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CUPRINS

THE USE OF IOMERON 350 IN CAT UROGRAPHY

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Abstract

The trend in modern medicine seeks to establish a diagnosis in the subclinical stage of the disease; it reveals the important role and place of contrast radiological exploration in the early diagnosis of certain pathological conditions (Adler and Carlton, 1994). The use of nonionic contrast substance Iomeron 350 in urography in cats provides extra safety on the patient's life and gives more quality to a radiological image (Skučas, 1989 Katzberg, 1992). Understanding in advance the working parameters of the machine for Roentgen diagnosis, as well as the amount of substance administered exposure and shutter speed, are a certainty in obtaining and interpreting image quality (Douglas, and Williamson, 1970, Wilkinson, 1985; Thrall, 1998; Kealy and Mcallister, 2000). The aim of this study was to evaluate the tolerance of cats to the nonionic contrast substance Iomeron 350 and to establish administration dose, the working parameters of the device during exposure and position in order to determine good quality radiographic images of the urinary tract. At the same time we followed the side effects of the contrast substance in the patient by monitoring the major functions before and after administration of the substance. Normal pulse in the cat is 110 - 130/minute, the respiratory rate 20 - 30/min, and temperature 38.5 ° C-39, 2 ° C (Papuc et al. 2009).

Keywords: *nonionic contrast substance, urography, cat, Iomeron 350.*

Material and method

The biological material on which the research was made, was 10 cats, male and female, of European breed, clinically healthy. The lot has been homogeneous, cats were aged 1.5 to 4.5 years, weighing between 2-8 kg. For testing the contrast substance Iomeron 350, the group consisting of the 10 cats was subdivided into four subgroups. Two subgroups had a total of 2 cats and two subgroups had a total of 3 cats. Each subgroup received different doses of nonionic substance to track the patient's tolerance to the substance administered and quality of images obtained. Radiological exposures were performed with different operating parameters for each subgroup. The study was conducted in the Laboratory of Radiology of the Faculty of Veterinary Medicine Cluj-Napoca.

The examined cats underwent a 24-hour food diet with water ad libitum. Tranquilization was achieved by means of neuroleptanalgesia, using Acepromazine at a dose of 0.5 mg / kg and Ketamine 20 mg / kg body weight. Preferred site for administration of the substance was the cephalic vein. The region of the forearm was prepared by trimming, shaving, disinfection with medicinal alcohol. For the venous puncture, a special catheter, Protectiv plus-Radiopaque, was used, with a diameter of 0.75 mm. The contrast substance was administered intravenously slowly over 1-2 minutes. The doses used were different depending on the body weight. Radiation exposure was performed serially, on average 4 exposures for each case, the first exposure was carried out in 30 seconds, the second exposure at about 1minute, the third and fourth exposure 3 minutes and 5 minutes after the administration of the substance. For the radiological imaging, the device used was a RX Temco model GRX, kilovoltage values were between 40 and 55 Kv and miliamperage values ranged between 20 and 25 mAs. Patient position was latero-lateral, with a side exposure.

Results and discussions

The first subgroup consisting of 3 cats, the 350 Iomeron dose was administered slowly IV in 0.3 - 0.5 ml / kg body weight. The radiological parameters used were 40 kV and 20 mAs (Fig. 1, Fig. 2). For each patient, the physiological constants were monitored before and after substance administration (Table 1). In this subgroup, side effects during administration of the contrast agent were observed: slight muscle tremor and tachycardia.

Table 1. Physiological values before and after Iomeron 350

		Before administration	1 minute from administration	In 10 minutes	In 20 minutes
CASE 1.	Heartbeat	120	165	150	110
	Breathing	28	27	27	28
	Temperature	38,2	38,2	38,1	38,0
CASE 2.	Heartbeat	110	150	155	130
	Breathing	25	25	26	27
	Temperature	38,0	38,1	38,2	38,2
CASE 3.	Heartbeat	117	145	140	120
	Breathing	32	32	32	32
	Temperature	38,5	38,5	38,5	38,5

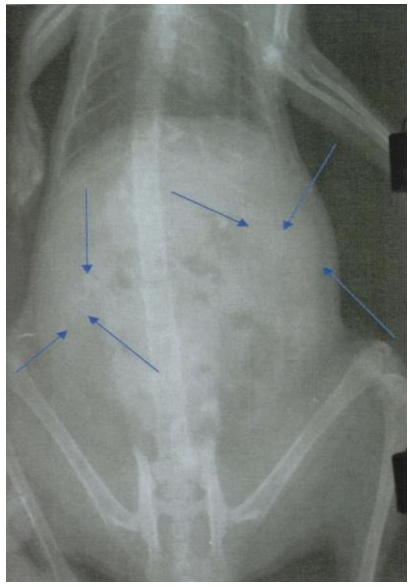

Fig. 1 Ventrodorsal exposure with a vague evidentiation of the kidneys after 1 minute., 40 Kv and 20 mAs

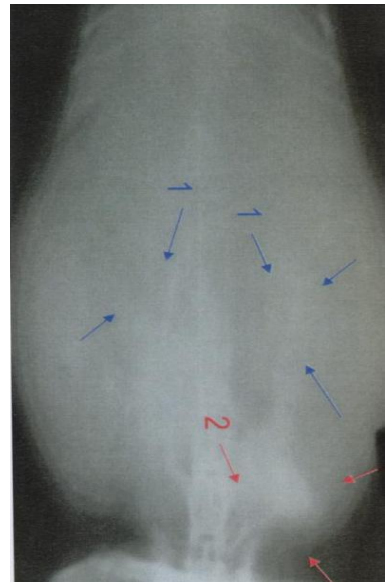

Fig. 2 Ventrodorsal exposure with a vague evidentiation of the kidneys (1) and urinary bladder (2) in 3 minutes, 40 Kv and 20 mAs

The second subgroup consists of 2 cats weighing 1.3 kg and 1.5 kg, aged 1 year, 1 ml of Iomeron 350/kg body weight was administered, iv, slowly, making three serial exposures, using parameters of 45 kV and 25 mAs (Fig. 4, Fig. 5). Side reactions were mild hyperthermia and tachycardia (Table 2).

Table 2. Physiological values before and after Iomeron 350 administration

		Before administration	1 minute after administration	In 10 minutes	In 20 minutes
CASE 4.	Heartbeat	120	165	150	108
	Breathing	26	28	25	28
	Temperature	37,9	38,8	39,3	39,5
CASE 5.	Heartbeat	130	160	180	168
	Breathing	28	30	32	30
	Temperature	38,5	38,5	39,2	39,5

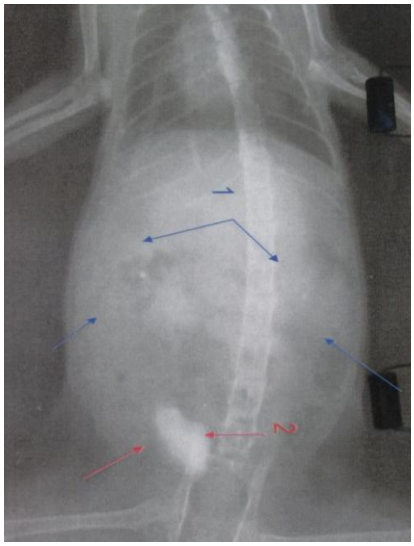

Fig. 3 Ventrodorsal exposure with a vague evidentiation of the kidneys (1) and urinary bladder (2), 45 Kv and 25

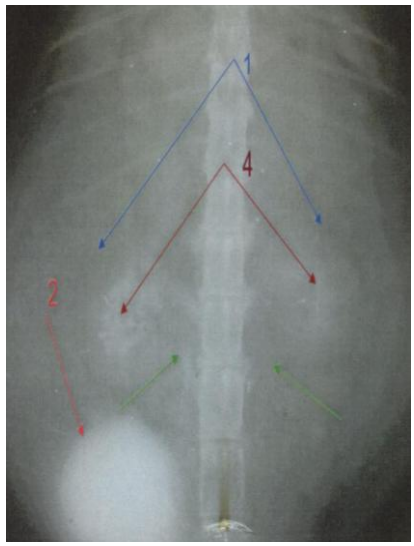

Fig. 4 Ventrodorsal exposure with a vague evidentiation of the kidneys (1, 4), of the urethers (3) and urinary bladder (2) in 1 minute, 45 Kv and 25 mAs

The third subgroup consists of 2 cats weighing between 1.5 - 2.5 kg, two years old, the Iomeron 350 dose used was 1.5 and 1.8 ml / kg body weight. Exposure parameters used were 48 kV and 25 mAs to achieve 4 serial exposures: 30 seconds, 1 minute, 3 minutes and 5

minutes after the administration of contrast. The images obtained in the first two exposures may reveal the kidney with two areas quite well: cortex and medulla. (Fig. 5). At 3 minutes from the start of administration a part of the ureters, urinary bladder, have been revealed, but with moderate intensity (Figure 6). After 5 minutes there is very weak evidence of the kidneys, but the bladder was evident (Fig. 7). Adverse reactions were expressed by tachycardia and tachypnea of moderate intensity (Table 3).

Tabel 3. Physiological values before and after Iomeron 350 administration

		Before administration	1 minute after administration	In 10 minutes	In 20 minutes
CASE 6.	Heartbeat	115	150	167	130
	Breathing	27	35	39	30
	temperature	38,3	38,5	38,0	38,0
CASE 7.	Heartbeat	130	160	167	135
	Breathing	32	36	40	35
	Temperature	38,0	38,1	38,1	38,5

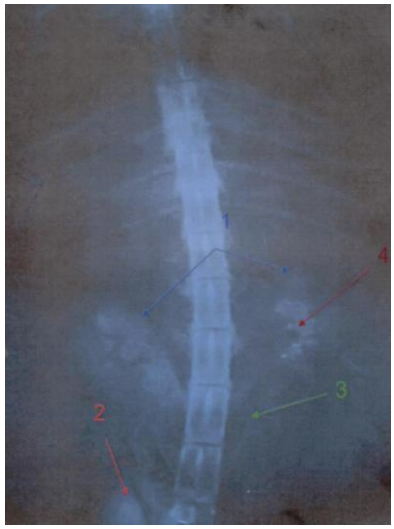

Fig. 5 Ventrodorsal exposure with a vague evidentiation of the kidneys (1), ureters (3) and urinary bladder (2) after 1 minute, 48 Kv and 25 mAs.

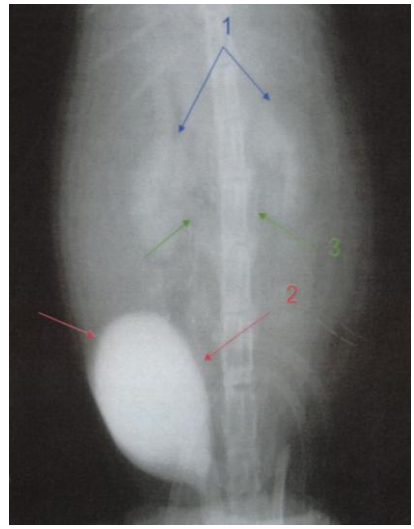

Fig. 6 Ventrodorsal exposure with evidentiation of the kidneys (1), a ureters (3) and urinary bladder (2) in 3 minutes, 48 Kv and 25 mAs.

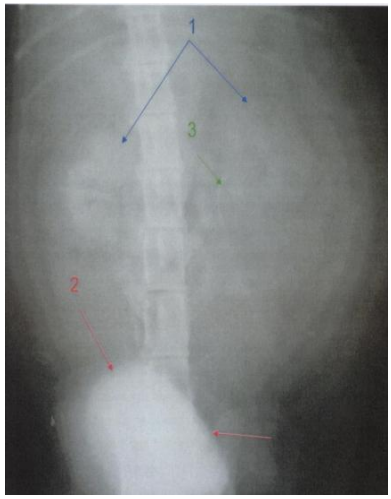

Fig. 7 Ventrodorsal exposure with a weak evidentiation of the kidneys (1), ureters (3) and urinary bladder (2) in 5 minutes, 48 Kv and 25 mAs.

The fourth subgroup consists of three cats weighing between 2.5-4 kg, with the age of 2-4 years; 2 ml / kg were administered in the first two cats and 2.5 ml / kg in the cats weighing 4 kg. Exposure parameters used were 55 kV and 25 mAs doing serial exposure at the established intervals. After the first exposure, 30 seconds after administration, the kidney is emphasized with two clearly defined areas (Fig. 8), second exposure was after 1 minute, it highlights the great part of the ureters and renal pelvis (Fig. 9), after 3 minutes the kidney, pelvis, ureters and bladder are revealed (Fig 10) and after 5 minutes , just the bladder is highlighted (Fig. 11).

Adverse reactions observed were tachycardia and regional muscle tremor with low intensity, which disappeared in 25 minutes after the administration of contrast substance. In the 10th case, the 4 years old cat in which the dose of contrast agent was 2.5 ml / kg body weight, the adverse reactions have been tachycardia, tachypnea, and muscle tremors with an elevated intensity compared to the other cases (Table 4).

Table 4. Physiological values before and after Iomeron 350 administration

		Before administration	1 minute after administration	In 10 minutes	In 20 minutes
CASE 8.	Heartbeat	125	128	150	138
	Breathing	29	32	32	32
	Temperature	37,9	38,0	38,0	38,0
CASE 9.	Heartbeat	130	135	160	140
	Breathing	28	29	35	37
	Temperature	38,2	38,5	38,5	38,5
CASE 10.	Heartbeat	132	145	160	180
	Breathing	32	36	40	38
	Temperature	38,1	38,2	38,2	38,2

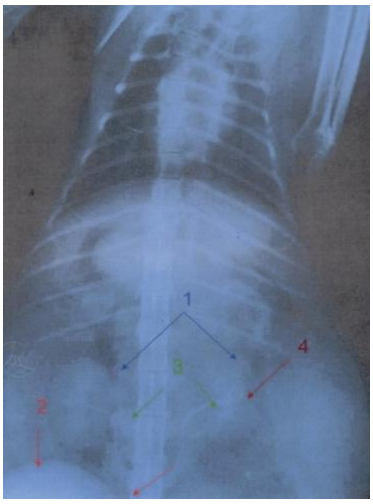

Fig. 8 Ventrodorsal exposure with evidentiation of the kidneys (1, 4), ureters (3) and urinary bladder (2) at 30 seconds, 55 Kv and 25 mAs.

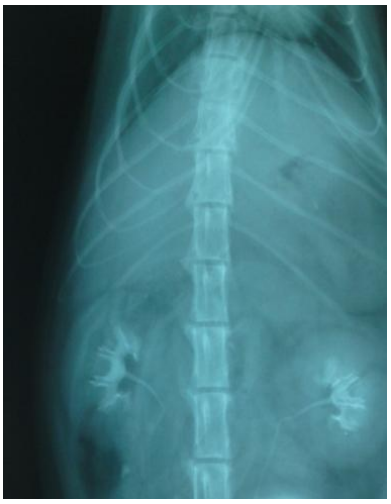

Fig. 9 Ventrodorsal exposure with evidentiation of the kidneys at 1 minut, 55 Kv and 25 mAs.

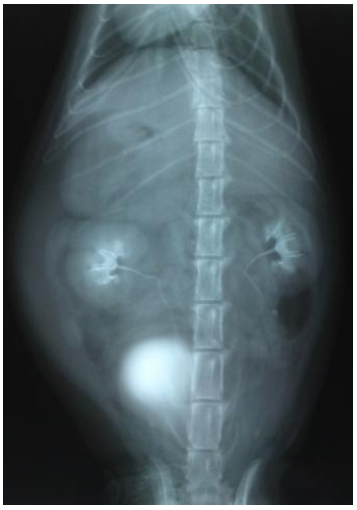

Fig. 10 Ventrodorsal exposure with evidentiation of the kidneys and urinary bladder at 3 minutes, 55 Kv and 25 mAs.

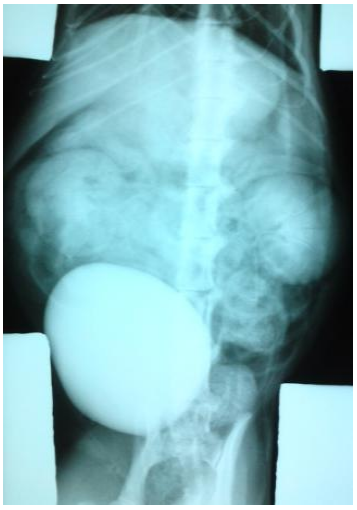

Fig. 11 Ventrodorsal exposure with a weak evidentiation of the kidneys and good evidentiation of the urinary bladder at 5 minutes, 55 Kv and 25 mAs.

Conclusions

The nonionic injectable substance Iomeron 350 contains 714.4 mg Iomeprol equivalent to 350 mg iodine which can be used in urography in cats because it has a better tolerance compared to ionic contrast agents, especially for the nervous system. The recommended dose of Iomeron 350 is of 2-2.5 ml / kg body weight for urinary radiography. Side reactions were of low intensity. There were no losses in the cats in the study group.

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A STUDY ON THE RELATION BETWEEN THE SIZE AND SEX OF DOG, AND THE TSH , THYROXINE AND TRIIODOTHYRONINE SERUM CONCENTRATION IN CANINE POPULATION IN MUSCEL AREA

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Abstract

This study is aimed to investigated the eventual correlation between the TSH, thyroxine and triiodothyronine serum concentration in dogs, according to the size and sex, in Muscel area. The TSH, thyroxine and triiodothyronine serum concentration are influenced by the size of dogs , while their sex has no major impact on the thyroid function. The dogs chosen to be observed were divided in two groups according to their sex, and three categories acording to their weight. Dogs present TSH, total thyroxine and triiodothyronine specific serum concentrations, dependent on the metabolic features of each group. Unlike in humans, the sex of the dogs does not influence significantly the thyroid hormones and TSH average serum levels.

Key words*: size, sex, thyroxine and triiodothyronine*

Introduction

The features of the thyroid function in dogs raise a set of problems depedent on settling optimum levels of TSH and thyroxine serum concentations. This is due to the influence of many environmental and inner factors that make difficult ranging the thyroid hormones values for both cases euthyroidism and hypothyroidism. Beside this, each geographical area puts its mark on thyroid hormone secretion, both by the iodine level available for ingestion and by the presence of some contaminats, which interfere to the thyroid iodine uptake.

The size of dogs generates a range of metabolic features induceded by the specific requirements related to the body growth, maintaining a constant body temperature, iodine ingestion related to metabolised energy, all leading to variations of thyroid hormones secretion.

In humans, the antagonism between the thyroid and estrogen hormones creates a much greater caseload related to female gender (women are six times more frequently affected than men), which is not found in canine thyroid pathology.

Materials and Methods

In order to obtain an overview of thyroid hormone and TSH serum levels in canine, dogs of different ages in Muscel area and Campulung were studied. They were fed both by commercial food and home-made food. In this study, the dogs selected to be observed were devided in two groups according to their sex and three categories according to their weight:

1. Group I - small sized dogs, weghting between 3-10Kg (32 individuals);
2. Group II - medium size dogs , weghting between 10-25 Kg (59 individuals);
3. Group III - large size dog, weghting over 25 Kg (61 individuals);

4. Males group (60 individuals);
5. Females group (72 individuals).

The average age of the female dogs under study was 3.5 years while the average age of males was 2.3 years.

Blood samples, were taken in the morning to prevent the fluctuations caused by the diurnal variations, by puncturing the cephalic vein, were analyzed by ELISA method on serum samples. All blood samples were centrifuged after the clot had been formed, and the serum was stored in small tubes at a temperature of -18^{o}C until they were analyzed.

Results and Discutions

The avarage of thyroid hormones and TSH blood levels, according to size and sex are presented in tables 1-2 and in graphs 1-4.

Table 1. TSH, TT_4 andTT_3 avarage values according to size

Size	TSH ng/ml	TT4 µg/dl	TT3 ng/dl
Small size	0.3233 ± 0.09	1.8477 ± 0.49	99.55 ± 23.07
Medium size	0.2950 ± 0.08	1.7613 ± 0.59	108.20 ± 24.73
Large size	0.3143 ± 0.06	1.4876 ± 0.52	104.46 ± 21.71
X	0.31	1.7	104

Table 2. TSH, TT_4 and TT_3 avarage values according to sex

Sex	TSH ng/ml	TT4 µg/dl	TT3 ng/dl
Female	0.308 ± 0.07	1.65 ± 0.52	106.94 ± 23.68
Male	0.298 ± 0.08	1.72 ± 0.66	105.02 ± 24.93

TSH average serum concentration registered (0.3233 ± 0.09 ng/ml) was higher in small sized dogs compared to the levels found in medium sized dogs (0.2950 ± 0.08 ng/ml), and to those obtained in large sized dogs (0.3143 ± 0.06 ng/ml). It was also noticed that in case of medium size dogs the average TSH serum concentration was lower compared to the levels registered in the other two size-groups.

In this study, it was not found significant differences of TSH serum concentrations in females (0.308 ± 0.07 ng/ml) compared to males (0.298 ± 0.08 ng/ml).

