivity and average cost per scientific outcome (ISI, BDI articles, conferences indexed in 2006-2010). The evaluation reports allowed me to calculate these values because the area is the same and I had a access to the number of people involved in research and also the financial resources attracted by national and international research projects.

How data is organized in international databases is one of the main limitations of this research. Because in the university name appears Veterinary Medicine, the results identification is slightly more difficult because the search engine will return almost all items of that university. Thus, the researchers chose to select the subject of articles corresponding to the main areas of interest of that faculty.

In terms of methodology, was used the statistical interpretation of the results of analyzed faculty and also the time series analysis.

RESEARCH RESULTS

In the period 2005-2012, were indexed in Web of Science database a total of 268 publications (articles, conference proceedings, book reviews or abstracts of articles). Looking at Figure 1 we can observe certain imbalances between years regarding the number of publications, meaning that in some years have been indexed a large number of articles and the following year a tiny percentage of the last year total. In general, the number of scientific results indexed in the Thomson ISI (Web of Science) is decreasing, the trend equation is logarithmic of the type $y = -30.0 (\ln x) + 73.33$ and $R^2 = 0.572$, which means that there is a average 57.2% chance that future results on the number of publications of veterinary medicine faculty can be mathematically modeled through the described Eq.

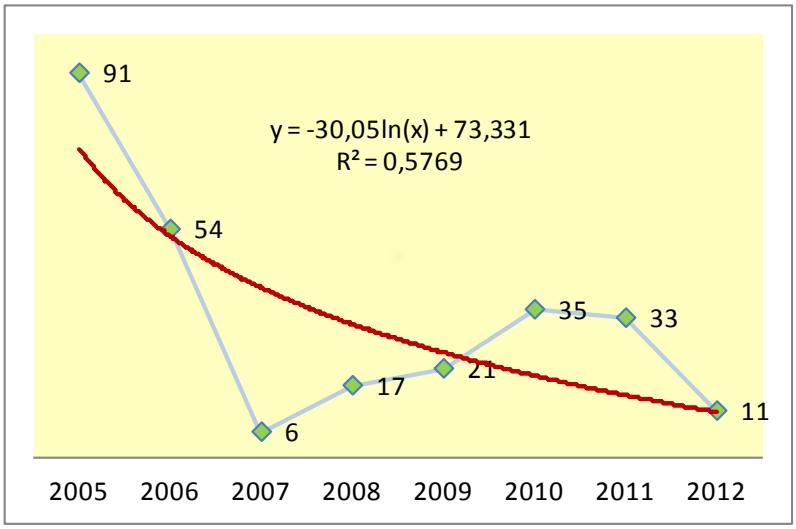


Figure 1 Evolution of the total number of the analyzed faculty publications in the period 2005-2012 (Source: Web of Science)

After a significant drop in 2007, the number of publications began to grow and we can say that the trend is ascendant given that the article is made in mid-2012.

Distribution of ISI articles on each faculty is detailed in Table 1. In the period under review were made on average 33.5 publications per year, the maximum being in 2005 when were indexed several important technology magazines in Romania, and the minimum in 2007 as the Assessment systems at that time did not clearly indicate which are the ways of promotion. Faculties of Veterinary Medicine of Bucharest and Cluj have adapted their strategies on publishing scientific articles in international journals faster compared with those from Iasi and Timisoara; from this point of view we consider necessary the development and implementation of new objectives with targets adapted to new requirements.

Table 1 - Number of publications indexed in WoS during 2005-2012

	All	VM Iasi	VM Cluj	VM Timisoara	VM Bucuresti
Articles/total	268	15	145	20	88
2005	91	1	59	10	21
2006	54	3	42	4	5
2007	6	1	4	1	
2008	17	2	3	1	11
2009	21	2	7	1	11
2010	35	3	10	2	20
2011	33	3	13	1	16
2012	11		7		4
Mean	33.5	2.14	18.12	2.85	12.57
SD	27.82	0.90	20.73	3.33	6.75
Max	91	3	59	10	21
Min	6	1	3	1	4

Source: Web of Science

In Table 2 are shown the main areas in which researchers from the faculties of veterinary medicine publish articles. It is noted that all four analyzed faculties have a large number of results in the area of veterinary medicine, followed in some cases by biotechnology or animal science.