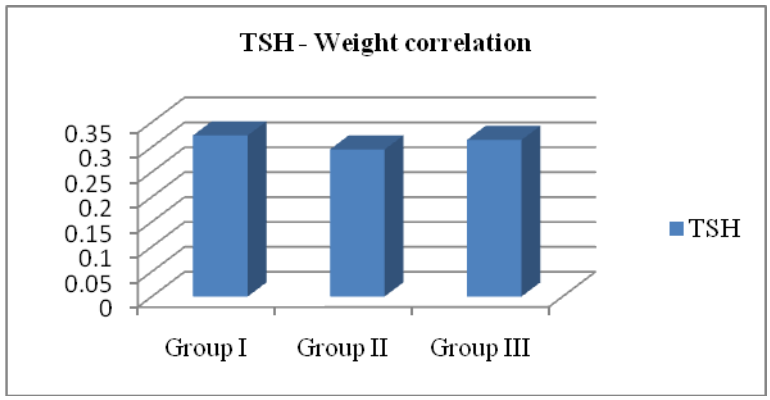

Fig. 1. TSH average serum levels dynamics in dogs dependent on size

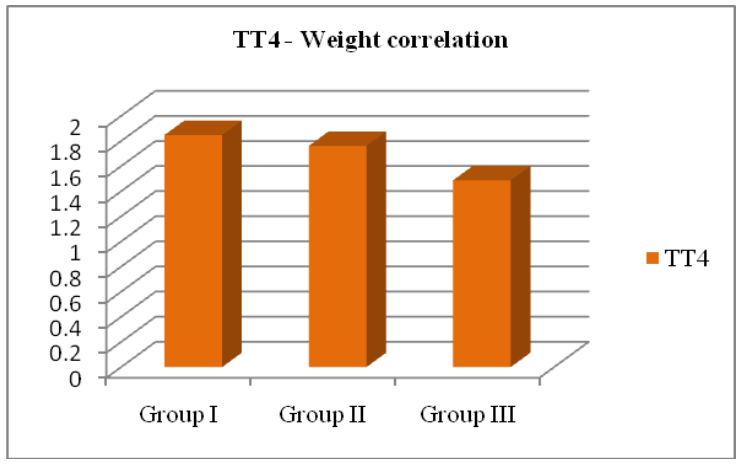

Fig. 2. TT4 average serum levels dynamics in dogs dependent on size

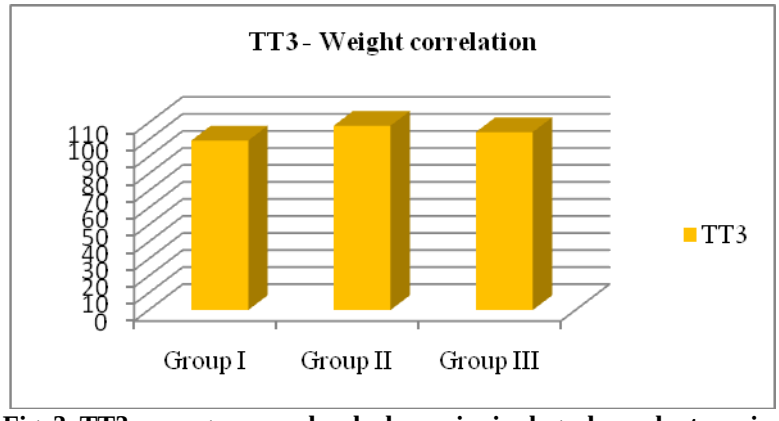

Fig. 3. TT3 average serum levels dynamics in dogs dependent on size

Body weight had a certain effect on thyroxine blood levels in dogs. (6). In this study, the average of total thyroxine serum concentration was higher in small size dogs (1.8477 ± 0.49 µg/dl), compared to the levels registered in medium size dogs (1.7613 ± 0.59 µg/dl) and large size ones (1.4876 ± 0.52 µg/dl) in which the lowest average concentration was found.

In medium and large size dogs were found lower TT4 serum concentration levels than those observed in small size dogs.(2) The values determined for thyroxine in females (1.65 ± 0.52 µg/dl) and males (1.72 ± 0.66 µg/dl) are alike.

The average triiodothyronine serum concentration registered in medium size dogs was 108.20 ± 24.73 ng/dl, higher than the average levels found in small size dogs (99.55 ± 23.07 ng/dl) and large size ones (104.46 ± 21.71 ng/dl).

In this study there were not found important differences between the average TT3 serum concentration in females (106.9 ng/ml) and males (105 ng/ml).

It was also noticed that the variation of the analyzed parameters was influenced both by the physiological characteristics of each study group of dogs, and, possibly, by the available iodine amount, thus the thyroxine serum levels tended to decrease in response to an insufficient iodine uptake, while triiodothyronine serum concentration was within normal limits.(5) Moreover, the attempt to define the thyroid function, based only on TSH determinations, was disappointing.

In 18-36% of dogs suffering from primary hypothyroidism was registered a TSH serum concentration within the reference limits, and in 14% of euthyroid dogs was found a high TSH serum concentration.(4)

Possible explanations for normal values of TSH blood levels in hypothyroid dogs, imply the secondary and tertiary hypothyroidism, the side effects of some medicine or diseases, diurnal fluctuations of TSH concentration etc.(1)

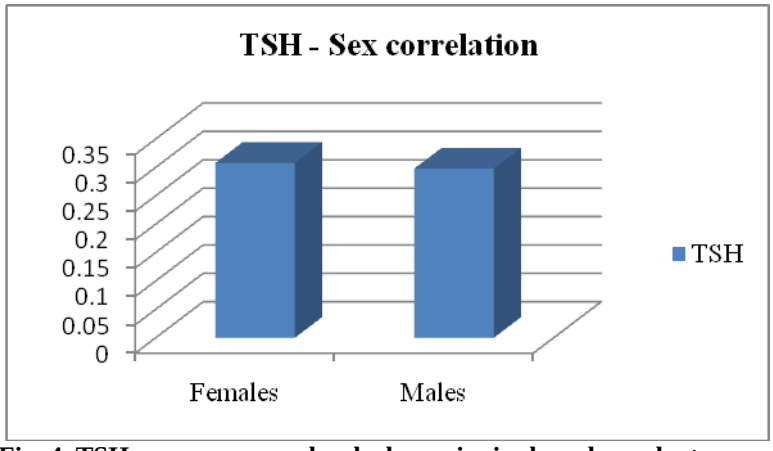

Fig. 4. TSH average serum levels dynamics in dogs dependent on sex

Conclusions

1. In this study, TSH average value dynamics dependent on size of dogs suggests that as weight increase, the thyroxine serum concentration decrease.

2. The level of pituitary TSH secretion reach higher values in small size dogs, and the lowest values in medium size dogs; in case of large size dogs the values are in between the two other weight groups.

3. In this study, in medium size dogs was found a triiodothyronine average serum concentration higher than the levels registered in the other two size groups.

4. In this study, the larger the size of dogs was, the lower the average thyroxine serum concentration tended to be.

5. The triiodothyronine average serum concentration was increased in large and medium size dogs, more likely as an adjusting mechanism to a possible lack of iodine or small size dogs are less prone to thyroid endocrine diseases.

6. In this study, there were no major differences in thyroxine, triiodothyronine synthesis and secretion by thyroid, and neither in TSH levels produced by the pituitary gland, thus it seems that the estrogen hormones did not interfere significantly in the thyroid function in dogs.

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THE INCIDENCE OF TOPOGRAPHIC GASTROINTESTINAL DISORDERS AT PET CARNIVORES

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Abstract

In 2009-2011, of the 5320 carnivores presented at the consultation, only 14 were diagnosed with topographic gastrointestinal disorders, which represents less than 1%, most exactely 0.26%. Compared to the overall morbidity by other medical illnesses, the result is 0.27%.All topographic gastrointestinal disorders were diagnosed in dogs; that is directly related to the species, feeding mode and temperament of the dog versus cat, without being able to make a direct link between certain triggers and their scope.Most topographic gastrointestinal disorders diagnosed in dogs were hernia and intestinal obstruction, with 5 cases and 35.71%, followed by 2 cases of diagnosed gastric torsion, respectively 14.28% and intestinal intussusception and intestinal volvulus, each with one case diagnosed, which represents each 7.14%. These results don't have statistical value, given the low incidence of topographic gastrointestinal disorders and their sporadic intestinal character in dogs, compared to other diseases.

Keywords: *topographic gastrointestinal disorders, dog, incidence*

Material and method

Topographic gastrointestinal disorders diagnosed in carnivores were gastric torsion, intestinal intussusception, intestinal volvulus, hernias and intestinal obstruction. For their diagnosis it was used the clinical examination, supplemented by special methods such as ultrasound and radiologic examination,peritoneal puncture and intestinal laparotomy for diagnostic and intestinal therapeutic.

Based on the data included in the Register of Consultations and Treatments present at the Medical Clinic of the Faculty of Veterinary Medicine, it has been studied the incidence of various topographic gastrointestinal disorders in dogs and cats in the period 2009-2011, compared to some variation factors such as the type of thetopographic disorders diagnosed, the year in which it were diagnosed, the incidence of other medical illnesses, species and total number of cases examined.

Results and Discussion

Topographic gastrointestinal disorders diagnosed in dogs over the years 2009-2011 are shown in Table 1 and Figure 1.

Of the topographic gastrointestinal disorders diagnosed, most were hernias and intestinal obstructions with 5 cases, meaning 35.71%, followed by 2 cases of diagnosed gastric torsion, respectively 14.28% and intestinal intussusception and intestinal volvulus, each with onediagnosedcase, which represents 7.14% of the total of topographic disorders diagnosed during the 3 years. This distribution can not be said to have any statistical value, taking into account the low incidence of topographic gastrointestinal disorders in dogs, compared to other diseases.

Table 1. The incidence of various topographic gastrointestinal disorders in dogs during the years 2009-2011

Year / Disease condition	2009	2010	2011	Total
Gastric Torsion	-	1	1	2
Intestinal intussusception	-	1	-	1
Intestinal Volvulus	1	-	-	1
Various types of hernias	2	2	1	5
Intestinal Obstruction	1	2	2	5
Total	4	6	4	14

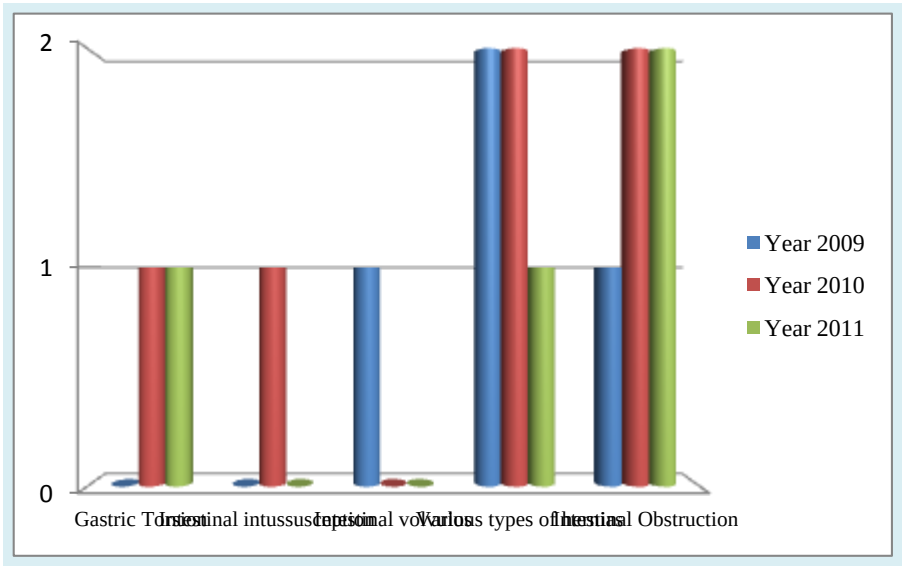

Figure 1. Diagram of the annual incidence of various types of topographic gastrointestinal disorders in dogs during 2009-2011

The diagram of the total incidence oftopographic gastrointestinal disorders in dogs during the years 2009-2011 is shown in Figure 2.

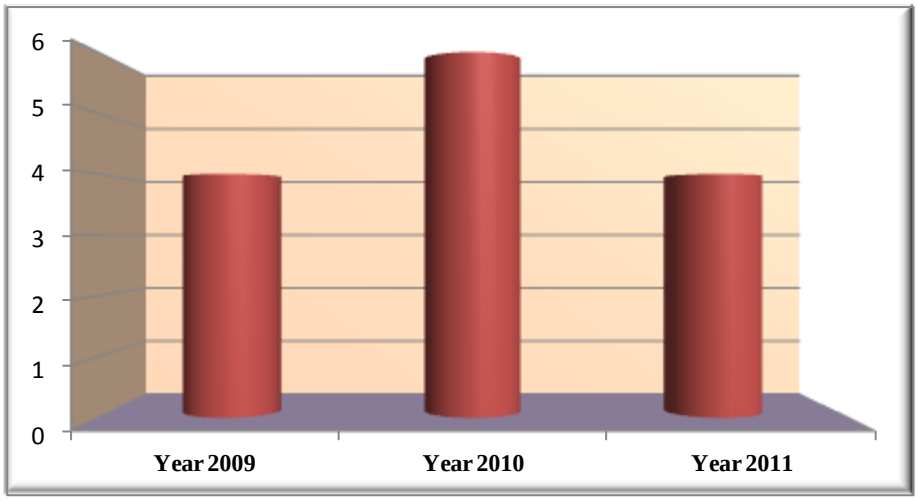

Figure 2. The overall incidence oftopographic gastrointestinal disorders in dogs during the years 2009-2011

It is noted that in 2009 and 2011 there were identified 4 cases with varioustopographic gastrointestinal disorders, respectively 28.57% of all cases diagnosed during the three years studied. In 2010 there was an increase, 6 casesbeing diagnosed, respectively 42.85%.

The incidence of topographic gastrointestinal disorders at pet carnivores in the period 2009-2011 is presented in Table 2, Figure 3 and Figure 4.

Table 2. The incidence of topographicgastrointestinal disorders during the years 2009-2011

Year / Disease Condition	Specia	2009	2010	2011	Total
Animals submitted to consultation	**Dogs**	431	1402	750	2583
	Cats	476	1498	763	2737
	Total	**907**	**2900**	**1513**	**5320**
Medical illness diagnosed	**Dogs**	427	1345	740	2512
	Cats	445	1501	748	2694
	Total	**872**	**2846**	**1488**	**5206**
Topographic gastrointestinal disorders	**Dogs**	4	6	4	14
	Cats	-	-	-	-
	Total	**4**	**6**	**4**	**14**

It can be seen that during the 3 years under study, of the 5320 cases submitted to consultation, only 14 were diagnosed with topographic gastrointestinal disorders, representing less than 1% , more exactely 0.26%. If we compare the total morbidity by topographic gastrointestinal disorders during the years 2009-2011to the incidence of other medical illnesses, the result is still below 1%, or 0.27%.

In addition, all topographic gastrointestinal disorders were diagnosed in dogs. This is directly related to species, method of feeding and general temperament of the dog versus cat.

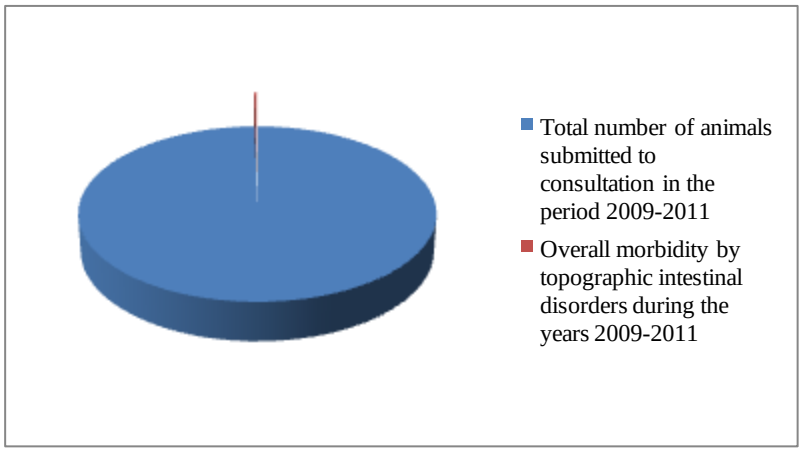

Fig. 3. Diagram of the total morbidity by topographic gastrointestinal disorders reported to the total number of animals submitted to consultation in the period 2009-2011

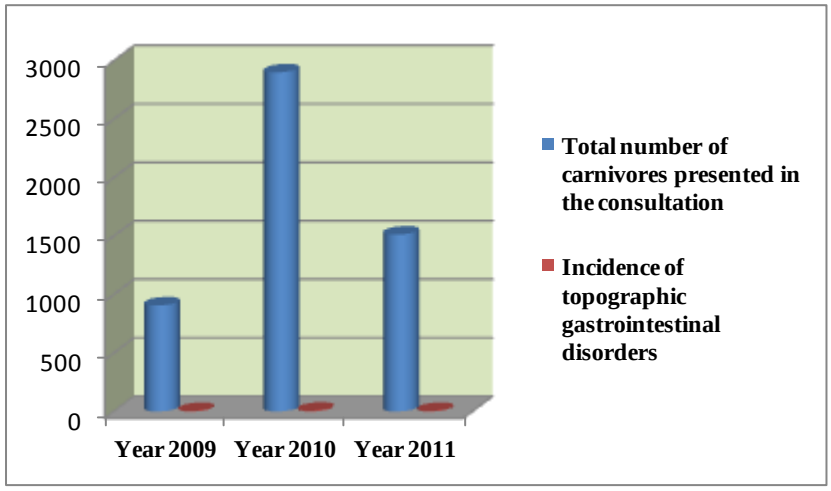

Fig. 4. Annual morbidity chart by topographic gastrointestinal disorders reported to the total number of animals present in the consultation in the period 2009-2011

During 2009, the total morbidity determined by topographic gastrointestinal disorders reported to the total number of animals present in the consultation, was 4 of 907 cases examined, representing 0.44% and if we relate it to other medical conditions diagnosed , it was 0, 46%. In 2010, the total morbidity by topographic gastrointestinal disorders was 6 cases of 2900 examined, representing 0.20% and reported to the morbidity by other medical diseases was 0.21%. In 2011 the total morbidity by topographic gastrointestinal disorders was 4 cases of 1513 diagnosed, representing 0.26% and if we reporte it to the incidence of other medical diseases, it was 0.27%.

Graphical representation of the evolution of topographical disorders gastrointestinal morbidity in carnivores is shown in Figure 5.

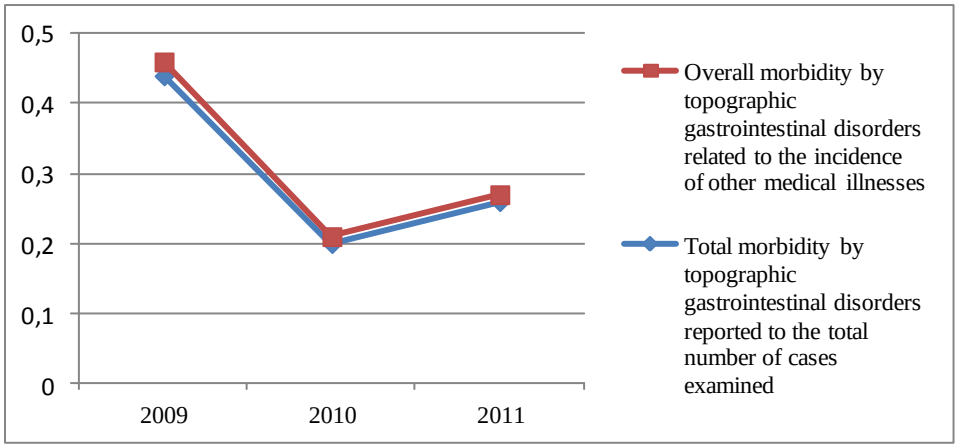

Fig. 5. Evolution of topographic gastrointestinal morbidity disorder in dogs

From Figure 5 it can be seen that the evolution of total morbidity by topographic gastrointestinal disorders in dogs, however, was different during the years 2009-2011, being higher in 2009 and lower in 2010. This can be explainedthrough a larger number of animals submitted to consultation in 2010 compared to 2009 and even 2011, while the number of cases with topographic gastrointestinal disorders remained relatively constant.

From these results we conclude that topographic gastrointestinal disorders occurred sporadically in dogs, but may be a direct link between certain triggers that acted during the years under study and their incidence.

Conclusions

1. Of the 5320 carnivores presented at consultation in 2009-2011, only 14 were diagnosed with intestinal topographic disturbances, which represents less than 1% and 0.26%. If we compare the total morbidity by topographic gastrointestinal disorders to morbidity by other medical illnesses, the result is still below 1%, or 0.27%.
2. Most topographic gastrointestinal disorders diagnosed in pet carnivores were hernia and intestinal obstruction with 5 cases and 35.71%, followed by 2 cases diagnosed with

gastric torsion, respectively 14,28 and intestinal intussusception and intestinal volvulus with one case diagnosed, representing 7.14% each.

3. This distribution can not be said to have any statistical value, taking into consideration the low incidence oftopographic gastrointestinal disorders in dogs, compared to other diseases.
4. All topographic intestinal disorders were diagnosed in dogs; it is directly related to species, method of feeding and general temperament of the dog versus cat.

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CLINICAL AND PARACLINICAL ASPECTS IN TOPOGRAPHIC GASTROINTESTINAL DISORDERS IN DOG: GASTRIC TORSION, INTUSSUSCEPTION AND INTESTINAL VOLVULUS

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Abstract

In gastric torsion, clinical signs suddenly started shortly after the consumption of food, when the animal began to play. Ultrasound was inconclusive due to the fermentation gases accumulated in the stomach, which made it impossible to obtain clear images. The Hemoleucograme revealed a deficient, normocytic, hypochromic anemia, (Hb = 11.2 g / dl, MCH = 20.7 pg, MCHC = 33.2 g / dL and MCV NRH x103/mm3 6.13 = 64.8 µ ³) and dehydration (Ht = 51.8%) while the blood biochemical profile indicated the existence of hepatocytolisis pathophysiological syndrome with increased serum enzymes (AST = 98 IU / L, ALT = 86 IU / L, ALP = 152 IU / L) and initiation of renal and hepatic impairment (BUN = 25.4 mg / dl, CRTN = 1.5 mg / dl). In intestinal intussusception and intestinal volvulus, the clinical signs were of acute abdomen, cortical inhibition, vomiting and the lack of defecation, and the peritoneal punction shown a sero-hemorrhagic fluid. Ultrasound gave circular hyperechoic images, alternating with circular hipoecogene images with a target aspect. The Hemoleucograme revealed the existence of a deficient, normocytic, hypochromic anemia, and dehydration (NrH=5,0x10^3/mm^3, Hb=11,8 g/dl, HEM=20,9 pg, CHEM=31,9 g/dl, VEM=67 µ³, Ht=53%), and the blood biochemical profile diagnosticated an autointoxication process, with renal and hepatic impairment (AST=110,4 UI/L, ALT=98,6 UI/L, BUN=25,8 mg/dl).

Keywords: *topographic gastrointestinal disorders, dog, hematology, biochemistry*

Materials and methods

The research was conducted in dogs of different ages and races, which presented topographic clinical signs of gastrointestinal disorders. In all the cases examined, it was performed in addition to clinical examination some laboratory tests: complete blood count (CBC) and blood biochemical examination. And as a special diagnostic method it were used ultrasound, peritoneal puncture and high enema.

Results and Discussion

Clinical signs of gastric torsion are presented in Fig. 1 (dog, Carpathian Shepherd, 3 years)

Clinical manifestations started suddenly, shortly after eating food, when the animal began to play. On examination it was found then restlessness, loss of appetite, congested mucous appearance, increased tenderness to palpation and there is a 'gap' between the stomach and diaphragm, abdominal rapid expansion in the epigastric area. In addition to these, it appeared mixed dyspnea, shortness of breath and vomiting, immediately after water drinking, and the inability of executing the survey of the stomach.

Ultrasound was inconclusive due to the fermentation gases accumulated in the stomach which made it impossible to obtain clear images.

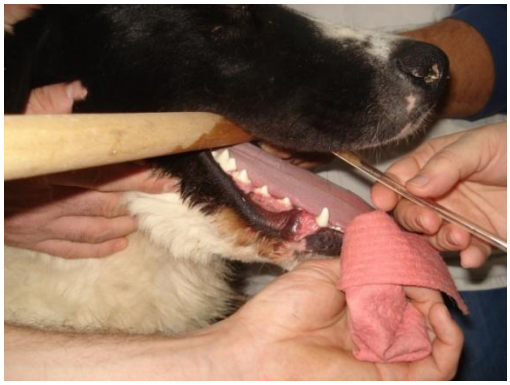
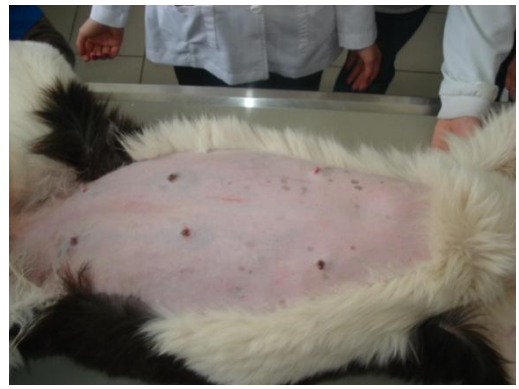

Fig. 1. Failure of the survey stomach. The existence of a "blank" between the stomach and the diaphragm. Abdominal expansion

Complete blood counts performed on blood samples taken on anticoagulant (EDTA) is shown in Table 1.

Table 1. Haematological profile in dogs, Carpathian Shepherd, 3 years Gastric torsion

Determined parameter	Number of red blood cells	Ht	Hb	VEM	HEM	CHEM
Unit expression	$x10^3/mm^3$	%	g/dl	µ³	pg	g/dl
Reference values(Merck)	5,4-7,8	37-54	13-18	64-74	22-27	34-36
Obtained values	6,13	51,8	11,2	64,8	20,7	33,2

In Table 1 it can be seen that hemoglobin, mean erythrocyte hemoglobin and mean erythrocyte hemoglobin concentration were slightly below the lower limit of the reference medium, Hb = 11.2 g / dl, MCH = 20.7 pg and MCHC = 33,2 g / dl, while the hematocrit values tend towards the upper limit of the average reference (Ht = 51.8%). The number of red blood cells and mean corpuscular volume was within the limits of the average reference, respectively x103/mm3 and MCV NRH 6.13 = 64.8 µ ³.

These values indicate a deficient, normocyte, hypochromic anemia , not directly related to gastric torsion. At the same time the tendency of increasing hematocrit may be associated with dehydration that accompanies gastric torsion.

In Table 2 are given the values of some blood biochemical parameters.

From Table 2 it is found elevated serum enzymes: AST = 98 IU / L, ALT = 86 IU / L, ALP = 152 IU / L, which indicates the onset of pathophysiological hepatocytolisis syndrome, subsequent to gastric torsion. On the other hand, the values of protein profiles are similar to the reference average values, meaning TP = 6.8 g / dl, White = 3.7 g / dl. The values of blood urea nitrogen (BUN) were 25.4 mg / dl and creatinine (CRTN) were 1.5 mg / dl, they tend to

the upper limit of the average reference values, which implies the occurrence of renal and liver insufficiencies.

Tabel 2. Serum concentration of biochemical parameters in dogs
Gastric torsion

Determined parameter	AST	ALT	ALP	TP	ALB	BUN	CRTN
Unit expression	UI/L	UI/L	UI/L	g/dl	g/dl	mg/dl	mg/dl
Reference values (Merck)	5-47	7-56	44-147	5,5-7,5	2,6-4,0	8,8-26	0,5-1,6
Obtained values	98	86	152	6,8	3,7	25,4	1,5

Clinical and ultrasound signs in intestinal intussusception and volvulus are presented in Fig. 2 (dog, German Shepherd, 5 years).

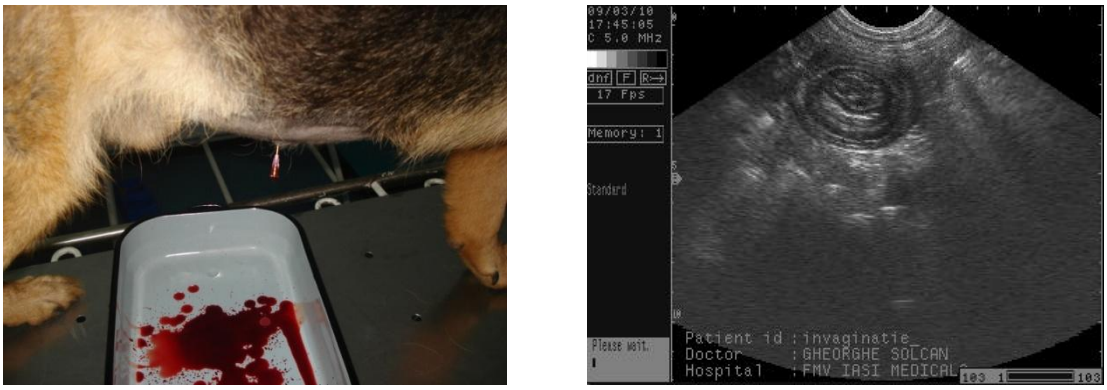

Fig. 2. Serohemoragic fluid in the peritoneal cavity. Acute abdomen.
Circular images, hypo and hyperechoic with the aspect of a target

As general signs were found dehydration, dyspnea, acute abdomen, cortical inhibition, high frequency pulse (82 where pulsatile / minute). As digestive signs, there was loss of appetite, vomiting and lack of defecation.

At peritoneal punction, sero-hemorrhagic fluid was obtained, and at the ultrasound there were observed circular hyperechoic images, alternating with circular hipoecogene-looking target.

Table 3 presents the values of the parameters that have made hematologic profile.

Tabel 3. Haematological profile in dogs with intestinal volvulus

Determined parameter	Number of red blood cells	Ht	Hb	VEM	HEM	CHEM
Expression unit	$x10^3/mm^3$	%	g/dl	μ^3	pg	g/dl
Reference values (Merck)	5,4-7,8	37-54	13-18	64-74	22-27	34-36
Obtained values	5,0	53	11,8	67	20,9	31,9

From Table 3 we observe a lower number of red blood cells (NRH = 5.0 x103/mm3), hemoglobin (Hb = 11.8 g / dl), mean erythrocyte hemoglobin (MCH = 20.9 pg) and mean erythrocyte hemoglobin concentration (MCHC = 31.9 g / dl). To the mean corpuscular volume it was assigned a value within the reference medium values (MCV = 67 μ^3) and the hematocrit value was close to the maximum value of the average reference (Ht = 53%). These data show the presence of deficient, hypochromic, normocytic anemia and dehydration.

Biochemical blood test results are shown in Table 4.

Tabel 4. Serum concentration of blood biochemical parameters in the intestinal volvulus in dogs

Determined parameter	AST	ALT	ALP	TP	ALB	BUN
Expression unit	UI/L	UI/L	UI/L	g/dl	g/dl	mg/dl
Reference values (Merck)	5-47	7-56	44-147	5,5-7,5	2,6-4,0	8,8-26
Obtained values	110,4	98,6	116,0	6,2	3,2	25,8

From Table 4 it is observed that serum transaminases value was increased (AST = 110.4 IU / L and ALT = 98.6 IU / L). The upper limit value of the average reference was obtained also to the blood urea nitrogen (BUN = 25.8 mg / dl). The amount of alkaline phosphatase (ALP) was 116.0 IU / L, falling within the average values of reference. The total serum protein and albumin values were included in the average values of the reference limits, (TP = 6.2 g / dl, ALB = 3.2 g / dl). These results show the existence of a process of intoxication, with renal and hepatic insufficiency, biochemical expressed mainly by increased BUN and serum transaminases.

Conclusions

1. The clinical signs of gastric torsion started suddenly, shortly after the animal food consumption, when he began to play.

2. Ultrasound examination was inconclusive due to the landfill gas accumulated in the stomach, which made it impossible to obtain clear images.
3. Blood counts in dogs with gastric torsion showed a deficienct, normocytic, hypochromic anemia (Hb = 11.2 g / dl, MCH = 20.7 pg, MCHC = 33.2 g / dL and MCV NRH 6.13 x103/mm3 ³ μ = 64.8) and dehydration (Ht = 51.8%).
4. Blood biochemical profile in dogs with gastric torsion indicates the hepatocytolisis pathophysiological syndrome with increased serum enzymes (AST = 98 IU / L, ALT = 86 IU / L, ALP = 152 IU / L) and the initiation of renal and hepatic impairment (BUN = 25.4 mg / dl, CRTN = 1.5 mg / dl).
5. The intestinal intussusception and volvulus clinical signs were acute abdomen, cortical inhibition, vomiting and lack of defecation, and at the peritoneal puncture it was achieved sero-hemorrhagic fluid.
6. Ultrasound examination in dogs with intussusception showed hyperechoic circular image, alternating with circular hipoecogene-looking target images.
7. Blood counts in dogs with intestinal intussusception and volvulus revealed the existence of a deficienct, normocytic, hypochromic anemia and dehydration (NRH = 5.0 x103/mm3, Hb = 11.8 g / dl, MCH = 20.9 pg, MCHC = 31.9 g / dl, MCV = 67 μ ³, Ht = 53%).
8. In dogs with intussusception and intestinal volvulus, blood biochemical profile diagnosed a process of intoxication , with renal and hepatic insufficiency (AST = 110.4 IU / L, ALT = 98.6 IU / L, BUN = 25.8 mg / dl).

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RABBIT GENERAL ANESTHESIA FOR ENUCLEATION. CASE STUDY

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Abstract

The aim of this paperis to present a case study regarding general anesthesia in a one year old rabbit for surgery enucleation. Rabbit was premedicated using medetomidine250µg/kg and buprenorphine 30µg/kg subcutaneous. Ten minutes after premedication anesthesia was induced using ketamine 15 mg/kg subcutaneous. Subject was then intubated by nasal access using a 2 mm uncuffed endotracheal tube. A mixture of oxygen and 1-2% isoflurane was administered via endotracheal tube. Cardio-respiratory parameters were recorded each 5 minutes using a capnograph,EKG and a pulse-oximeter. During anesthesia rabbit received a mixture of Hartmann solution, colloids and glucose at an infusion rate of 20 ml/kg/h intravenous. Before surgery the subject also received enrofloxacine 5 mg/kg and meloxicam 0,6 mg/kg subcutaneous. After surgery atipamezole 0,5 mg/kg and metoclopramide 0,5 mg/kg were administered intramuscular to promote recovery and regaining appetite. This case study concluded that this protocol may provide sufficient analgesia and narcosis for this kind of surgeries.

Keywords: *rabbit, enucleation, buprenorphine, medetomidine, isoflurane*

Introduction

Injectable anesthetic protocols are frequently used in rabbits because demand no specific anesthetic machines and in many cases are cheaper. There are only two other articles in literature that present general anesthesia using this protocol (Difilippo et al., 2004; Murphy et al., 2010). Difilippo et al. (2004) assessed this protocol during cardiothoracic surgery while Murphy et al. (2010) study tested the protocol on rabbits that did not suffer any surgery. This research will help the implementation of this protocol in clinical practice for routine and special surgeries.

Materials and methods

For this case study we used one rabbit subject that came to Clinique Vétérinaire Universitaire de Liège for enucleation by conjunctive access. It weighted 1,7 kg and it was not starved before anesthesia. The subject was risk classified as ASA 2.

For premedication it received medetomidine (Sedator® 1mg/ml, Eurovet animal health) 250 µg/kg subcutaneous and buprenorphine (Vetergesic® 0.3 mg/kg, Ecuphar) 30 µg/kg subcutaneous. Induction was realized using ketamine (Ketamine 1000® 100 mg/ml, CEVA) 15 mg/kg subcutaneous 10 minutes after premedication.

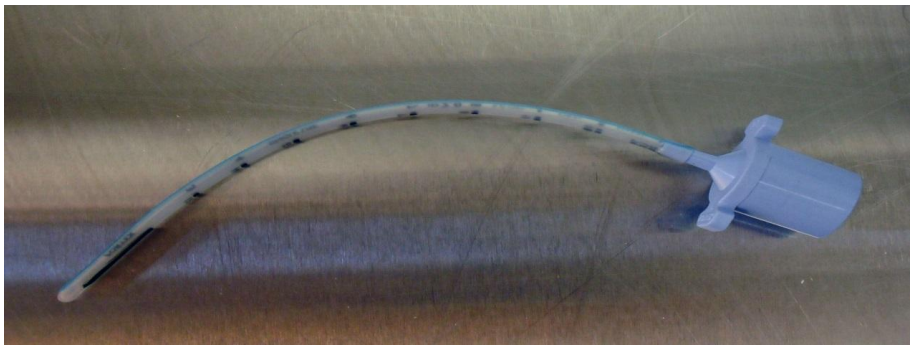

Fig. 1.Uncuffed endotracheal tube used for nasal-tracheal intubation

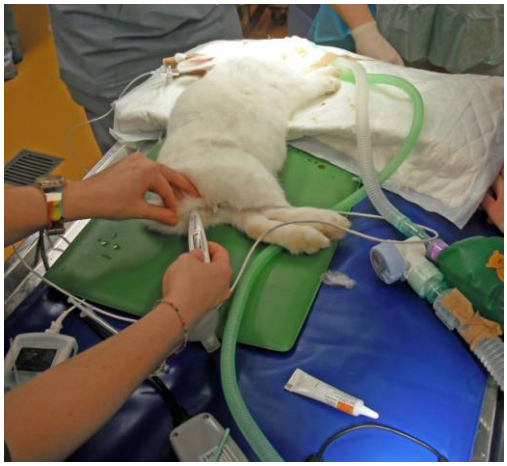

Fig. 2. Pre-operatory aspects of anesthesia

After induction subject was placed in sternal position with head in forced extension and a2mm uncuffed endotracheal tube (figure 1)was passed in the trachea by the nose. The placement of the tube was checked using a capnograph. Pure oxygen and isoflurane (iso-ver® 1000mg/g, eurovet animal health) 1-2% was then administered by endotracheal tube to maintain anesthesia.

During anesthesia rabbit was not mechanically ventilated. An intravenous catheter was placed in the marginal ear vein and we administered a mixture fluid 20 ml/kg/h using an injection pump: colloid (Voluven® 6%, Fresenius KabiDeutschland gmbh) 44%, lactated ringer's (Excel®, b. Braun medical inc.) 44% and glucose (glucose 30% Lavoisier fl 500ml, Chaix et du Marais®) 12% of the mixture.

Cardio-respiratory parameters were recorded each five minutes in an anesthetic sheet (figure 2; figure 3). Respiratory parameters were assessed using direct clinical examination, using a capnograph and a pulse-oximeter. Cardio-circulatory parameters such as pulse rate, heart rate and capillary refill time were assessed by direct clinical examination, EKG and using a pulse-oximeter.

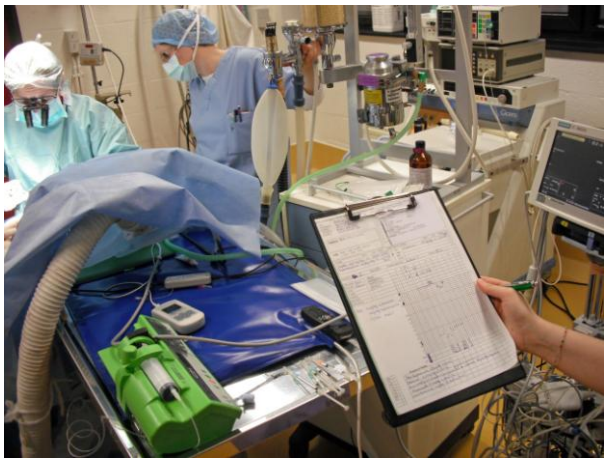

Fig. 3. Intra-operatory aspects of anesthesia

Rabbit received enrofloxacine (Baytril® 5 %, Bayer Animal Health GmbH, D-51368 Leverkusen, Germania) 5 mg/kg subcutaneous to prevent surgery infections.Meloxicam (Metacam® 5 mg/kg, BoehringIngelheim) 0,6 mg/kg subcutaneous was administered to stop local inflammatory reaction and to limit intra-operatory and post-operatory pain.

For recovery subject received atipamezol (Revertor® 5 mg/ml, Virbac) 1 mg/kg intramuscular as antidote for medetomidine and metoclopramide (Vomend® 5mg/ml, Dechra veterinary products) 0.5 mg/kg intramuscular to stimulate appetite and digestion after surgery.

Results and Discussions

Premedication, induction and recovery took place smooth as inMurphy K.L. et al. (2010) study. After medetomidine premedication the subjectwas sedated but it did not lose standing position or any reflexes. Ketamine administration produced smooth induction with losing sternal position. Ocular reflexes and pedal pinch reflex were also abolished after ketamine induction. Buprenorphine did not change anything in the anesthetic status after administration. After induction rabbit was successfully intubated by nasal access. Subject recovered gently from anesthesia after stopping isoflurane and atipamezol inoculation.

A venous catheter was applied in the lateral ear vein (TutunaruA.C., et al. 2012). The study showed that this protocol is efficient to desensitize external ear and to apply a venous catheter.

During anesthesia heart rate had a mean value of 218,3 beats/minute (180-240) equal to those recorded by Murphy K.L. et al. (2010) and higher to those recorded by DifilippoS.M. et al. (2004). Heart rate variation was not correlated with surgery pain stimulation. Rabbit did not suffer bradycardia during anesthesia. The use ofmedetomidine might produce a fall in heart rate (TutunaruA., et al. 2011; TutunaruA., et al. 2010).

Hemoglobin tissue oxygen saturation did not pass lower than 95%, with a mean value of 98%. Respiratory rate was relatively constant during surgery with a mean value of 36

breath/minutes (20-50). Apnea was not recorded, not even in the induction phase. Apnea might not be recorded even if higher doses were used (DifilippoS.M. et al., 2004).

Buprenorphine reduces ventilation and tissue oxygenation by his depressive action on the respiratory function in conscious rabbits but do not influence blood pressure or heart rate (ShaffordH.L. and SchadtJ., 2008). The protocol achieved a level of analgesia sufficient for this kind of surgerie.

Conclusions

This protocol was successfully used for general anesthesia in rabbit for ophthalmic enucleation. The protocol produced a smooth induction facilitating nasal intubation. The degree of analgesia facilitated a good anesthetic maintenance and a fast post operatory recovery.

The study also showed that maintaining inhalatory anesthesia by nasal intubation can be a simple and safe technique that should be used in clinics were direct endotracheal intubation might be a problem.

Acknowledgements

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CHIARI-LIKE, MENINGOENCEPHALITIS AND ATLANTOAXIAL SUBLUXATION IN A YORKSHIRE TERRIER DOG

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Abstract

Chiari-like malformation involves occipital malformation that allows cerebellum herniation thru foramen magnum. Mostly is a characteristic disorder of the Cavalier King Charles Spaniel breed but the literature also mention the York Shire breed. Atlantoaxial subluxation is a disorder that affects the first 2 cervical vertebrae and in consequence they are not well attached due to the lack o ligament support between them or the dens. Necrotizing encephalitis is arather rare inflammatory disorder of the brain that affects mostly the adult York Shire Terrier dogs. No sex predisposition has been mentioned. There was one dog, York Shire Terrier, radiographic and MRI examined. It presented sever neurological clinical signs. There were diagnosed three pathologies: Chiari-like malformation, encephalitis/meningoencephalitis and atlantoaxial subluxation. Romanian literature doesn't mention, describes or diagnose these types of disorders. The objective of this study is to describe and characterize the imaging findings of these diseases.