Table 2 – Research area articles distribution 2005-2012

VM Iasi		VM Cluj		VM Timisoara		VM Bucuresti	
VETERINARY SCIENCES	4	VETERINARY SCIENCES	115	VETERINARY SCIENCES	13	VETERINARY SCIENCES	43
ENVIRONMENTAL SCIENCES	3	BIOTECHNOLOGY APPLIED MICROBIOLOGY	7	AGRICULTURE DAIRY ANIMAL SCIENCE	2	BIOTECHNOLOGY APPLIED MICROBIOLOGY	21
GENETICS HEREDITY	3	PATHOLOGY	7	PATHOLOGY	2	PARASITOLOGY	11
BIOCHEMISTRY MOLECULAR BIOLOGY	2	DEVELOPMENTAL BIOLOGY	3	BIOCHEMICAL RESEARCH METHODS	1	PATHOLOGY	9
INFECTIOUS DISEASES	2	FOOD SCIENCE TECHNOLOGY	3	BIOTECHNOLOGY APPLIED MICROBIOLOGY	1	CHEMISTRY MULTIDISCIPLINARY	6
BIOLOGY	1	MEDICINE RESEARCH EXPERIMENTAL	3	ETHICS	1	BIOCHEMISTRY MOLECULAR BIOLOGY	4

Source: Web of Science

Articles distribution on types of publications is presented in Table 3. It is noted that the Faculty of Veterinary Medicine of Cluj Napoca has published a significant number of articles in ISI conferences compared with the Faculty of Veterinary Medicine of Bucharest which has published several articles in journals indexed in the Web of Science database. Previous remark on the fact that a significant percentage of articles were published during 2005, when some indexed magazines were is confirmed for the Faculty of Veterinary Medicine Timisoara magazine that has in the University Bulletin of Agricultural Science and Veterinary Medicine a total of 13 articles out of 20 for the entire period 2005-2012.

Tabelul 3 – Type of publication 2005-2012

VM Cluj		VM Timisoara		VM Bucuresti	
PROCEEDINGS PAPER	102	BULLETIN OF THE UNIVERSITY OF AGRICULTURAL SCIENCE AND VETERINARY MEDICINE VOL 62 2005	13	ARTICLE	44
ARTICLE	33	VIRCHOWS ARCHIV	2	PROCEEDINAGS PAPER	35
MEETING ABSTRACT	8	ACTA VETERINARIA HUNGARICA	2	MEETING ABSTRACT	17
REVIEW	2	BULLETIN OF THE UNIVERSITY OF AGRICULTURAL SCIENCES AND VETERINARY MEDICINE VOL 63 2006	1	REVIEW	1

Sursa: Web of Science

According to data reported by each school for the institutional hierarchy during 2006-2010 is observed that there aren't significant differences compared to the international database Web of Science. Since 2005 was removed from the analysis, the publications trend is upward after a fall in 2007, the equation is $y = 10.12 \cdot \ln(x) + 11.06$ both calculating variables are positive, however the value $R^2 = 0.373$ means that 37% of future values can be modeled by the equation.

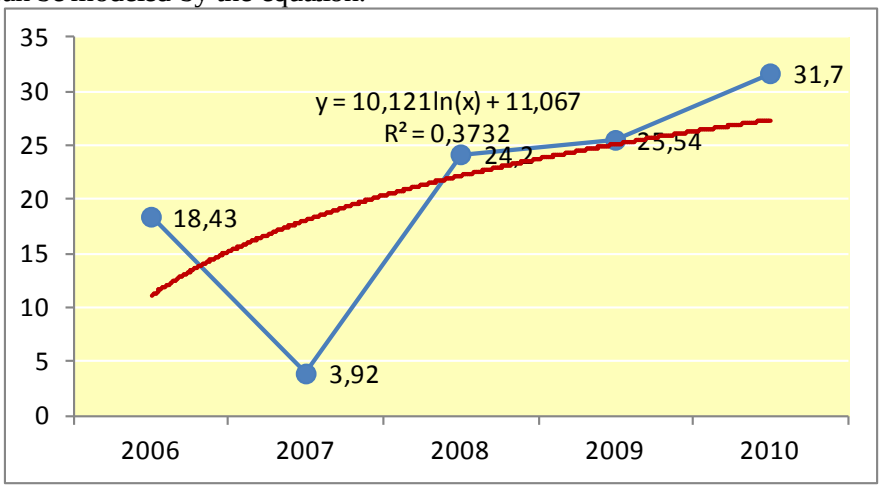


Figure 2 Evolution of the total number of publications for the analyzed faculties in the period 2005-2012 (Source: Reports of hierarchy)

The results obtained by each faculty are presented in Table 4, the maximum for 3 of the four colleges were obtained in 2010. Faculty of Veterinary Medicine Timişoara has maximum values in 2006, the results reported in future periods being unsatisfactory. Standard deviation values are not very high, meaning that are no large discrepancies in the number of annual publications compared with the average. Perhaps only in 2007 when the criteria for performance evaluation of field in Romania were not clearly defined, the values are low. Changing the national approach implemented by the Research, Development and Innovation Strategy 2007-2013, has forced universities to understand the importance of indicators for assessing scientific research and adapted their strategies to publications with international relevance.

Table 4 – Number of publications reported during 2006-2010

	All	VM Iasi	VM Cluj	VM Timisoara	VM Bucharest
Articles/total	103.79	14	54.26	13.532	22
2006	18.43	1	5.97	10.46	1
2007	3.92	-	2.99	0.93	-
2008	24.20	2	15.2	-	7
2009	25.54	8	13.54	-	4
2010	31.70	3	16.56	2.14	10
Mean	20.76	2.80	10.85	2.71	4.40
SD	10.52	3.11	6.01	4.421	4.16
Min	3.92	1	2.99	0.93	1
Max	31.70	8.00	16.56	10.46	10.00

Source: Self-evaluation reports

Impact factor of journals where are published the scientific results is one of the important indicators in the evaluation. The trend is to publish articles in magazines with a factor as high in order for the researcher to be quickly recognized. From the analyzed faculties, the Faculty of Veterinary Medicine of Cluj published articles in journals with high impact factors, the maximum being in 2010 when the published articles of researchers obtained 22.54 points. Compared with other faculty is noted that the Faculty of Iasi had a high factor in 2009 compared to the years 2008, 2010, the value being 6.25.

Table 5 – Impact factor of scientific publications 2006-2010

	VM Iasi	VM Cluj	VM Timisoara	VM Bucuresti
2006	0.00000	1.76000	1.33024	0.38911
2007	0.00000	7.49000	0.91335	0

2008	0.93300	12.40000	0.00000	2.97349
2009	6.25000	8.04000	0.00000	1.93139
2010	0.88500	22.54000	0.92249	1.44561
Mean	0.63322	10.44600	1.61360	1.34792

Source: Self-evaluation reports

Evaluation reports highlighted an interesting indicator: articles published in indexed conferences, only that evaluating the main database, some of these articles are not indexed, and that raises some question marks. We believe that it is possible for the items to appear in other databases such as SCOPUS or one specific for the domain. In compliance with specific studies on the differences between Scopus and Web of Science, about 2/3 of all articles are in both.

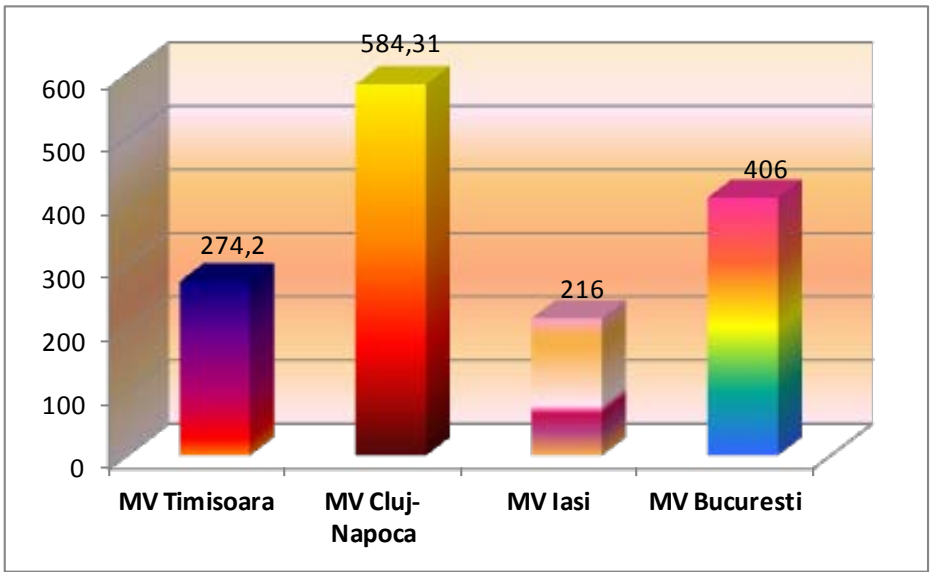


Figure 3 –Number of articles published at indexed conferences

High performances on this indicator have the Faculties of Medicine of Cluj-Napoca and Bucharest while Iasi and Cluj have approximately half of the performance of the first two (Figure 3).

If until now were analyzed the output indicators, interesting and beneficial for final conclusions is the analysis of the input indicators. For the number of full-time researchers and staff in faculties, the data are taken from the self-evaluation reports as seen in Table 6. Faculty of Veterinary Medicine of Iasi has the lowest number of employees, approximately half of those who are in Bucharest and 60% less that in Cluj-Napoca.

Table 6 – Number of full-time staff 2006-2010

	VM Iasi	VM Cluj	VM Timisoara	VM Bucuresti
2006	51	65	68.28	87
2007	49	66	71.67	93
2008	52	68	64.21	90
2009	47	74	62.85	91
2010	44	70	59.04	92

Source: Self-evaluation reports

Funds gained by the four faculties through national or international projects competitions are plotted in Figure 4. Thus, the faculties of Iasi and Bucharest have managed to attract significant amounts, in some cases, as the self-evaluation reports mention, three to four times higher than those obtained by their colleagues in Timisoara and Cluj.