Keywords: *meningoencephalitis, Chiari-like, atlantoaxial subluxation, MRI*

Materials and methods

There was one dog, York Shire Terrier, 2 years old female that had the following clinical signs: ataxia, astasia, tumbling abnormal positioning of the head and neck. The neurological exam couldn't have been performed because of the severe neurological signs.

Radiological exam was performed in two projections LT and VD at the level of the cervical spine. A digitalized Maxivet 300HF X-ray machine was used for the radiological projections and the MRI (magnetic resonance imaging) head and cervical exams were performed using a 0.2 Tesla Esaote machine. The MRI exam was performed under inhalatory anesthesia. We obtained T1, contrast T1 and T2 weighted images in all three planes: sagittal, transversal and dorsal.

Results and discussions

Clinical nervous sign made the neurological exam impossible to be performed.

Radiological exam revealed increased gap between the dorsal spinous process of the C2 vertebra and the C1 vertebra. The dens is not visible on the VD view. There is bending of the spine in the VD view that could be positional. Doming of the calvarium typical of the breed (Fig. 1). MRI study confirms lack of the dens. There are at least two T2 hyperintensities in the brain and one at the spine, level of C1-C2 vertebrae. There is ,,Z,, kink of the medulla oblongata and pointy cerebellum but not herniating. Mildly dilated 3rd and 4th ventricles. Large lateral ventricles typical of the breed.

All the data are compatible with atlantoaxial subluxation secondary to hypoplasia/aplasia of the dens. Intramedullary lesion at C1 and C2 vertebrae may reflect hemorrhage. Multifocal central nervous system disease - compatible with encephalitis/meningoencephalitis. Due to the breed there is high suspicion of necrotizing encephalitis that has grave prognosis. Evidence of Chiari-like malformation.Prognosis has to be very guarded to poor due to the high possibility of necrotizing encephalitis.

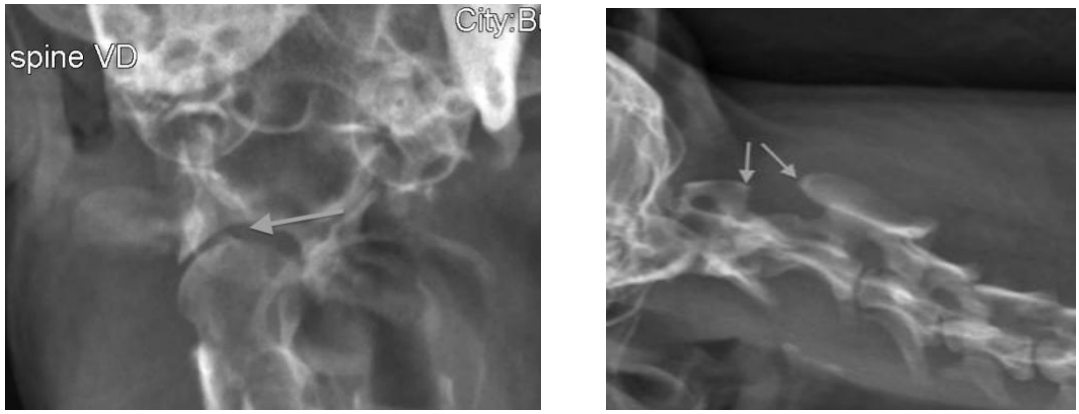

Fig. 1. Increased gap between the dorsal spinous process of the C2 vertebra and the C1 vertebra.
The dens is not not visible on the VD view

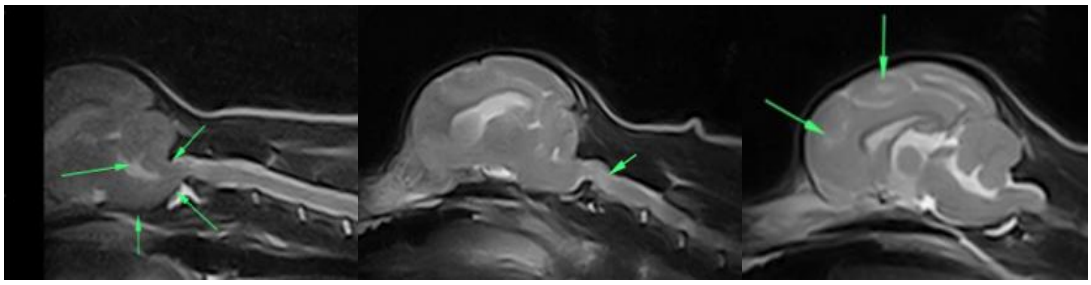

Fig.2. MRI images compatible with atlantoaxial subluxation secondary to hypoplasia/aplasia of the dens. Intramedullarlesions at the C1 and C2 level may reflect hemoragia. Multifocal lesion from CNS are compatible with encephalitis/meningoencephalytis- by the breed it could also be necrotic encephalitis. The cerebellum is slightly in sharp angle toward the foramen magnum but not herniated though- Chiari malformation. The 3rd and 4thVentriculi are moderate dilated. The lateral ventriculi are also dilated but it is a normal finding for the breed

Conclusions

Images in different perpendicular planes acquisition and superior anatomical details of MRI makes it a useful imaging diagnosing tool by the ability to describe the lesions leading as well to a more close to reality prognostic. Radiographic imaging is a useful diagnostic method but it has its limits at the level of the brain and spinal cord.

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OBSERVATIONS REGARDING VAGINAL PROLAPSE IN BITCH – CASE STUDY

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Abstract

Vaginal prolapse, also called vaginal ptosis or vaginal hyperplasia is characterized by a portion of edematous tissue of the vaginal canal protruding in the vaginal and vulvar opening, usually in proestrus and estrus stages of the sexual cycle. Vaginal prolapse may also occur near parturition or shortly after, due to lower levels of progesterone and increased seric concentration of oestrogen but this type of prolapse occurs rarely in dogs. This case study presents a Cane Corso breed bitch aged 2 and a half years old and weighing 37.3 kg, who developed a III degree vaginal prolapse. The affection debuted as a type II vaginal prolaps and transformed later on into a type III vaginal prolapse. The prolapsed formation, with an approximately oval shape and a diameter of 22/20 cm, presented numerous areas of necrosis. According to the abdominal radiograph, the bladder was not present in the abdominal cavity and the ultrasound revealed a globular formation, nonechogenic, filled with liquid, which was actually the urinary bladder that herniated at this level. It is to be noted that the condition occurred during oestrus and debuted as a second degree vaginal prolapse, transforming later into a third degree vaginal prolapse. The large size of the prolapsed mass was determined on one hand by the venous stasis and inflammation that determined vaginal oedema and on the other hand by the herniated urinary bladder. Since the owner did not agree to the ovariohysterectomy procedure after the reposition of the vaginal wall, the prolapsed formation was resected and the vulvar labia were sutured for 7 days postoperation. We may conclude that vaginal prolapse of I or II degree, that occurr during proestrului or oestrus can often turn into to a third-degree vaginal prolapse that might complicate with bladder herniation in the next sexual cycle or even in the same sexual cycle.

Key words: *vaginal prolapse, oestrus, bitch*

Introduction

Vaginal prolapse is a affection encountered in many species of domestic animals such us ovines, bovines and goats (Johnston și col., 2001). In this species, we refer to a true vaginal prolapse, involving the entire vaginal wall and sometimes other organs such as the bladder, the uterus and the distal part of the colon (McNamara et al., 1997).

True vaginal prolapse or uterine prolapse are rarely seen in bitches. These types of prolapse often ocurrs during parturition or shortly after, due to the decreased levels of progesterone and increased seric concentration of oestrogen (Schaefers-Okkens, 2001 ; Johnston și col., 2001 ; Konig și col. 2004 ; Rani și col., 2004).

In complete vaginal prolapse, the cervix is externalized while in partial prolapse, the cervix is non-externalized (Wykes, 1986). In bitch, this type of true vaginal prolapse is an affection rarrely encountered (Okkens, 2001). Some of the causes that contribue to the apparition of true vaginal prolapse in bitch are constipation, forced separation during coitus and sexual dimorphism (Purswell, 2000).

Sometimes the prolapse of the vaginal folds represent the exteriorization of the edematous vaginal tissue through the vulvar opening and occurs during proestrus and estrus phases of the sexual cycle. The vaginal oedema is a consequence of the oestrogen action (Johnston și col., 2001). This affection was first called vaginal ptosis or vaginal hyperplasia (Mialot J.P., 1984). Due to the absence of true hyperplasia or true prolaps and the presence

edematous tissue, the most appropriat term for this affection is prolaps of the vaginal folds (Purswell, 2000).

Depending on the degree of the prolapse, Schutte (1967) realised a clasification that included all three types of prolapse. Type I prolaps is characterized by a mild to moderate protruding of the vaginal mucosa originated from the vaginal floor, cranial to the urethral opening. In type II vaginal prolapse, the vaginal mucosa, originated aswell from the vaginal floor, is externalized at the level of the vulvar labia. In females with type III vaginal prolapse, the entire circumference of the vaginal mucosa is externalized and may be often accompanied by externalization of the urethral orifice.

Through this case study we intended to treat the condition by resecting the prolapsed tissue and preserving the reproductive function of the animal, since the owner wanted to continue using the female for breeding.

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Material and method

Observations were made on a Cane Corso breed bitch, aged 2 years and a half and weighing 37.3 kg which was presented at the Clinic of Reproduction and Obstetrics, Faculty of Veterinary Medicine. The owner affirmed that three weeks ago an oval shape fleshy formation, covered in vaginal mucosa with dimensions of 4/5 cm, protruded through the vulvar labia. The formation increased in volum suddenly in the past 4 days and the general condition of the female has modified, the bitch presenting during consult a temperature of 37,4 ^{0}C, apathy, lack of urination and loss of appetite. The prolapsed formation visible at the level of the vulvar labia had an oval shape, with a diameter of 22/20 cm and presented areas with oedema and necrosis (fig. 1)

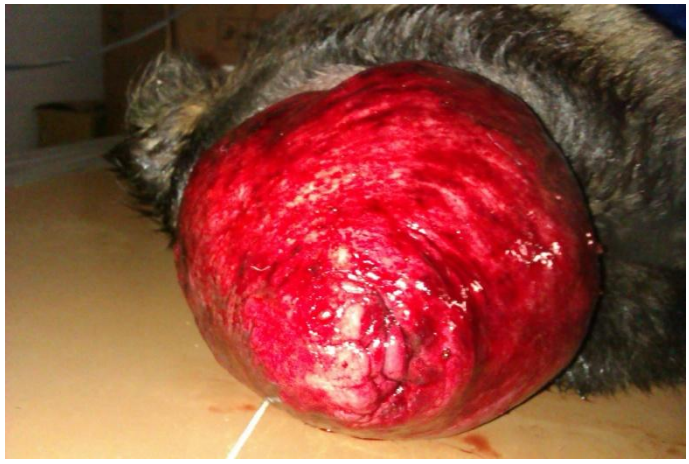

Fig. 1 Aspect of III degree vaginal prolaps in bitch

An abdominal radiography and an ultrasound were performed in order to establish if at the level of the prolapsed formation are any herniated structures such as urinary bladder, uterine body or intestinal loops.

According to the owner, the female was in oestrus at the time the formation appeared and in the previous cycle the female presented a II degree vaginal prolapse which was resolved without treatment in about 4 weeks after the end of oestrus.

After repositioning the vaginal wall, ovariohysterectomy operation was proposed as a solution in order to avoid recurrence. The owner wanted to use the female for breeding opting for surgical resection of the vaginal prolapsed.

Results and discussions

The vaginal prolapse has been reported in all species of domestic animals (Mc Namara et al., 1997) but comparative with other vaginal disorders it is not a common affection in bitch.

While Memon și col. (1993) showed that prolapse of the vaginal folds appear mainly in proestrus and at the begining of the estrus, Schaefers-Okkens, (2001) affirmed that true vaginal prolaps appears during or at short time after parturition. It appears that the incidence of this affection is higher in *brachycephalic dog breeds* such as Boxer, Bull Mastif and Mastino Napolitano (Schaefers-Okkens, 2001). We encountered this affection in other breeds such as Caucasian Shepherd, Romanian Mioritic Shepherd and Cane Corso.

Three types of vaginal prolapse have been described. Type I represented by a mild to moderate protruding of vaginal floor (Johnston si col., 2001) accompanied in some cases by the presence of a redish pyriform formation between the vulvar labia when the female is lying (Mialot J.P., 1984). Type II prolapse is characterized by the prolaps of the cranial vaginal floor and the lateral walls of the vagina, between the vulvar labia, resulting a tongue or pear shape formation (Johnston si col., 2001) or a three-lobe tumefaction under the inferior commissure of the vulva (Mialot J.P., 1984). Type III prolapse is represented by the prolapse of the entire vaginal mucosa circumference as a a donut-shaped mass with a lumen in the center (Johnston et al., 2001).

Mialot J.P., 1984 describes the vaginal ptosis as a hyperplasia of the vaginal mucosa which can be classified as type I, II or III and is considered to be a partial prolapse that debutes during estrus.

Since the vaginal prolapse originates in the vaginal floor, cranial to urethral papilla, the external urethral orifice is visible on the ventral surface of the prolapsed mass (Johnston si col., 2001).

In the present case, it has been established after consultation that the bitch presented a type III vaginal prolapse that occured during estrus. The large size of the prolapsed mass was determined on one hand by the venous stasis and inflammation that determined vaginal oedema and on the other hand by the herniated urinary bladder.

The radiographyc exam (fig.2) evidentiated the absence of the urinary bladder in the abdominal cavity and the ultrasound exam confirmed the presence of a globular non-echogenic formation, filled with liquid, which was in fact the herniated urinary bladder (fig.3).

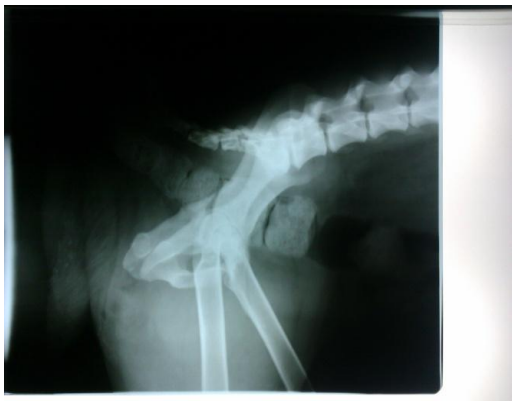

Fig.2 Radiography of the posterior portion of the abdominal cavity

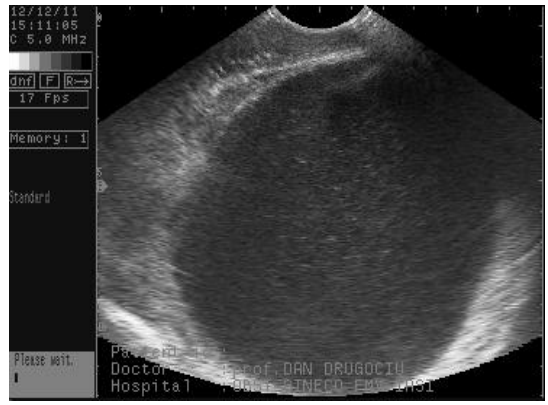

Fig.3 Ultrasound of the herniated urinary bladder

The female has undergone surgery which aimed to reduce the prolapsed formation, followed by suture of the vulvar labia. The general anesthesia was performed with Atropine sulfate (0.04 mg / kg sc), Xylazină (2 mg / kg, im) and Ketamine (10 mg / kg im). After the female was anesthetized, the formation was properly toileted and the urethral orifice was identified on the ventral in order to be catheterized. Since the introduction of the urinary chateter in the herniated urinary bladder was not possible, the bladder was evacuated using a neddle and a syringe.

After repositioning the urinary bladder by digital manipulation, a urethral catheter was introduced in the bladder and mainted during surgery and 2 days postoperation. The bitch was placed in sterno-abdominal decubitus and the prolapsed tissue was resected following the clasical technique. The bleeding was stopped by compression, forcipressure and simple sutures with resorbable thread, Polyglycolicacid (PGA 1-0). (fig.4).

Fig. 4 Aspect of the vaginal suture

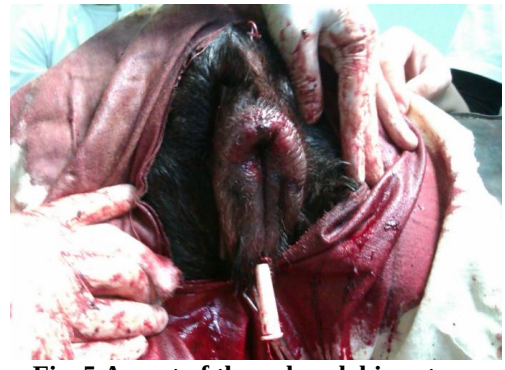

Fig. 5 Aspect of the vulvar labia suture

The operation ended with the U suture vulvar labia. The urinary catheter was kept for another two days after surgery and vulvar labia was maintained sutured for 7 days. (Figure 5.) There were no postoperative complications or incidents after recovery from anesthesia. The

postoperative therapy focused on the volemic and hydroelectrolyte equilibration using glucose serum 5% (150 ml iv), vitamin therapy and antibiotics for 5 days postoperatively (Amoxycilină trihydrate 8.75 mg / kg / day).

Conclusions

1. This case report represents a case of type III vaginal prolapse observed in a bitch that presented in the previous sexual cycle a type II vaginal prolapse.
2. The vaginal prolapse appeared during estrus and the impressive dimensions were due to venous stasis and inflamation that emphasized the vaginal oedema on one hand and on the other hand due to the herniated urinary bladder.
3. The profound modified general condition of the female was determined by anuria caused by the herniated urinary bladder.
4. Since the owner did not agree with the ovariohysterectomy intervention, the surgical approach was represented by the resection of the prolapsed formation after the clasical tehnique and the suture of the vulvar labia.

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PROSPECTIVE STUDY REGARDING INCIDENCE OF HIP DYSPLASIA IN FIVE BREEDS IN ROMANIA

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Abstract

Hip dysplasia is one of the most common orthopaedic diseases in dogs, which leads to chronic pain and functional impairment. It occurs principally in large dogs but also affects small dogs and cats. Usually the condition is bilateral but unilateral hip dysplasia has been reported in dogs radiographed using the extended ventrodorsal projection.This retrospective study was undertaken to estimate prevalence of canine hip dysplasia (CHD) in five different breeds (Golden Retrievers, Rottweilers, German Shepherds, Labrador Retrievers and Cane Corso) in Romania and identify sources of bias in published reports. There were 551 dogs examined for CHD that belong to 5 different breeds (German Shepherds-156, Labrador Retrievers-138, Rottweilers-111, Golden Retrievers-103 and Cane Corso-43). The percentage was established by total number of positive CHD dogs and by its unilaterality or bilaterality. From 156 German Shepherd dogs 57,04% were positive, 37,67% of the 138 Labrador retrievers, 46,83% of the 111 Rottweilers, 42,81% of the 103 Golden retrievers, and 41,86% of the 43 Cane corso. Prevalence of CHD in these 5 breeds Romania resembles very much with the studies that we have found on the subject. The condition is mostly bilateral but unilateral (left or right) is also encountered in this study. Most of the positive dogs for CHD were bilateral affected and the unilateral incidence between left and right is similar.

***Keywords** : hip dysplasia, incidence, canine, radiology*

Introduction

Hip dysplasia is one of the most common orthopaedic diseases in dogs, which leads to chronic pain and functional impairment. It occurs principally in large dogs but also affects small dogs and cats. Usually the condition is bilateral but unilateral hip dysplasia has been reported in dogs radiographed using the extended ventrodorsal projection. Hip dysplasia is a developmental age-related disorder; it is not present at birth. Radiographic assessment is the election technique for diagnosing CHD. A variable amount of time must elapse before radiographic changes are manifest. Once present, these radiographic changes usually progress as the affected animal ages.

Objective

This retrospective study was undertaken to estimate prevalence of canine hip dysplasia (CHD) in five different breeds (Golden Retrievers, Rottweilers, German Shepherds, Labrador Retrievers and Cane Corso) in Romania and identify sources of bias in published reports.

Materials and Methods

The screenings were performed at the 4Vet X-ray Laboratory, from Bucharest, Raspantiilor No 30, that uses an Maxivet 300 HF x-ray machine with a Toshiba tube. Survey radiographs were performed on the sedated pacient. Sedation has been made with Domitor (0.1mg/kg) and Antisedam. The projections were obtained using the ventrodorsal extension method.

There were 551 dogs examined for CHD that belong to 5 different breeds (German Shepherds-156, Labrador Retrievers-138, Rottweilers-111, Golden Retrievers-103 and Cane Corso-43). The percentage was established by total number of positive CHD dogs and by its unilaterality or bilaterality.

Results

From 156 German Shepherd dogs 57,04% were positive, 37,67% of the 138 Labrador retrievers, 46,83% of the 111 Rottweilers, 42,81% of the 103 Golden retrievers, and 41,86% of the 43 Cane corso. The condition is mostly bilateral but unilateral (left or right) is also encountered in this study. Most of the positive dogs for CHD were bilateral affected and the unilateral incidence between left and right is similar (see table 1, chart 1).

Table 1. Bilateral, right and left CHD percentages on five breeds

	German Shepherd	Labrador retrievers	Rottweilers	Golden retrievers	Cane corso
Bilateral	60,37%	74,53%	50,78%	82, 46%	54, 72%
Right unilateral	27, 62%	19, 43%	26, 09%	14, 32%	27, 16%
Left unilateral	12,01%	6,04%	23,13%	3,22%	18,12%

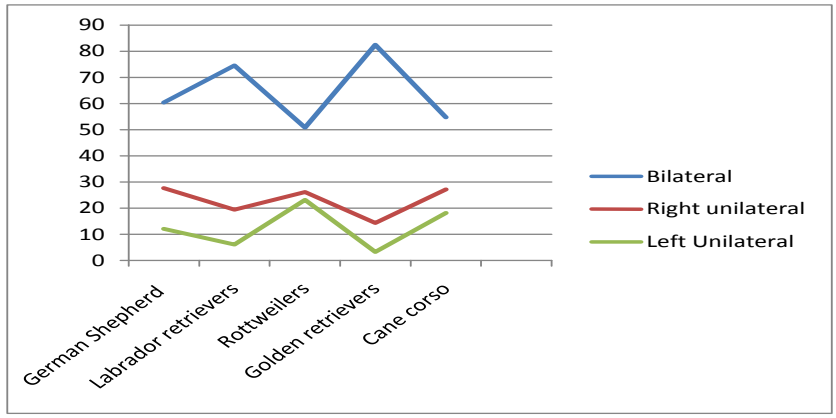

Fig. 1. Chart representation on CHD percentages

In "Estimates of prevalence of hip dysplasia in Golden Retrievers and Rottweilers and the influence of bias on published prevalence figures" study the prevalence of CHD in Golden Retrievers ranged from 53% to 73% and in Rottweilers ranged from 41% to 69%. In

this study they examined 200 clinically normal Golden Retrievers and 140 clinically normal Rottweilers between 24 and 60 months of age referred for hip evaluation (group 1) and 93 clinically normal dogs evaluated for Orthopedic Foundation for Animals (OFA) hip certification (group 2).

In another retrospective study the authors reviewed pelvic radiographs of 891 dogs, to determine the incidence of Unilateral Canine Hip Dysplasia (UCHD). Results show that 149 (16.7%) dogs had UCHD.

The study "Incidence of Canine Hip Dysplasia: A Survey of 272 Cases" bilateral hip dysplasia was found to be more (88.60 percent) than unilateral. Among the 2 unilateral hip dysplasia, left side was found to be more (54.83 percent) than right. In most cases, dysplasia will occur bilaterally and approximately 7% occurs unilaterally.

Conclusions

Prevalence of CHD in these 5 breeds in Romania resembles very much with the studies that we have found on the subject. The condition is mostly bilateral but unilateral (left or right) is also encountered in this study. Most of the positive dogs for CHD were bilateral affected and the unilateral incidence between left and right is similar.

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EFFECTS OF PUERPERAL ENDOMETRITIS ON THE OVARIAN ACTIVITY OF MILK COWS DURING THE POST PARTUM PERIOD

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Abstract

The efficiency of reproduction of milk cows diminishes sometimes due to the occurrence of endometritis, which determine great economic loss. After partition there is the risk of bacterial contamination of the uterine lumen and infections. From the clinical point of view the disease can be diagnosed by the occurrence of vaginal secretions which reflect the presence of pathogen bacteria, such as: Escherichia Coli, Fusobacterium necrophorum, Prevotella melaninogenicus. "In vitro" Herpes virus 4 (BOHV-4) has a tropistic action on the endometrial cells, infecting the epithelial cells and especially the stromal cells causing a strong cytopathic effect. The elimination of the patogene agents by the innate immune system dependent on pattern recognition receptors binding. The epithelial and stromal cells, uterine express receptors such as, Toll -like Receptor 4,theat binds lipopolysaccharide from the E. coli membrane, (LPS) being a major constituent of the cell wall of the negative Gram bacteria, which is an immunogenic with the capacity to enlarge the number of immune responses to soluble antigen. The viruses role in the uterine diseases is relatively unexplored although Herpes 4 virus was isolated from several animals with endometritis. The infertility associated with uterine diseases is caused by demage to the endometrium and disruption of ovarian cyclic activity.The presence of bacteria from the uterine level can affect the endometrial prostaglandin secretion and perturb ovarian follicle growth and function. The knowledge and understanding of the molecular base of the uterine disease will lead to novel approaches to treating infertility.

Keywords*: uterus, ovary, disease, endometritis*

Introduction

Uterine diseases can be classified as puerperal metritis, clinical metritis, clinical and subclinical endometritis. (Sheldon I.M. et al.,2006).

These diseases are highly prevalent in high producing dairy cows and have been associated with the decreased of, pregnancy per AI, extended interval to pregnancy, increased culling, and economic loss. (Gilbert R.O. et al., 2005). Metritises affects about 20% of the milk cows population with the incidence ranging from 8-40% in some farms. (Huzzey J.M. et al., 2007; Galvão K.N. et al., 2009), clinical endometritis olso affects about 20% of the milk cows population, a prevalence between 5-30% in some herds.(McDougall S. et al., 2007 ; Galvão K.N. et al., 2009). Subclinical endometritis is more abundant and affects about 30% of the milk cows population during lactation, with a prevalence of 11-70%. (Barlund C.S.et al., 2008; Galvão K.N. et al., 2009).

Retention of fetal membranes is a condition where the cow fails to release the placenta 12 or 24 hours after calving.. Although retention of fetal membranes is not a disease per se, many researchers have tried to treat (systemically or intrauterine) this condition because it is a major risk factor for metritis. (Drillich M .et al., 2006). Puerperal mertritis is characterized by the presence of an abnormally enlargen uterus, a fetid watery red-brownish uterine discharge associated with sings of systemic illness, and fever (> 39.4 Celsius) within 21 days in milk.Animals without systemic sings but an enlarged uterus and

a purulent uterine discharge within 21 may be classified as having clinical metritis. (Sheldon I.M et al.,2006).

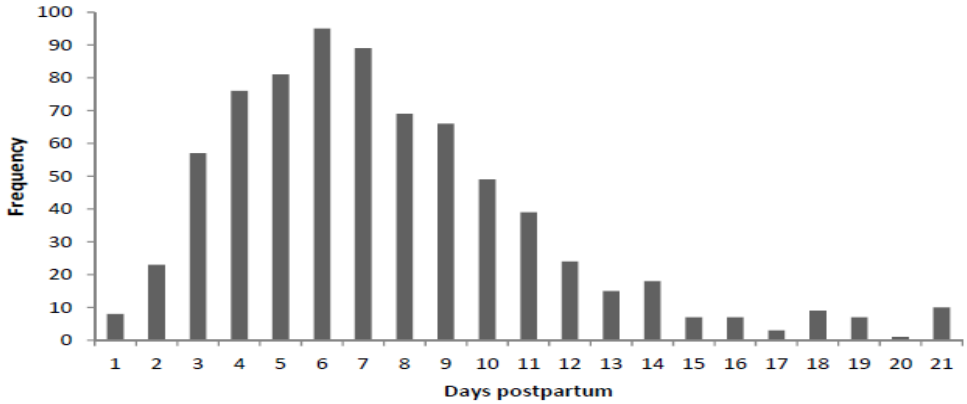

Fig. 1. Frequency distribution of metritis incidence by day postpartum in a sample of 753 metritis causes that occurred over one year period in dairies in Ohio, New York and California (Galvão K.N. et al., 2009)

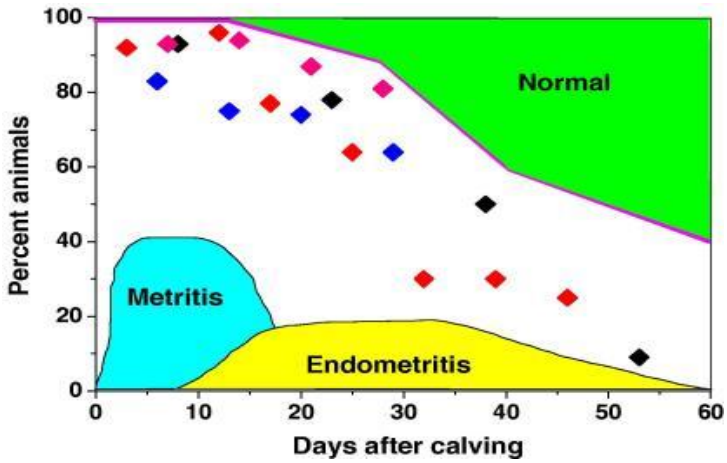

Fig. 2. Almost all cows have bacteria within the cavity of the uterus during the first 2 weeks of calving and uterine disease is very common
(Sheldon I.M. et al., 2007)

Each marker ◆ indicates the percent of animals with bacteria isolated from the uterine cavity; data are from four different studies, with animals sampled at various times between calving and 60 days after parturition (Sheldon,I.M. et al., 2002b; Williams,E.J. et al., 2005). The shaded areas represent the proportion of animals with metritis within 2 weeks of calving and endometritis 3–5 weeks after calving. The solid line indicates the percent of animal with histological evidence of inflammation of the endometrium *(*Gilbert,R.O. et al., 2005)

Metritis is diagnosed by a complete physical examination of the cow including attitude, hydration status, rectal temperature and palpation of the uterus per rectum to evalue uterine discharge. Evaluation of the rectal temperature performed before the palpation per rectum. (Benzaquen M.E. et al., 2007). This author has noticed that 60% of the cows diagnosed with puerperal metritis do not present a temperature higher than 39,4 Cesius, the affection not always accompanied by fever.

The cows and especially cows raised in an intensive system can frequently present microbial infections of the uterus. Of all the animals examined during the study 80 to 100% present a series of bacteria at the level of the uterus in the first two weeks after parturition. (Sheldon I.M. et al., 2007).

Parturition often represents a risk for the mother as well as for the calf. Physical deterioration during parturition, retention of fetal membranes may cause the increase of the risk of bacterial infections. Sometimes animals may suffer infections of the uterus and the mammary gland even from the gestation period, infections which may cause premature parturition.

Although the largest impact on health and productivity of the milk cows is microbial activity after parturition. (Sheldon I.M. et al.,2008)

Bacteria which can contaminate the uterus can be classified according to their pathogenicity, bacteria which are specific with a tropism at the level of the uterus, bacteria potentially pathogen for the uterus and opportunist contaminated bacteria. (Williams E.J.et al., 2005).

The first line of defense against invading bacteria in the uterus in the innate immune system. Part of the innate response is the elaboration of pro-inflammatory cytokines, including as tumour necrosis factor alpha (TNF α), which induce the production of acute phase proteins such as α1-acid glycoprotein (AGP), serum amyloid A. (SAA) and haptoglobim . Periphral plasma concentrations of AGP are greater in animals from which recognized uterine pathogens are isolated. (Gayle D. et al., 1999).

Although the immune response eliminates in fact the microbes, up to 40% of the animals present bacterial infections up to 3 weeks after partition.(Sheldon I.M.et al., 2007) . The immune response of the uterus to different microbes leads to an afflux of neutrophils from the peripheral circulation into the endometrium and the uterine lumen. (Zerbe H. et al., 2003; Dhaliwall G.S. et al., 2001).

The severity of endometritis depends largely on the pathogenicity of the present bacterial agents. The resistance of the pathogen agents is influenced by the uterine environment , genetic factors, innate immunity or acquired immunity. The uterine environment in the postpartum period favours the development of a large variety of bacteria anaerobe and aerobe. Many of these bacteria are contaminated for the uterine lumen and removed by a range of uterine defence mechanism.

Table 1. Classification of anaerobe and aerobe bacteria, according to the pathogen potential, isolation from cellular cultures, gathered from the uterine environment

(Dobson H.et al., 2000 ;Sheldon I.M. et al., 2002)

Bacterial category		
1	**2**	**3**
Arcanobacterium pyogenes	*Bacillus licheniformis*	*Clostridium perfringens*
Prevotella melaninogenicus	*Enterococcus faecalis*	*Klebsiella pneumoniae*
Escherichia coli	*Mannhiemia haemolytica*	*Micrococcus species*
Fusobacterium necrophorum	*Pasteurella multocida*	*Providencia stuartii*
	Peptostreptococcus species	*Proteus species*
	Staphylococcus aureus	*Staphylococcus species, coagulase negative*
	Non-haemolytic Streptococci	*α-haemoltyic Streptococci*
		Streptococcus acidominimus
		Aspergillus species

1-Represent bacteria recognized as being associated with endometrial injuries.
2-Potential pathogen agents from the uterin lumen, but without producing frequent uterine injuries.
3-Opportunist contaminants transiently isolated from the uterine lumen but not usually associated with endometritis.

Infertility associated with uterine disease is the cause of the endometrium deterioration and, in consequence disturbes the activity of the ovarian cycle. Bacteria have the capacity to modulate prostaglandine secretion of the endometrium, the ovaries function, follicles development, also affecting the evolution of the corpus luteum. (Sheldon I.M. et al., 2007).

Uterine disease is frequent associated with E. coli, F. necrophorum, Prevotella Species, Arcanobacterium pyogenes, bacteria which proved to act synergistically, increasing the risk of uterine disease and occurrence clinic endometritis, as well as its gravity. (Olson J.D. et al.,1984).

Most common pathogen agents are : E.coli 37% from the total of isolated bacteria and A.pyogenes 49%. E.coli seems to precede and favour the infection with A. pyogenes. (Williams E.J. et al., 2007).

Although at the uterus level there are a very large number of bacteria, only the ones previously mentioned are pathogen for the genital tractus and will cause diseases with a severe inflammatory answer and obvious clinical manifestations.

Effects of the uterine infection on the ovarian functions

Contamination of the uterus with several pathogen agents is associated with ovarian disfunctions during the postpartum period. Plus the infection leads to the increase of the production of the inflammation mediators. However, it is not clear if the suppression of the ovarian follicular growth and the ovary function is influenced by the

presence of pathogen bacteria, potentially pathogene bacteria or opportunist bacteria, present in the uterus. (Williams E.J. et al., 2007).

Animals which present a large density of bacterial development in the uterine lumen, present a smaller dominant follicle and decreased estradiol concentrations in the plasma, compared to healthy cows from the postpartum period, fact which affects the reproductive functions, determinated abnormalities of the ovarian function. (Lavon Y. et al., 2008; Williams E.J. et al., 2007).

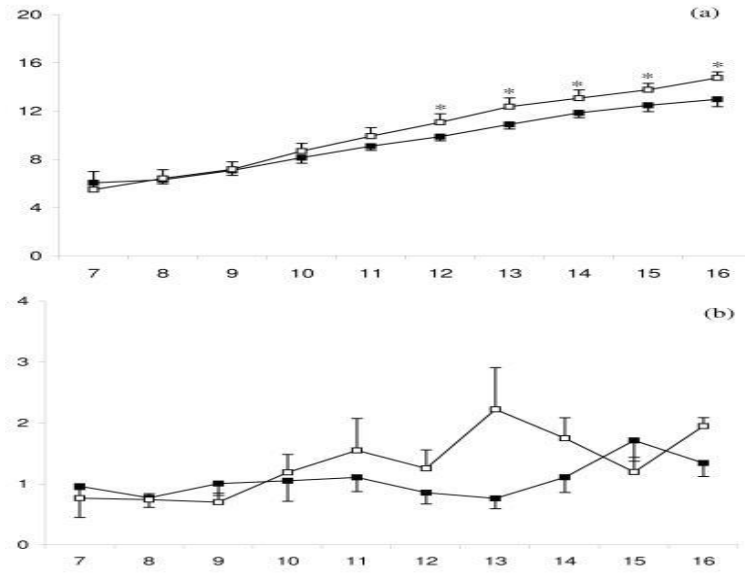

Fig. 3. The diameter of the first dominant follicle, (b) , the estradiol concentration from the peripheral plasma between days 7-16 postpartum

(Williams E.J.et al., 2007)

Mean ± SEM (a) diameter of the first dominant follicle, (b) peripheral plasma oestradiol concentration between days 7 and 16 postpartum for cows with high (■) or low (□) day 7 UPGD. Values differ between groups within day * P < **...**

Between days 7 and 16 post partum the internal diameter of the first postpartum dominant follicle increased in all animals ($P < 0.001$) as did peripheral plasma oestradiol concentrations ($P < 0.001$). However, in high day 7 UPGD cows, the first postpartum dominant follicle was smaller over days 6 and 11 (Fig 3a, $P < 0.05$) and peripheral plasma oestradiol concentrations were lower (Fig3b, $P < 0.05$) than in low day 7 UPGD cows. Mean growth rate of the first post partum dominant follicle tended to be slower in the high than the low day 7 UPGD cows (0.79 ± 0.08 vs. 1.03 ± 0.09 mm/day, respectively, $P = 0.05$).

Table 2. The location and timing of postpartum ovarian events for animals with high or low day 7 UPGD. Asterisks denote differences between the day 7 UPGD groups
(Williams E.J. et al., 2007)

Event	Low UPGD (n = 20)	High UPGD (n = 70)
Number of first wave follicles ≥ 4mm in diameter	2.1 ± 0.1	1.9 ± 0.1
Calving to dominance interval: first dominant follicle (days)	12.1 ± 0.4	11.1 ± 0.4
Number of animals with first dominant follicles on the ipsilateral ovary	6 (30 %)	8 (16.3 %)
Number of animals with first dominant follicles on the contralateral ovary	14 (70 %)	42 (83.7 %)***
Number of first dominant follicles ovulated	13 (65 %)	27 (54 %) *
Number of first dominant follicles regressed	2 (10%)	11 (22 %) *
Number of first dominant follicles persisted	5 (25 %)	12 (24 %)

*P < 0.05
***P < 0.001.
UPGD-uterine pathogen bacterial growth density

Also the size and function of the corpus luteum is affected, which is essential to gestation, so the uterine bacterial infection has the capacity to influence ovulation and gestation. (Green M.P. et al., 2011).

The effects of the bacterial uterine infection on the corpus luteum are clearly associated with premature regression or the failure of lutolysis, which causes an extended luteal phase. (Opsomer G. Et al., 2000)

Pathogen bacteria for the uterus, such as : E.coli, cause E.2 prostaglandin secretion, in cultures of endometrial cells and explant tissue "in vitro" , which can affect the corpus luteum function. (Herath S. Et al., 2007). All the same, the effect of the uterine infection on the development and function of the corpus luteum in the postpartum period remains unclear. (Williams E.J. et al., 2007).

Between days 17 and 26 post partum the internal diameter of the first postpartum CL increased in all ovulating animals ($P < 0.001$, Fig 4a) as did peripheral plasma progesterone concentrations, ($P < 0.001$, Fig 4b). However, in animals with a high day 7 UPGD, the first postpartum CL was smaller ($P < 0.05$) than in low day 7 UPGD animals. The growth rate of the corpus luteum did not differ between high and low UPGD cows (0.8 ± 0.4 vs. 1.3 ± 0.7 mm/day, respectively). Peripheral plasma progesterone concentrations tended to be lower in high day 7 UPGD versus low day 7 UPGD animals over the study period ($P = 0.09$), and a significant interaction between group and time was observed ($P < 0.05$). Therefore, comparisons were made between high and low day 7 UPGD groups when a CL was present between 21 and 26 days postpartum. Peripheral progesterone concentrations were lower in animals with a high versus low day 7 UPGD score over this time ($P < 0.05$).

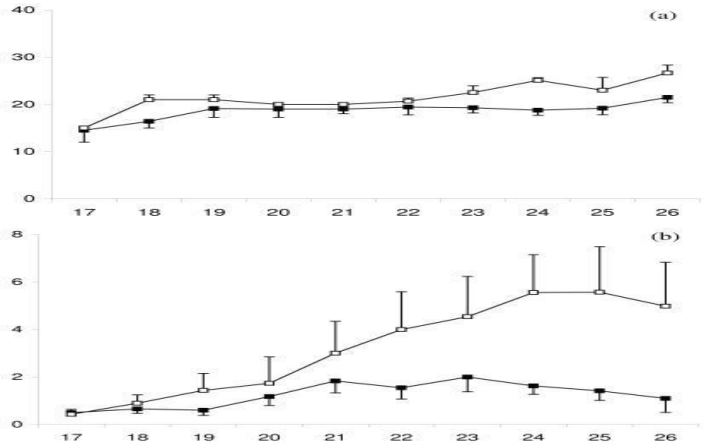

Fig. 4. Corpus luteum formation and progesterone production
(Williams E.J. et al., 2007)
Mean ± SEM (a) diameter of the corpus luteum and (b) peripheral plasma progesterone concentrations between days 17 and 26 post partum for cows with high (■) and low (□) day 7 UPGD

Mechanisms which are the basis of uterus disease and infertility

The effects of endometritis on the ovarian function are mediate probably at several levels: Ovarian, hypothalamus and pituitary. Similary, the low conception rates in cattle with subclinical endometritis ar after resolution of uterine disease are probably consequence of disruption of endocrine pathways and physiology as well as associated with uterine inflammation. (Sheldon I.M. et al., 2008)

It is assumed that a healthy endometrium is necessary for the nutrition of the blatsocyst and embryo, and the successful occurrence of pregnancy. Certainly, pathogen bacterial infection occurs to preclude conception. Furthermore there is embryo mortality if uterine infection occurs with these bacteria after conception. (Semambo D.K. et al., 1991).

If the infection is produced ‘ in vitro ‘, the result is that the uterine disease prolongs the luteal phase, by modulating the PGF2α secretion of the endometrium, which is also clinically observed. Furthmore, exogenous PGF2α can be an efficient treatment for this affection. (Lewis G.S. et al., 2006) . In vitro, lipopolysaccharide (LPS), major components of the cellular wall of Negative Gram bacteria stimulate the progesterone secretion of the mix populations of luteal cells (including types of steroidogenic, endothelial and immune cells). At a similar level with the luteal hormone (LH), but at higher concentrations of LPS, kills the luteal cells. (Grant E.J. et al., 2007)

Conclusions

Placental retention, uterine bacterial infections, uterine disease at cattle are frequent after parturition. Uterine bacterial infections stimulate a strong immune answer, but also modulate normal physiology of reproduction. Clinical and subclinical uterine diseases produced by known pathogen agents such as: E. coli, A. pyogenes, F. necrophorum, Proteus, negatively influence the ovaries functions causing a weak development of the ovarian follicles and, in consequence a reduced production of estradiol, one of the most important

estrogene hormones, which affects the installation of heat, so a prolonged period of anoestrum. Altogether the corpus luteum activity is affected, by the decrease of the PGF2α secretion, hormone responsible for the luteolysis. So a corpus luteum which persists will cause the continuation of the period of retaking the estrous cicles. In these conditions rather large economic loss is registered.