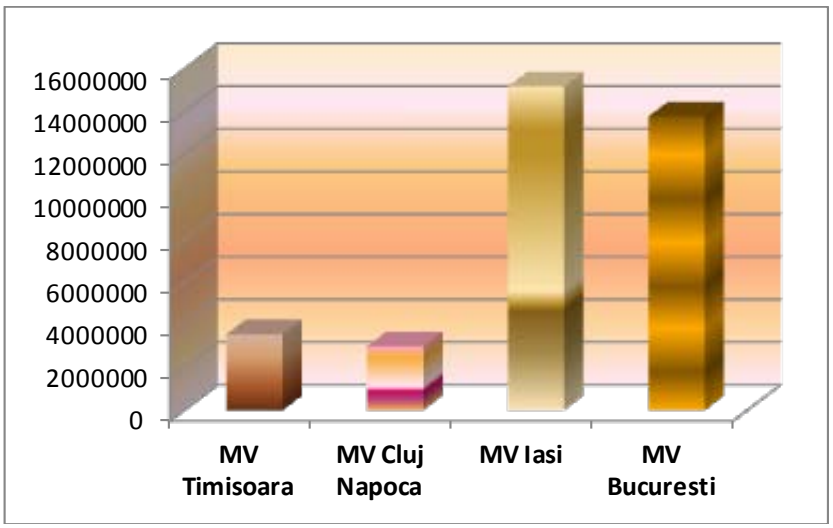


Figure 4 – The financial resources attracted by scientific research projects
(Source: Self-evaluation reports)

The ratio between the results identified as output (number of ISI articles, number of articles in conferences and the number of articles indexed in databases) and the input indicator (number of researchers) allows us to calculate scientific productivity of each person involved full time in research in the respective university.

Table 7 – Scientific productivity 2006-2010

	VM Iasi	VM Cluj	VM Timisoara	VM Bucuresti
2006	1.26	0.95	1.52	1.08
2007	1.61	1.63	1.79	1.05
2008	1.81	3.77	2.15	1.42
2009	1.62	4.37	2.25	1.57
2010	2.19	3.01	1.86	1.05

Source: Self-evaluation reports

Table 7 does not shows significant differences in scientific productivity between the analyzed faculties, only the University from Cluj sometimes exceeds the average with high values in 2009 and even 2010.

CONCLUSIONS

The purpose of the comparative analysis of scientific research performance of the Faculties of Veterinary Medicine in Romania is not to achieve their hierarchy. Presentation of evaluation results to decision-makers at each institution must convince them to develop and implement strategies aimed at improving the efficiency of scientific research.

The national strategy change that took place in 2007 by implementing a strategic plan with programs that followed electronic indicators achieved for each project and new criteria to promote teaching and research forces those involved into turning towards publishing articles in international journals.

Compared to other scientific fields, we can say that the development potential of research in veterinary medicine, with a continuous growth of importance at national level is high.

REFERENCES:

1. Abramo, G., D'angelo, C., & Pugini, F. (2008), *The measurement of Italian universities research productivity by a non parametric-bibliometric methodology*, Scientometrics, 76(2), 225–244;
2. Baskurt O. (2010) Time series analysis of publication counts of a university: what are the implications? Scientometrics DOI 10.1007/s11192-010-0298-1
3. Buss D., Providing *Stewardship and Leadership for the Future of Veterinary Medical Research: A Responsibility of the Veterinary Colleges*; JVME 32(3);
4. Gauffriau, M., Larsen, P., Maye, I., Roulin-Perriard, A., & Von Ins, M. (2008), *Comparisons of results of publication counting using different methods*, Scientometrics, 77(1), 147–176;

5. Groot T., T. Garcia-Valderrama, (2006) *Research quality and efficiency An analysis of assessments and management issues in Dutch economics and business research programs*, Research Policy 35 1362–1376;
6. Markusova V. A., A. I. Tsygankova, and T. A. Krylova, The Indices of Scientific Productivity and Ratings of Domestic Universities *Scientific and Technical Information Processing, 2009, Vol. 36, No. 4, pp. 229–233*
7. Andrew J. Nelson, (2009)*Measuring knowledge spillovers: What patents, licenses and publications reveal about innovation diffusion*, Research Policy 38 994–1005
8. Pritchard, A. (1969) *Statistical bibliography or bibliometrics*, Journal of Publication;
9. Zbigniew W. Kundzewicz, Demetris Koutsoyiannis (2005)„*The peer-review system: prospects and challenges*", Hydrological Sciences–Journal–des Sciences Hydrologiques, 50(4), p. 580;

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