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IMPORTANCE OF BLOOD INDICATORS OF HEPATIC FUNCTION ON THE REPRODUCTIVE PERFORMANCE AFTER TREATMENT OF CYSTIC OVARIAN DISEASE WITH GONADOTROPHIN-RELEASING HORMONE IN DAIRY COWS

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Abstract

The aim of this study was to investigate whether a relation exists between blood indicators of hepatic function and reproductive performance after treatment with gonadotrophin-releasing hormone of cows with clinical cystic ovarian. Reproductive performance after treatment was assessed from the interval between treatment and first insemination (ITFI) and the interval between treatment and conception (ITC). We found elevated glucose, cholesterol, total protein concentrations and lower values of aspartate transaminase, gamma-glutamyl transferase for the cows ho had better response for the GnRH treatment.

Key words*: Balţată cu Negru Românească, hepatic function, cystic ovarian disease, GnRH*

Introduction

Cystic ovarian disease (COD) is an important reproductive disorder in dairy cattle. It can be characterized as the persistence of an anovulatory follicular structure on one or both ovaries with a diameter of >2.5 cm in the absence of a corpus luteum (Kesler and Garverick, 1982; Lopez-Diaz and Bosu, 1992), resulting in an aberrant reproductive function.

The reported incidence of COD in dairy cows varies between 5 and 10%, although rates as high as 30% have been reported (Kesler and Garverick, 1982; Lopez-Diaz and Bosu, 1992; Laporte et al., 1994). In a longitudinal study, the overall incidence of COD, diagnosed after 30 days post-partum, increased from 3.0% per lactation in 1987 to 9.5% 9 years later (Hooijer et al., 2001).

Parturition results in an abrupt shift in metabolic demands from nutrient accrual (body reserves and foetal mass) to rapid mobilization of lipid and protein stores in support of the sudden onset of high milk production (Butler, 2000). At this stage, the cow's appetite is limited, the amount of energy exported in milk cannot be covered by dry matter intake, and most cows will experience a period of negative energy balance (NEB). The modern high genetic merit dairy cow prioritizes nutrient supply towards milk production in early lactation and this demand takes precedence over the provision of optimal conditions for reproduction (O'Callaghan, 2000).

Negative energy balance leads to lipomobilization with a high probability of fatty infiltration into the liver. Liver lesions caused by fatty infiltration as a consequence of lipomobilization are typically observed in high producing cows during the first stage of lactation (Ametaj, 2005). The main indicators of hepatic lesions and function are enzymes aspartate transaminase (AST), gamma-glutamyl transferase (GGT) and the metabolites glucose, cholesterol, and albumin (Wittwer, 1995).

It is still uncertain whether a relation exists between the occurrence of COD, hepatic function and NEB.

In a cohort study with 12 feeding trials, NEB significantly reduced the time until clinical COD was diagnosed (diagnosed from early lactation to conception) (Sovani et al., 2000). This finding appeared to be independent of age and other peri-parturient events.

Heuer et al. (1999) concluded that COD tends to be positively associated with the fat to protein ratio of the first milk test (average at day 18) postpartum ($P < 0.10$). However, Opsomer et al. (1998) stated, based on the results of a study focusing on delayed cyclicity post-partum, that a causative role of NEB with regard to COD is not indicative. This was confirmed in a study with 600 clinical COD cases (Hooijer et al., 2003), in which the daily change in milk fat during the first and second milk recordings after calving was not statistically related to the incidence of clinical COD.

Treatment of COD has been primarily focused on resumption of ovarian cyclicity which results in oestrus, and subsequently, in conception after insemination. For this purpose, several hormones have been used. Human chorionic gonadotrophin (hCG) has LH activity, inducing follicular growth. Gonadotrophin-releasing hormone (GnRH) induces also LH release, and oestrus usually occurs within 4 weeks after treatment (Dinsmore et al., 1990; Hooijer et al., 1999). The GnRH and hCG elicit equivalent endocrine and clinical responses, but for routine treatment GnRH has an advantage over hCG because of its minimal antigenicity (Drost and Thatcher, 1992).

The aim of this study was to investigate whether a (indirect) relationship exists between the blood indicators of hepatic function and reproductive performance of dairy cows with clinical COD after treatment with GnRH.

Materials and Methods

This study was conducted form January 2012 to March 2013 on a 36 Bălţată cu Negru Romanească cows from S.C.D.C.B. Dancu-Iasi. After calving the cows were kept in the same condition with the herd.

Blood samples were obtained by jugular venipuncture, into the coagulation-activated vacuum tubes (Vacutest, Kima – Italia), on day of diagnostic. Each blood sample was centrifuged with the Rotofix 32A (Hettich Lab. Tachnology – Germany) at 3000 rpm for 15 min. for an optimum serum yield. Serum samples were transferred to the capped Eppendorf tubes and analyzed right away with the Accent 200, an automatic clinical chemistry analyzer (Pz Cormay S.A., Poland), using the photometric absorbance and nephelometric work principles. The biochemical parameters glucose, cholesterol, total protein, AST (aspartate transaminase), GGT (gamma-glutamyl transferase) and LDH (lactate dehydrogenase) were determined. The values are presented as means ± standard deviation.

All cows were palpated per rectum, in the morning, between 30 and 60 days after calving. In some exceptional cases cows were examined before or after this period. An animal was diagnosed as COD positive if follicles with a diameter >2.5 cm were detected by palpation on one or both ovaries in the absence of a corpus luteum.

The COD-positive cows were treated with 5 ml Gonadotrophin-Releasing Hormone Analogue **(**Receptal, Intervet) at the day of diagnosis.

Reproductive performance after treatment was assessed the interval between treatment and first insemination (ITFI) and from the interval between treatment and conception (ITC).

The program Microsoft Office Excel 2003; t-Test: Two-Sample Assuming Unequal Variance was used in order to determine the degree of the statistical significance (coefficient "P").

Results and discussions

After selection 2 groups of cow were selected, group 1 (n = 17) with cows who presented estrus after 21 days after treatment and group 2 (n = 15) with cows in estrus before 21 days after tratament of COD. 4 cows did not show estrus after treatment.

The average interval between treatment and first estrus was 19.4 ± 1.2 days for group 2 and 34.6 ± 2.3 days for group 1. Also the average interval between treatment and conception was 39.4 ± 2.3 days for group 2 and 63.2 ± 3.1 days for group 1 (table 1).

Table 1. The average interval between treatment and first estrus (ITFE) and between treatment and conception (ITC)

	ITFE (days) $\bar{X} \pm \sigma_X$	**ITC (days)** $\bar{X} \pm \sigma_X$
Group 1	34.6 ± 2.3	63.2 ± 3.1
Group 2	19.4 ± 1.2*	39.4 ± 2.3*

* Means within a row with different superscripts differ between group 1 and 2 (P < 0.05).

Biochemical parameters

The main indicators of hepatic lesions and function are presented in table 2.

Table 2. Blood indicators of hepatic function

Biochemical parameters	**UM**	**Group 1** $\bar{X} \pm \sigma_X$	**Group 2** $\bar{X} \pm \sigma_X$	**Reference values (The Merk Veterinary Manual)**
AST	IU/l	113.32 ± 13.72	78.2* ± 12.32	60-125
GGT	IU/l	20.52 ± 5.25	12.5* ± 6.73	6-17.4
Cholesterol	mg/dl	69.68 ± 16.22	145.26* ±18.87	62-193
Glucoza	mg/dl	61.51 ± 6.52	93.6* ± 4.32	42-100
Proteine totale	g/dl	5.90 ± 0.62	7* ± 0.54	6.7-7.5
LDH	IU/l	383.17 ± 3.8	328.5 ± 4.3	309-938

* Means within a row with different superscripts differ between group 1 and 2 (P < 0.05).

Aspartate transaminase (AST) was significant higher in group 1 compared with group 2. Increased AST activity in the serum is a sensitive marker of liver damage, even if the damage is of a subclinical nature (Kauppinen 1984, Meyer et co. 1998). Gamma-glutamyltransferase activity also depended on the observed period. El-Ghoul et al. (2000) found that GGT activity in late pregnancy is much lower than in the first week after calving, and 6 weeks after delivery, the activity increased. In our study the values of GGT was significant lower for group 2 compared with group 1.

Both groups of cows presented normal values of LDH. LDH is not organ-specific, and it is found in high concentrations in muscles, the heart, the kidney and the liver. It is released to the blood on an acute damage to cells of those organs.

The increase in LDH activity was related to an increase in the degree of liver steatosis (Pechová et al. 1997). Asmare et al. (1999), on the other hand, found no significant changes in serum LDH activity linked to liver damage, although they were able to demonstrate a degree of influence in a study of LDH isoenzymes. Reduced LDH activity has been associated with increased reproductive performance of heifers (Looper et al., 2002).

Katoh, 2002 reported that cows with fatty liver, therefore, have decreased synthesis of cholesteryl esters, which are important precursors for the synthesis of steroids. In our study the cows of group 1 presented the smallest values of serum cholesterol compared with group 2. Also the cows from the group 2 presented the highest values of glucose and total protein values compared with cows from the group 1.

Because glucose is the main source of energy for the ovarian function (Rabie et. al. 1997) and influences bovine thecal cell steroidogenesis in vitro (Stewart et al. 1995), it may play a major role in the achievement of post treatment ovulation. Rabiee and Lean (2000) found that there was a positive and highly significant cross correlation ($r = 0.5$) between the uptake of glucose and cholesterol and suggested that glucose may promote cholesterol uptake into ovarian cells or vice versa.

We conclude that the elevated glucose, cholesterol, total protein concentrations, in correlation with lower values of AST, GGT to be sufficient to determine a better response on the treatment of cystic ovarian diseases with Gn-RH.

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SUBTOTAL INTRACAPSULAR RESECTIONOF PROSTATE ADENOMA WITH TOTAL ABLATIONOF THE INTRAPROSTATIC URETHRA IN DOGS

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Abstract

Subtotal intracapsular resection of prostate adenoma with total ablation of the intraprostatic urethra and urinary continence preservation in dogs is an operation that has a high degree of difficulty and it is done in severe cases, acute blockage of the urinary and digestive transit, consecutive to total prostate volume augmentation. Subtotal prostatectomy is indicated in valuable breeding dogs for benign prostatic hyperplasia in lieu of castration and in stable dogs with abscessation or cysts in lieu of drainage procedures.In the Clinic of Obstetrics and Gynecology of the Faculty of Veterinary Medicine Bucharest, we have performed this surgery in 2 male dogs, common breed, with prostate adenoma, age 8 and 9 respectively. Using the electroscalpel, we dissected all parenchyma except a 2 mm shell attached to the capsule. We resected all the urethra except a 1-2 cm dorsal strip, trying to maintain the integrity of the Seminal colliculus and not to reach the striate sphincter of the urinary bladder.Before closing the prostatic lodge and restoring all the anatomic layers, we had placed a drainage tube to collect all residual urine from the prostatic lodge.The drainage tube was removed after 5 days and the urethral catheter was maintained for 10 days.The urinary continence was not affected as we conserved the striated sphincter of the urinary bladder (Seminal colliculus). No blood has been seen into the urine starting with the 5th day post surgery in both patients.The defecation and the urination went form better to normal from day one to day 10 post surgery in both cases.Both patients became independent and autonomous after 10-12 days after surgery.After 25 days we have observed the shrinkage of the prostate lodge at ultrasound check in both cases.

Key words: *canine,prostate, adenoma, intracapsular, resection*

Introduction

Subtotal intracapsular resection of prostate adenoma with total ablation of the intraprostatic urethra and urinary continence preservation in dogs is an operation that has a high degree of difficulty and it is done in severe cases, acute blockage of the urinary and digestive transit, consecutive to total prostate volume augmentation. This is considered to be an emergency decision and a life saving operation.

Subtotal prostatectomy is indicated in valuable breeding dogs for benign prostatic hyperplasia in lieu of castration and in stable dogs with abscessation or cysts in lieu of drainage procedures (Fossum, Theresa Welch, 2002).

Materials and methods

The prostate is the accessory sex gland, the ducts of which (Ductuli prostatici) open beside the seminal colliculus. The prostate has a body (external part) with two glandular lobes and a slight disseminate part (internal part), the glandular lobules of which are located within the wall of the urethra and surrounded by the urethralis muscle. Prostate is the only gland in the dog and it is situated in the posterior abdomen in old dogs and in the pelvic cavity in young ones. This gland covers the neck of the bladder and it has two lobes.

In the Clinic of Obstetrics and Gynaecology of the Faculty of Veterinary Medicine Bucharest, we have performed this surgery in 2 male dogs, common breed, with prostate

adenoma, age 8 and 9 respectively. Blood tests, RX and ultrasound check have been performed for both of them before the surgery. The blood tests were normal, latero-lateral radiographs reveal the presence of a large mass in the posterior abdomen that moved the bladder and rectum dorso-cranially. In both dogs, the prostate covered more than 70% of the distance between the cranial edge of the pubis and the promontory. On ultrasound exam, prostate parenchyma was homogeneously, slightly hyperechoic.

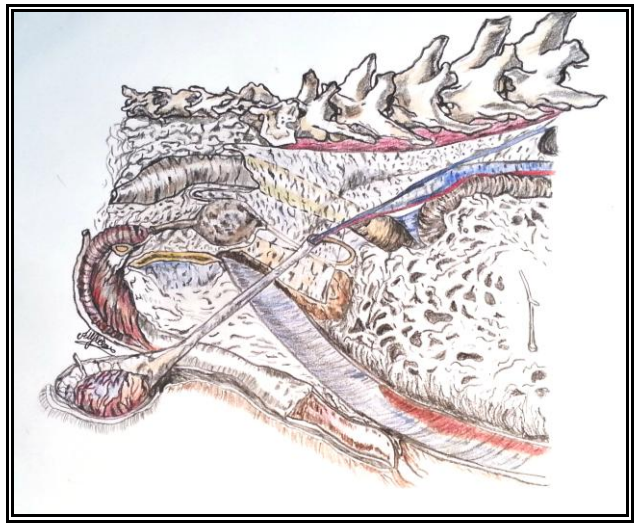

Fig. 1. The topography of the organs in the posterior abdomen and the pelvis in the dog
(orig., adapt. from Budras, K. D., 2007)

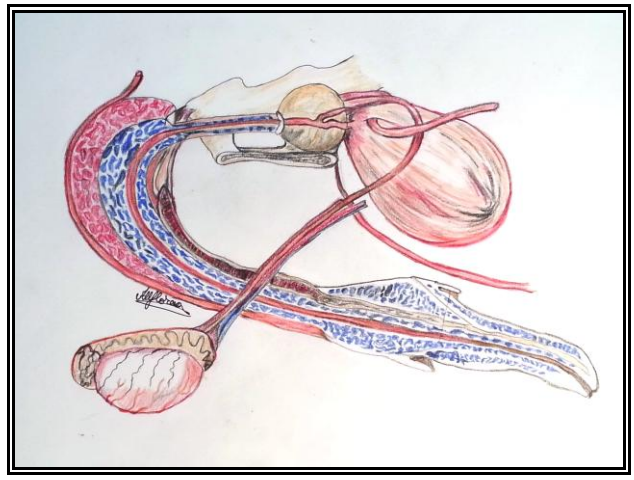

Fig. 2. Male genitalia in the dog
(orig., adapt. from Budras, K. D., 2007)

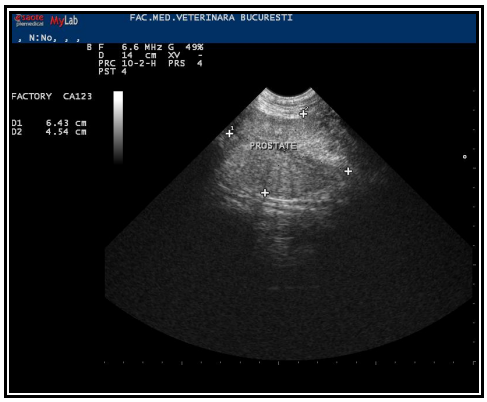

Fig. 3. Ultrasound exam, first case, shows prostate parenchyma homogeneously, slightly hyperechoic, 6,43/4,54 cm diameter (orig.)

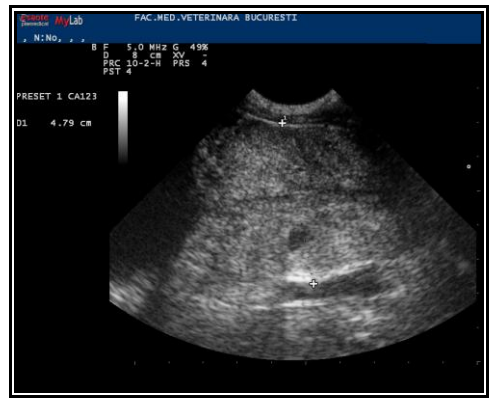

Fig. 4. Ultrasound exam, second case, shows prostate parenchyma homogeneously, slightly hyperechoic, 4,79 cm diameter (orig.)

Intracapsular subtotal prostatectomy starts, after laparotomy, with the incision of the ventral median septum with an electroscalpel. We used, for this incision, for the first time in veterinary medicine, the VIO 300D System which can be programmed for any individual work style, for a specific procedure or for any medical specialty.

Fig. 5. VIO 300D System using the Bipolar Soft (orig.)

We used the spray coagulation mode and then combined it with the VIO's BiClamp mode which reacts dynamically to the quality of the individual tissue and automatically adjusts the current output. Once the optimal degree of vessel or tissue fusion has been achieved, the electrosurgical activation is automatically switched off as an additional safety factor. We continued the incision through the parenchyma into the ventral urethra. Using the electroscalpel, we dissected all parenchyma except a 2 mm shell attached to the capsule. We resected all the urethra except a 1-2 cm dorsal strip, trying to maintain the integrity of the Seminal colliculus and not to reach the striate sphincter of the urinary bladder.

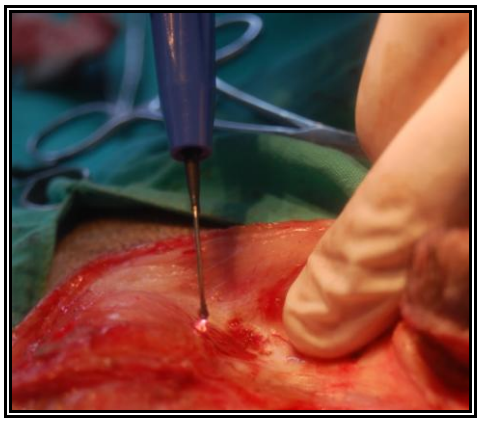

Fig. 6. The spray coagulation mode of the VIO's electroscalpel, closing all the vessels (orig.)

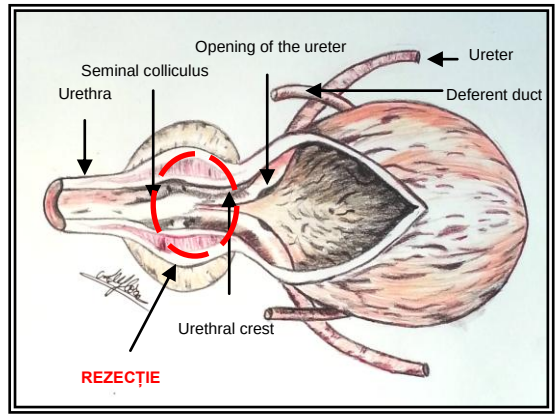

Fig. 7. The site of surgery. Anatomic view (orig. adapt. from Budras, K. D., 2007)

We performed the lavage of the prostatic shell, sealed all the vessels with the spray coagulation mode and closed the capsule over a urethral catheter positioned in the urinary bladder. We used an approximating pattern for the first layer and an inverting pattern for the second layer of closure with 3-0 polydioxanone. Before closing the prostatic lodge and restoring all the anatomic layers, we had placed a drainage tube to collect all the residual urine from the prostatic lodge.

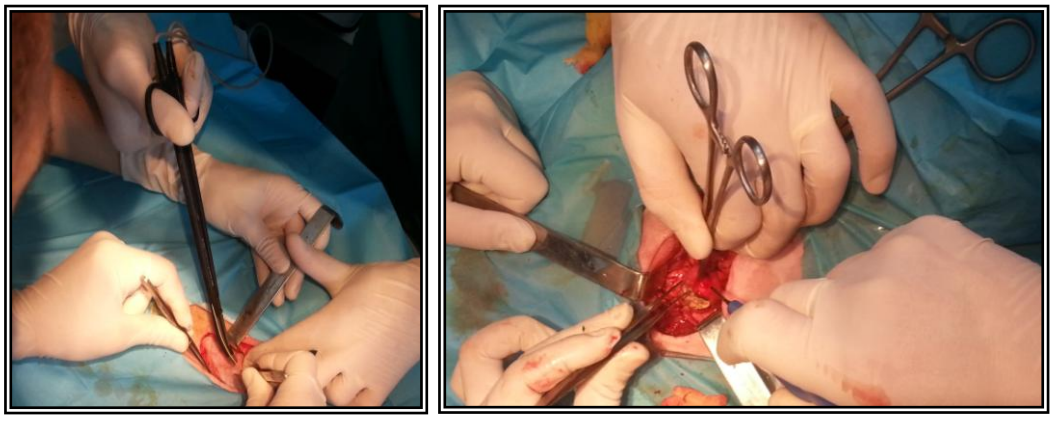

Fig. 8. VIO's BiClamp used to enter in the abdomen (left). **Using the electroscalpel, to dissect prostatic parenchyma** (right) (orig.)

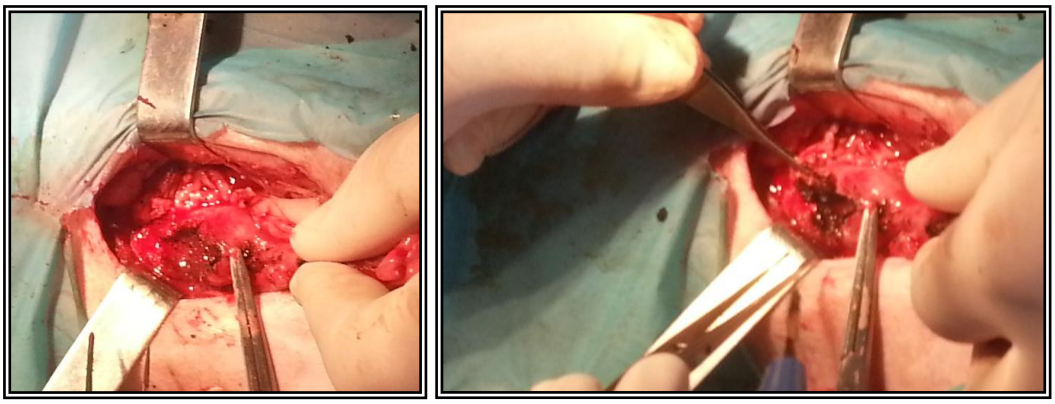

Fig. 9. Prostatic lodge after removing the prostatic parenchyma (orig.)

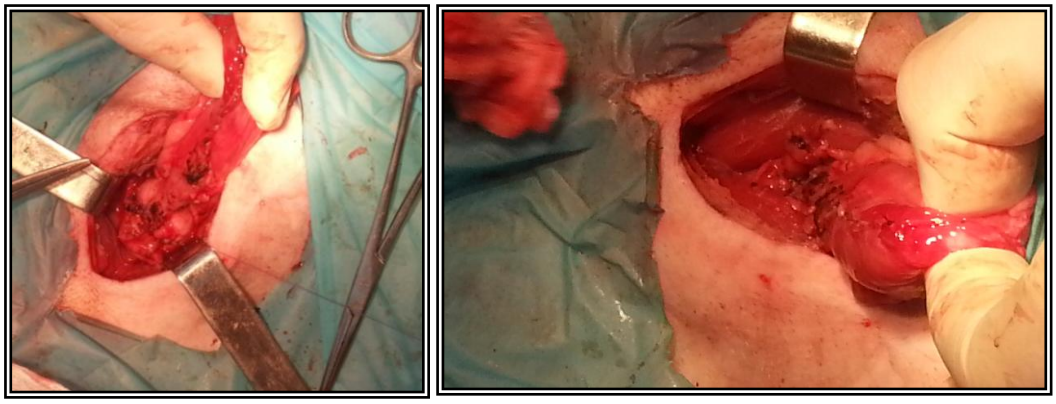

Fig. 10. Suture of the prostatic capsule (left).
Prostatic lodge after closing the prostatic parenchyma (right). (orig.)

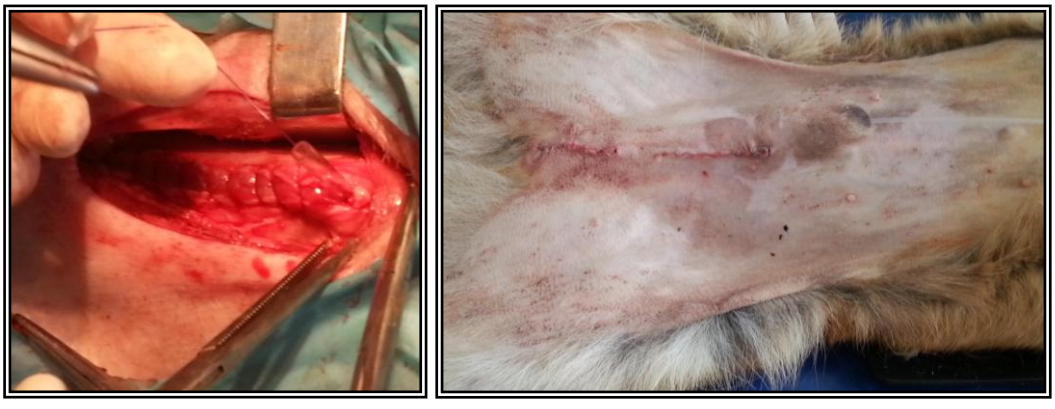

Fig. 11. The drainage tube placed to collect all the residual urine from the prostatic lodge (left). **Final image with the patient after the surgery** (right) (orig.)

The drainage tube was removed after 5 days and the urethral catheter was maintained for 10 days.

We have used Ceftriaxone for the intravenous treatment, 25 mg/kg every 12 hours, for 3 days, then we continued with oral administration for another 7 days, raising the total daily dose to 35 mg/kg.

Results and discussions

The general body temperature was normal in both cases, we have observed a slight increase, but within normal limits in the first 3 days.

The urinary continence was not affected as we conserved the striated sphincter of the urinary bladder (Seminal colliculus). No blood has been reported into the urine starting with the 5th day post surgery in both patients.

The defecation and the urination went form better to normal from day one to day 10 post surgery in both cases.

Both patients became independent and autonomous 10-12 days after surgery.

After 25 days we have observed the shrinkage of the prostate lodge at ultrasound check in both cases.

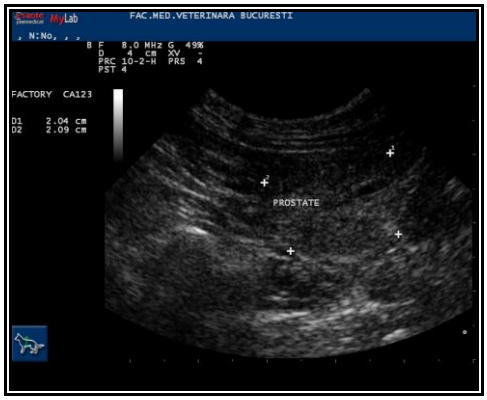

Fig. 12. Ultrasound exam, first case, shows prostate parenchyma homogeneously, slightly hyperechoic, 2,04/2,09 cm diameter (orig.).

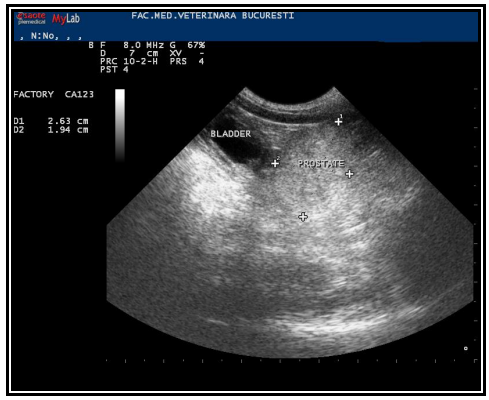

Fig. 13. Ultrasound exam, first case, shows prostate parenchyma homogeneously, slightly hyperechoic, 2,63/1,94 cm diameter (orig.).

Conclusions

Subtotal prostatectomy is the elective surgery in valuable breeding dogs for benign prostatic hyperplasia in lieu of castration and in stable dogs with abscessation or cysts in lieu of drainage procedures.

In dogs with bigger benign prostatic hyperplasia, it is easier to perform the subtotal prostatectomy due to the migration of the prostate to the abdominal cavity.

The spray coagulation mode combined with the VIO's BiClamp mode makes the operation easier to perform due to the lack of haemorrhage.

Maintaining the integrity of the Seminal colliculus and not reaching the striate sphincter of the urinary bladder leads to success in preserving the urinary continence.

A drainage tube placed to collect all the residual urine from the prostatic lodge, before closing the prostatic lodge and restoring all the anatomic layers, helps in maintaining the normal body temperature.

The defecation and the urination went form better to normal from day one to day 10 post surgery in both cases.

After 25 days we have observed the shrinkage of the prostate lodge at ultrasound check in both cases.

Acknowledgements

The author would like to thank the staff from the Hospital of Urology Professor Theodor Burghele (Dr. Ion Dragomirişteanu) for the help in performing and adapting the techniques from human medicine to dogs.

We are also grateful to Dr. Ana Doicescu from ELMED medical for providing the ERBE System.

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COMPARATIVE EVALUATION ON THE EFFICIENCY AND TOLERABILITY OF THREE NSAIDs USED IN LOCOMOTIVE OSTEOARTICULAR INFLAMMATIONS IN DOG

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) are efficient substances used in controlling pain and inflammation, especially of osteoarticular kind, but, alongside the health effects, they concurrently carry the risk of inducing side effects. The primary purpose of this study isto comparatively evaluate and quantize the anti-inflammatory and analgesic efficiency, alongside the tolerance, of three different-classes NSAIDs , frequently used in current veterinary practice, in decreasing and fighting osteoarticular inflammations of house pets. Studies were conducted on a total number of 30 dogs, 21 males and 9 females of different breeds and which were clinically and paraclinically diagnosed with articular inflammation. The patients were randomly grouped in three batches and administered per os 2.5 mg/kg b.w./ day of carprofen, 2mg/kg b.w./day of ketoprofen and 0.3 mg/kg b.w./day of meloxicam, in keeping with the therapeutic protocol specified by the producer. During the study, the idiosyncratic pain of the patients was clinically evaluated by appreciating the articular mobility, the hypalgesic effect of the considerate NSAIDs was quantized and the patient's general state and possible side effects were monitored. The results revealed a high anti-inflammatory and analgesic therapeutic effect of the substances in matter, alongside a significantly different tolerability.

***Keywords**: osteoarticular inflammation, carprofen, ketoprofen, meloxicam, efficiency, tolerability*

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) continue to occupy an important place in current therapy, being one of the most numerous groups of medicamentary substances used, as well as one of the oldest. They have the quality of diminishing the signs and symptoms of an inflammation, without discarding the initial causes (Nastasa *et al.*, 2010; Plumb, 2008). Their place in veterinary medicine is mostly related to the muscular-osteoarticular impairment, highly encountered in dogs. However, using NSAIDs on a large scale in acute or chronic affections is limited by the side effects (on the gastro-intestinal, renal, cardiac, hepatic departments). This proportional limited use in veterinary medicine, compared to human medicine, could be explained through the different pharmacokinetic operational factors in various animal species, therefore the possibility of limited therapeutic effect or of the appearance of an excessive toxicity.

The prescription of a NSAIDs treatment must take into account a series of precautions (Bonagura *et al.*, 2009; Boothe, 2001): the adjunction of NSAIDs is not allowed because the anti-inflammatory effects will not increase, only potentiate the side effects; the adjunction with steroids is recommended in severe forms of inflammation; prescribing an efficient dose (as recommended by the producing company) and further adjusting it according to the inflammation and pain intensity.

Choosing of the NSAIDs is made according to the anti-inflammatory and analgesic effect; also keeping in mind the pathology we are addressing (Goodman and Gilman's, 2006). Furthermore, we have to consider that small doses act like strong analgesics, while large doses have a powerful anti-inflammatory effect.

Materials and method

Experimental design

From the registered cases of two private veterinary offices, as well as from the Medical Clinic within the Faculty of Veterinary Medicine Iasi, we took into study a total of 30 dogs of different breeds and ages: 21 males and 9 females, ages between 3 - 15 and weight between 6 – 37 kg (± 0,5kg) which were in need of non-steroidal anti-inflammatory therapy.

After a thorough and individual clinic examination, as well as paraclinic tests, performed when presented for consultation, several osteoarticular inflammations processes were found. In the grouping of the animals a series of criteria was mandatory for the inclusion or exclusion in/from the study: dogs with limps, intense pain during movement, low paravertebral tone, sensitiveness to pain when applying pressure, muscular rigidity and impairment were included, this phenomena being accompanied by general state disorder (such as apathy, dormancy, refusing to move).

Were excluded from the study patients which have had recent operations, limb or spine fractures, and digestive, hepatic, renal, cardiac disorders, females in gestation and any other disease that could have interfered with the safety of the evaluation and the efficiency of the therapy.

The dogs were randomly grouped in 3 batches – "C", "K" and "M" - 10 dogs/batch, each batch having administrated a different substance during a 21 day period, in keeping with the dose recommended by the producer. The "C" group was given carprofen - 2,5 mg/kg b.w./day, the "K" group was given ketoprofen - 2mg/kg b.w./day and the "M" group was given meloxicam – 0.3 mg/kg b.w./day. Substances, under the form of troche, were administered per os, in the morning, only after the dogs were fed. Because therapy was in progress for a longer period of time, the direct implication of the owners was needed in providing the dogs with the medicaments, prior to a briefing.

The owners accepted the terms after the disclosure of the risks of a long term NSAIDs therapy and of the possible side effects. Concurrently, they were informed about the experiment's stages, the importance of permanently keeping an eye on the patient and the necessity of following the prescription exactly.

Monitoring clinical aspects

Evaluation of the therapeutic efficiency of the considered NSAIDs was appreciated on a numeric scale of four points, respective 0, 1, 2, 3, where 0 = the absence of pain, 1 = moderate pain, 2 = increased pain, alongside general state modification, 3 = intense pain, immobilized patient and general state modification. Therefore, following the criteria described by Pinals in 1981 (citat de Stephen *et al*., 2005), referring to the remission of the inflammatory process, we compared the therapeutic activity of the NSAIDs in matter with the help of a visual-analogic scale, a current measure used in human rheumatology, evaluating the inflammation and pain intensity.

Parameters taken into consideration were: inflammation, pain intensity, local hyperemia, eventual modifications and deformations, mobility and functional depreciation (limitation), as well as the patient's general state modifications.

Evaluation of the NSAIDs taken into consideration was conducted through daily general examination of the patient by the owners and by the clinician on day 5 – 10 – 15 – 21. By monitoring the appearance of side effects, a tolerability degree of the NSAIDs was assessed, recorded information pointing to symptoms with gastro-intestinal localization,

gastric sensitiveness to pain shown while palpation on the abdominal area, queasiness, diarrhea, inappetence, weight loss or to the evolution of other symptoms, such as modified clotting time (high hemorrhage risk), hypersensibility reaction (allergy), edema, general pain (diffuse), sleeping disorder, physiologic parameters modifications, renal and hepatic disorders, metabolism disorder.

At the beginning and end of the trial, feces samples were collected for an early detection of blood, encountered in a long term therapy (more than 7-10 days) with NSAIDs. Hemoccult test was used in the detection of hemoglobin and hemoglobin-haptoglobin complex. This test uses filter paper with ingrained guaiac, a reagent that, in case of a positive probe, turns the paper's color into blue. The analysis tracks traces of blood (blood coming, usually, from the gastro-intestinal segment) in feces. If the test turns out to be positive, it means that a pathologic quantity of blood reaches the feces. Samples were individually collected in anhydrous plastic recipients, with a volume of 2–3 cm^3 (1–2 spoons). Involvement of urine or any other biological materials was averted.

The quantification of occult hemorrhages represents a very important screening test in determining digestive hemorrhage which may occur in intoxications with diverse substances, after NSAIDs therapy or after a digestive disorder; this test is used in human science in detecting colorectal cancer.

Results

The efficiency of carprofen, ketoprofen and meloxicam treatment was analyzed based on the clinical aspects registered during the study period.

It is considered that patients responded favorable to these three NSAIDs treatment if pain and inflammation ameliorated, alongside the remission of the registered parameters and the recovery of the locomotion function.

Evaluation of the patients and the data registered, proving the anti-inflammatory and analgesic effects of carprofen, ketoprofen and meloxicam, showed considerable improvement with each and every 5 – 10 – 15 – 21 day of clinical examination (figure 1). Total remission of painful phenomena and inflammation process associated with osteoarticular disorders was present at the end of the 21-days treatment in over 92.8 % of the cases (n = 28), subjects going on with their daily activities: running, jumping, climbing up and down the stairs etc.

Thus it has been observed that pain after pressure – physical pressure on the affected area, as well as intervening pain associated with active dynamics: moving, climbing up and down or with passive dynamics, which were obvious at start, reaching a level 3 on the pain scale, an intense pain, started to diminish step by step, as seen after a 7 to 10 days of treatment. Same NSAIDs benefic result, diminishing of pain, was highlighted in paravertebral tone – very high (tensed up), limited function – manifested through the immobilization of the animal in the same position (dogs were embracing a calming position, usually lateral decubitus), inflexibility at awakening – that determined a difficult circulation, dogs even refused movement, all of which were of great intensity from the beginning.

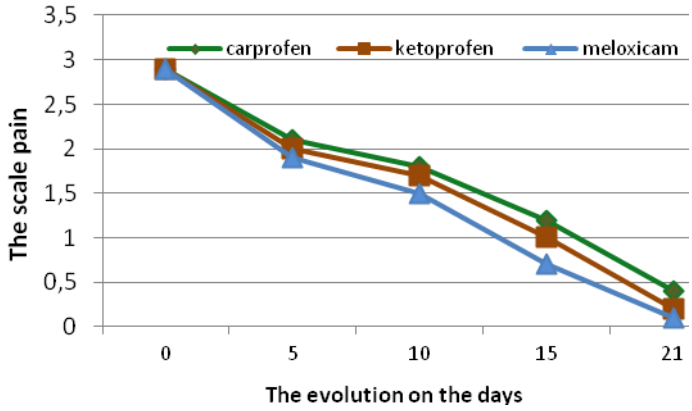

Fig. 1. Evolution of the analgesic and anti-inflammatory efficiency (pain points/days) during carprofen, ketoprofen and meloxicam therapy

Between the three substances – carprofen, ketoprofen and meloxicam – different grades of therapeutic activity were observed, of insignificant statistic aspect. During the trial, the highest factor in diminishing pain and inflammation was observed in meloxicam (90 %), followed by ketoprofen (75 %) and carprofen (68 %) (figure 2).

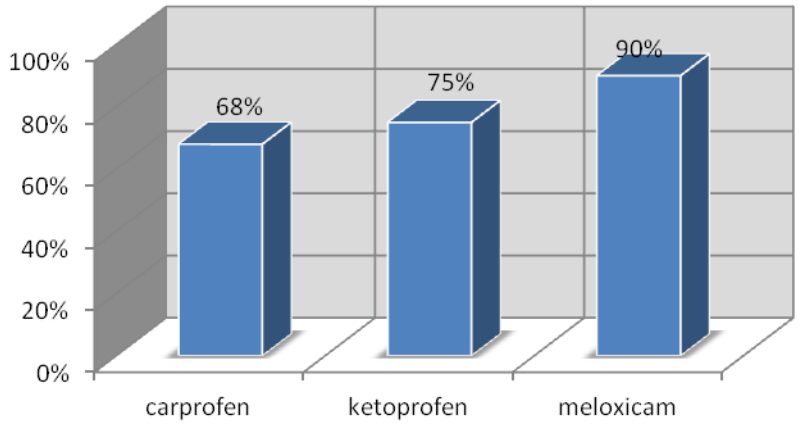

Fig. 2. Therapeutic efficiency of the substances taken into consideration

Regarding the *tolerability* of carprofen, ketoprofen and meloxicam, clinicians appreciated it as being *decent* in 30% (n = 9) of the cases, meaning that approximately 70% (n = 21) of the patients indicated side effects. It has been kept a check especially on digestive tolerability, being well known that NSAIDs are responsible of inducing canker and digestive hemorrhages, both in humans and in animals, shown forth by: misbehavior, loss of appetite,

queasiness, diarrhea or bloody queasiness and diarrhea, referred to high sensitiveness at abdomen palpation (in the stomach region).

Better most tolerability was observed in meloxicam treated patients, where a small number of dogs showed signs of digestive side effects, followed by ketoprofen, then carprofen. The reason is that it is a selectiveCOX-2 inhibitor with fewer digestive side effects, because it inhibits prostaglandins arrived at the inflammatory spot, in comparison to carprofen, a selective COX-1 inhibitor and to ketoprofen, a non-selective anti-inflammatory.

Side effects such as misbehavior, bloody queasiness and diarrhea have erratic come into sight after 6-7 days of therapy in every batch taken into consideration and continued to appear throughout the whole treatment period. The highest rate of incidence was manifested in the "C" group whom were treated with carprofen, reporting 7 out of 10 cases. Same effects, only on some of the subjects, were observed in the "K" group, whom were given ketoprofen, reporting 5 out of 10 cases, followed by the meloxicam treated "M" group, reporting only 3 cases (table 1). Patients were given needed treatment for these specific symptoms (digestive protectors, anti-diarrheal, anti-nausea treatment), supposing a higher treatment cost. The goal was to prevent the NSAIDs treatment arrest, eliminating the risk of an exacerbated osteoarticular inflammation which usually appears after a sudden NSAIDs treatment suspension.

Table 1. Therapeutic tolerability of substances taken into study

Tolerability	carprofen	meloxicam	ketoprofen
	Cases/animals (batch percentage)		
weak (side effects: apathy,innapetence, nausea, diarrhea)	70 %	30 %	50%
good (without considerable changes in the general state of the patients)	30 %	70 %	50%

Meloxicam has proven to be best tolerated by dogs, the rate of side effects and digestive disorders being diminished up against the ketoprofen treated group and considerably up against the carprofen treated patients. However, all patents suffered from digestive disorders determined by usage of these three substances, of different intensity levels. This determined us to ascertain that the tolerability of these substances – carprofen, meloxicam and ketoprofen ranges from moderate to weak.

The Hemoccult test consisted in placing 2 drops of reagent over a paper filter imbued with guaiac, onto which the feces probe was placed. Blue coloring of the paper indicates the presence of blood (the test having a distinctiveness of 80 – 85%). Normally, the amount of blood passing through the gastro-intestinal tract is insignificant and does not mismatch the test.

Results from the Hemoccult test performed before starting treatment were negative.

Probes gathered at the end of the treatment showed the lack of blood in feces in 12 patients (negative test results) and positive test results in 18 dogs, as deduced by the blue coloration of the filter paper imbued with guaiac, hence the digestive microhemorrhages (the blue stripe validated the test results).

Highest rate of blood in feces was shown in carprofen treated group (group "C"), followed by the ketoprofen treated group and the meloxicam treated one (table 2).

Table 2. Microhemorrhages rate as shown in the study batches

Total number of dogs	Negative probes	Positive probes
30	12 cases (36 %)	18 cases (54 %)
Rate in dog groups		
carprofen	Meloxicam	ketoprofen
8 cases (80 %)	4 cases (40%)	6 cases (60%)

Thus, the test revealed digestive lesions caused by the non-steroidal anti-inflammatory treatment, especially when the duration exceeds 7 – 10 days.

Discussion

Treatment of acute or chronic inflammation, as well as pain caused by it, in patients suffering from osteoarticular problems supposes, according to authors such as Robbins and Cortran, 2004, taking into consideration some rules, factors and general and particular conditions: a detailed anamnesis regarding the admission and manifestation of the inflammatory processes, the necessity of a radiologic examination, the necessity of an adequate pharmacotherapy, readjustment of the posology with careful attention over the side effects, close ups with the patients during this whole period. Inflammatory pain is mostly susceptible to NSAIDs. Several studies have proven the efficiency of this pharmaceutical class, both in pain relieve in muscular-osteo-articular inflammations (Goodman and Gilman's, 2006) and in controlling post operatory pain in dogs and cats (Grisneaux *et al*., 2003).

In our study we kept track of NSAIDs effects on the osteoarticular affections and, after accumulating data from the patient's owners and from our personal studies, usage of carprofen, ketoprofen and meloxicam has proven to have a very good analgesic and anti-inflammatory quality and to be a very efficient treatment in inflammations. Benefic results of the administrations were shown even from the first evaluation (after 5-7 days of treatment). Improvement of locomotive function and general state of the dogs could be seen, as well as a high rate of remission of the symptoms, pain and inflammation, progressively, with each day of treatment, observed and described phenomena by many experts in their studies (Santana, 2001; Ostensen *et al*., 2004).

Evaluation of the efficiency and tolerability of the three substances taken into study – carprofen, ketoprofen and meloxicam – has led to a generalization of the obtained data and to the observation of both the different degrees of improvement of the physical state of the treated animals and the side effect apparitions.

Meloxicam turned out to have the greatest anti-inflammatory efficiency, better that ketoprofen and carprofen, based on its rapid action in diminishing inflammatory processes and on its powerful influence on limiting pain, registered fact in other comparative studies that showed meloxicam as acting quickly on inflammatory processes in dogs and cats, in comparison to other substances from the NSAIDs class: carprofen, etodolac, ketoprofen or piroxicam (Craven *et al*., 2007; Slingsby *et al.*, 2000; Deneuche *et al.*, 2008). Furthermore, in 2005, Stephen *et al.*, noticed the fact that animals treated with meloxicam needed a shorter amount of time for pain remission to kick in, in comparison to dogs treated with other non-steroidal anti-inflammatory drugs, keeping in mind that pain from the spine level could be limited by the animal itself by adopting a less painful position.

Significant results of the anti-inflammatory quality of ketoprofenvis- à-vis that of carprofen were recorded by Grisneaux *et al.*, 2003, in their comparative study on controlling postoperation pain, demonstrating a higher and quicker effect of ketoprofen, than ofcarprofen. The anti-inflammatory activity of carprofen on different osteoarticular inflammations in dogs was described in some studies as being very agreeable (Fox *et al*., 2006), though comparative studies on the side effects of NSAIDs show carprofen as having a weaker action than that of other same class substances (Luna *et al*., 2007), in keeping with the fabrication protocols regarding doses.

Although recorded results in the usage of non-steroidal anti-inflammatory display benefic results, risks of side effects cannot be ignored. Administrating to animals (especially carnivores) efficient doses is still accompanied by multiple and significant failings because of the side effects that come into sight as reversible lesions, or even irreversible ones, in some vital organs, causing death. By studying carprofen, ketoprofen and meloxicam on a number of 30 dogs, tolerability appears to be reduced, with approximately 70% of the patients showing signs of digestive disorder, thus revealing a high rate of side effects appearing after anti-inflammatory use. NSAIDs activity was followed by nausea and alternate diarrhea, accompanied by general state alterations, thus needing to be added specific medication for patients with clinical symptoms, therefore raising the therapy costs for the owners. Meloxicam had the best tolerability, 70% of dogs from that group getting along with the treatment, followed by ketoprofenand, with a lower rate, carprofen. As demonstrated by several clinical studies, gastro-intestinal side effects rate, in keeping with the recommended doses, were lower when using meloxicam, rather than other standard anti-inflammatories (Craven *et al*., 2007; Luna *et al*., 2007). Explanation relies in the fact that meloxicam in a selective NSAID, mostly inhibiting COX-2 – an inductive enzyme, present in a large quantity in inflammatory focuses and least COX-1 – a constituent enzyme.

Ketoprofen, known as an unspecific COX inhibitor and carprofen, known as a COX-1 inhibitor, are used with caution in dog treatment because of their less secured specific COX-2 inhibition. Existent proof reveals that after inhibiting COX-2, therapeutic effects of the NSAIDs are maintained while the inhibition of COX-1 is responsible of renal and gastro-intestinal side effects (Dubois *et al*., 2004; Grecu *et al.*, 2007). Other studies highlighted that using ketoprofen and carprofen more than 5 days led to digestive tract, renal and thrombocytes disorders (Raekallio *et al*., 2006). Following that line of investigation, it is fair to suppose that reducing the dosage, in muscular and osteoarticular inflammations, improves the product's safety, gainsaid by some authors who took into consideration that reducing the dose (as recommended by the producer) will be closely followed by a low anti-inflammatory effect (Laine, 2006).

By reason that we could not perform an endoscopy (lack of an endoscope) needed to track disorders at a digestive level, as a result of non-steroidal anti-inflammatories, we appealed to identifying occult hemorrhaging by examining feces probes. For this purpose we used the Hemoccult test, blood being detected in 18 out of 30 cases, revealing a pretty high rate of digestive lesions produced by the NSAIDs taken into study. Older or actual consequent studies following side effects after NSAIDs therapy in dogs showed blood presence in feces in all patients, as well as severe gastric ulceration while performing endoscopy (Gibbons *et al.,* 2006).

Reckoning these major disadvantages on NSAIDs usage, a question remains: *is it necessary to have a new safety level in NSAIDs therapy for patients presenting pain caused by different inflammatory processes, awakening doctor's concerns about gastro-intestinal bleeding risks?*

This are reasons why, on a global scale, a rising interest in research of side effects of anti-inflammatory therapy, with a large and diverse spectrum is conducted, as well as finding new solutions to fix this problem.

Conclusions

Comparative studies between carprofen, meloxicam and ketoprofen revealed a higher efficiency and tolerability in meloxicam, remarked by its quick anti-inflammatory-analgesic activity debut and lower percentage of side effects within the groups.

As clinical advantages we remarked the rapid anti-inflammatory – analgesic activity debut of carprofen, meloxicam and ketoprofen, good therapeutic efficiency, shadowed by secondary side effects of the NSAIDs on the organism.

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VARIATIONS ON CYATHOSTOMINS POPULATION AMONG HORSES FROM DIFFERENT PARTS OF IAŞI AND NEAMŢ COUNTIES

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Abstract

From gastrointestinal parasitoses of horses, cyathostomosis (trichonemosis) is considered to be the most common, there is a high diversity of nematode species involved. The biodiversity was studied to identify the parasites species in three groups of horses from Iaşi and Neamţ county. Two groups were formed from equine growth in semi-intensive system, and one consisted from horses growth in extensive system. Following coproparasitological analysis we identified five species of cyathostomins: Cylicostephanus longibursatus (Trichonema longibursatum) Cylicostephanus calicatus, Cyathostomum catinatum, Cyathostomum pateratum and Coronocyclus labiatus. The increased prevalence was found for Cylicostephanus longibursatus species in horses of all three groups. Species of Cylicostephanus calicatus and Cyathostomum catinatum had a medium extensivity in both systems, the species Cylicostephanus calicatus being slightly higher in horses from extensive system. A lower prevalence was obtained for Cyathostomum pateratum and Coronocyclus labiatus species, the latter being most common in semi-intensive system. It was found as predominant species of the genus Cylicostephanus.

Key words: cyathostomins, identification, species, prevalence

Introduction

Digestive tract of horses is often the seat of many parasitic diseases, of which cyathostomosis occupies a proeminent place. The clinical importance of this parasitosis derives mainly from its wide spread and is seen in horses worldwide, virtual, and can be considered that 100% of horses are infested with cyathostomin helminths (Lyons E.T. et al., 1999).

The etiologic agents, called cyatostomins or "small strongyls" are a large group of parasitic species, so the horse parasites population diversity of an area can be increased. Cyathostomosis is caused by nematodes from Family *Strongylidae*, Subfamily *Cyathostominae*, being known so far 19 genera and 64 species (Lichtenfels J.R., 2008). Worldwide is considered that cyathostomins are the most common parasites affecting horses and they are also the main pathogens of horses, due to decrease of infestation prevalence with large strongyls following of measures taken, and to emergence of resistant cyathostomin populations to various anthelmintic substances (Traversa D. et al., 2010).

The presence of cyathostomins is associated with various forms of cramps (Murphy D., 1997; Mair T.S. et al., 2000), reduced performances, strengthening of hair and debility (Uhlinger C.A., 1991). The simultaneous reactivation of encysted larvae in the intestinal wall has clinical importance and is leading to the onset of clinical syndrome called "larvae cyathostomosis", consisting of a severe inflammatory enteropathy localized in cecum and colon. This disease is a colitis characterized by weight loss, severe diarrhea, decrease of protein, subcutaneous edema, and a mortality rate which can reach 50%, even if establish an antihelmintic therapy (Giles C.J. et al., 1985; Eysker M. et al., 1989, 1990; Love S. et al., 1997).

In order to develop effective measures to control cyathostomin infestations in horses, numerous studies have been undertaken both international and national, being analyzed cyathostomin population of equines from various areas. In this study was aimed at identifying small strongyls species in horses from Iaşi and Neamţ County area.

Materials and methods

Study of cyathostomin populations was achieved by coproparasitological examinations on faecal samples of horses from extensive and semi-intensive systems. The horses from extensive system, belonged to diverse populations from different commons of Iassy County, being examined a total number of 29 animals, aged between 9 months and 26 years (*tab. 1*). The horses from semi-intensive system came from Equestrian Base Iassy, a number of 6 copies aged betwen 2 to 16.5 years, and the remaining 24 were from the Stallions Deposit Dumbrava, Neamt County, aged between 3 and 20 years (*tab. 2*).

From each animal were collected two faecal samples per day, freshly removed (about 150 g each), in morning and evening, for three consecutive days, after antihelmintic treatment. Identification of visible macroscopic helminths from faecal samples was done by collection of samples, followed by fixation in 70% alcohol solution, clarification by including in lactic acid and subsequent microscopic examination of morphological characters.

The research was conducted during 2010 and 2011; tests and analyzes were conducted in the laboratory of parasitology at the Faculty of Veterinary Medicine Iassy.

Table 1. Data on horses from the extensive system

Crt. No.	Breed	Age	Sex	Growth mode	Provenance
1	Metis	13 years	♂	Loose housing and grazing	Grajduri commune, Iassy county
2	Metis	6 years	♂	Loose housing and grazing	Grajduri commune, Iassy county
3	Metis	7.5 years	♂	Loose housing and grazing	Grajduri commune, Iassy county
4	Metis	7 years	♂	Loose housing and grazing	Grajduri commune, Iassy county
5	Metis	4 years	♂	Loose housing and grazing	Grajduri commune, Iassy county
6	Metis	8.5 years	♀	Loose housing and grazing	Grajduri commune, Iassy county
7	Metis	9 months	♀	Loose housing and grazing	Grajduri commune, Iassy county
8	Metis	3 years	♀	Loose housing and grazing	Grajduri commune, Iassy county
9	Metis	19 years	♀	Loose housing and grazing	Grajduri commune, Iassy county
10	Metis	8.5 years	♀	Loose housing and grazing	Grajduri commune, Iassy county
11	Metis	20 years	♂	Loose housing and grazing	Popești commune, Iassy county
12	Metis	15 years	♂	Loose housing and grazing	Popești commune, Iassy county
13	Metis	13.5 years	♂	Loose housing and grazing	Popești commune, Iassy county
14	Metis	10 months	♂	Loose housing and grazing	Popești commune, Iassy county
15	Metis	3 years	♂	Loose housing and grazing	Popești commune, Iassy county
16	Metis	5 years	♂	Loose housing and grazing	Popești commune, Iassy county
17	Metis	4.5 years	♂	Loose housing and grazing	Popești commune, Iassy county
18	Metis	7 years	♂	Loose housing and grazing	Sinești commune, Iassy county

19	Metis	10 years	♀	Loose housing and grazing	Sineşti commune, Iassy county
20	Metis	13 years	♀	Loose housing and grazing	Sineşti commune, Iassy county
21	Metis	17 years	♂	Loose housing and grazing	Sineşti commune, Iassy county
22	Metis	9 months	♂	Loose housing and grazing	Sineşti commune, Iassy county
23	Metis	2.5 years	♀	Loose housing and grazing	Sineşti commune, Iassy county
24	Metis	8 years	♀	Loose housing and grazing	Miroslava commune, Iassy county
25	Metis	1 years	♂	Loose housing and grazing	Miroslava commune, Iassy county
26	Metis	25 years	♀	Loose housing and grazing	Miroslava commune, Iassy county
27	Metis	26 years	♂	Loose housing and grazing	Miroslava commune, Iassy county
28	Metis	8 years	♂	Loose housing and grazing	Miroslava commune, Iassy county
29	Metis	6 years	♂	Loose housing and grazing	Miroslava commune, Iassy county

Table 2. Data on horses from the semi-intensive system

Crt. No	**Breed**	**Age, years**	**Sex**	**Growth Mode**	**Provenance**
1	Frisian	4.5	♂	Loose housing	Equestrian Base, Iassy
2	Frisian	4	♂	Loose housing	Equestrian Base, Iassy
3	Sport roumanian horse	2	♂	Loose housing	Equestrian Base, Iassy
4	Sport roumanian horse	18	♂	Loose housing	Equestrian Base, Iassy
5	English thoroughbred	16.5	♂	Loose housing	Equestrian Base, Iassy
6	Metis	5	♀	Loose housing	Equestrian Base, Iassy
7	Semihard	10	♂	Loose housing	Stallions deposit - Dumbrava, Neamt
8	Semihard	10	♂	Loose housing	Stallions deposit - Dumbrava, Neamt
9	Semihard	6	♂	Loose housing	Stallions deposit - Dumbrava, Neamt
10	Semihard	8	♂	Loose housing	Stallions deposit - Dumbrava, Neamt
11	Semihard	3	♂	Loose housing	Stallions deposit - Dumbrava, Neamt
12	Semihard	3	♂	Loose housing	Stallions deposit - Dumbrava, Neamt
13	Sport roumanian horse	8	♂	Loose housing	Stallions deposit - Dumbrava, Neamt

14	Sport roumanian horse	5	♂	Loose housing	Stallions deposit - Dumbrava, Neamt
15	Sport roumanian horse	9	♂	Loose housing	Stallions deposit - Dumbrava, Neamt
16	Sport roumanian horse	8	♂	Loose housing	Stallions deposit - Dumbrava, Neamt
17	Sport roumanian horse	13	♂	Loose housing	Stallions deposit - Dumbrava, Neamt
18	Sport roumanian horse	15	♂	Loose housing	Stallions deposit - Dumbrava, Neamt
19	Lipițan	20	♂	Loose housing	Stallions deposit - Dumbrava, Neamt
20	Lipițan	10	♂	Loose housing	Stallions deposit - Dumbrava, Neamt
21	Lipițan	10	♂	Loose housing	Stallions deposit - Dumbrava, Neamt
22	Lipițan	7	♂	Loose housing	Stallions deposit - Dumbrava, Neamt
23	Lipițan	4	♂	Loose housing	Stallions deposit - Dumbrava, Neamt
24	Lipițan	6	♂	Loose housing	Stallions deposit - Dumbrava, Neamt
25	Lipițan	9	♂	Loose housing	Stallions deposit - Dumbrava, Neamt
26	Lipițan	10	♂	Loose housing	Stallions deposit - Dumbrava, Neamt
27	Lipițan	10	♂	Loose housing	Stallions deposit - Dumbrava, Neamt
28	Lipițan	18	♂	Loose housing	Stallions deposit - Dumbrava, Neamt
29	Bucovina Horse	3	♂	Loose housing	Stallions deposit - Dumbrava, Neamt
30	Bucovina Horse	3	♂	Loose housing	Stallions deposit - Dumbrava, Neamt

Results and discussions

Research conducted, leading to the identification of the following species of cyathostomins:

- *Cylicostephanus longibursatus (Trichonema longibursatum)*, most prevalent - 92.5%;
- *Cylicostephanus calicatus* - 52%;
- *Cyathostomum catinatum* - 44 %;
- *Cyathostomum pateratum* - 32%
- *Coronocyclus labiatus* – 27%.

The proportion of these species has undergone some variations depending on the horse populations examined. The species present were those of *G. Cylicostephanus*,

especially species of *Cylicostephanus longibursatus (Trichonema longibursatum*) was found in a percentage of 92.5% of horses examined (*fig. 1*).

On horses from semi-intensive system belonging of Equestrian Base Iassy, we found the presence of the following species: *Cylicostephanus longibursatus* (100%), *Cylicostephanus calicatus* (50%), *Cyathostomum pateratum* (33%), *Coronocyclus labiatus* (33%) (*fig. 2*).

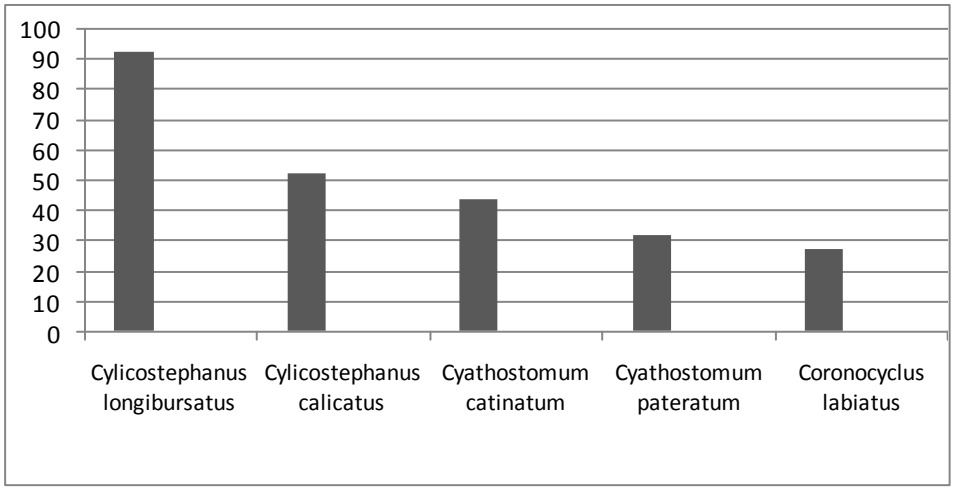

Fig. 1. Prevalence of cyathostomins species identified

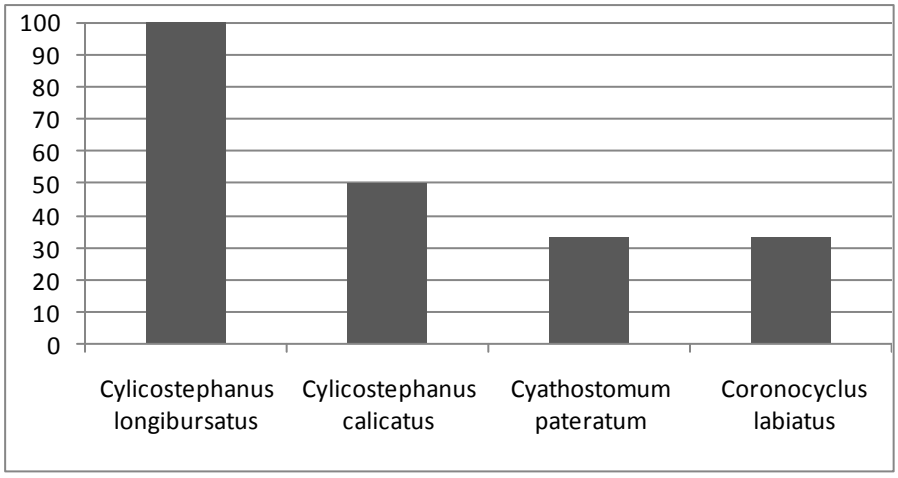

Fig. 2. Prevalence of cyathostomins species identified in horses from Equestrian Base Iassy

Species found in the Stallions Deposit Dumbrava Neamt, were represented by all 5 identified the following proportion: *Cylicostephanus longibursatus* (87.5%), *Cylicostephanus calicatus* (45%), *Cyathostomum catinatum* (45%), *Cyathostomum pateratum* (25%), *Coronocylcus labiatus* (41.5%) (*fig. 3*).

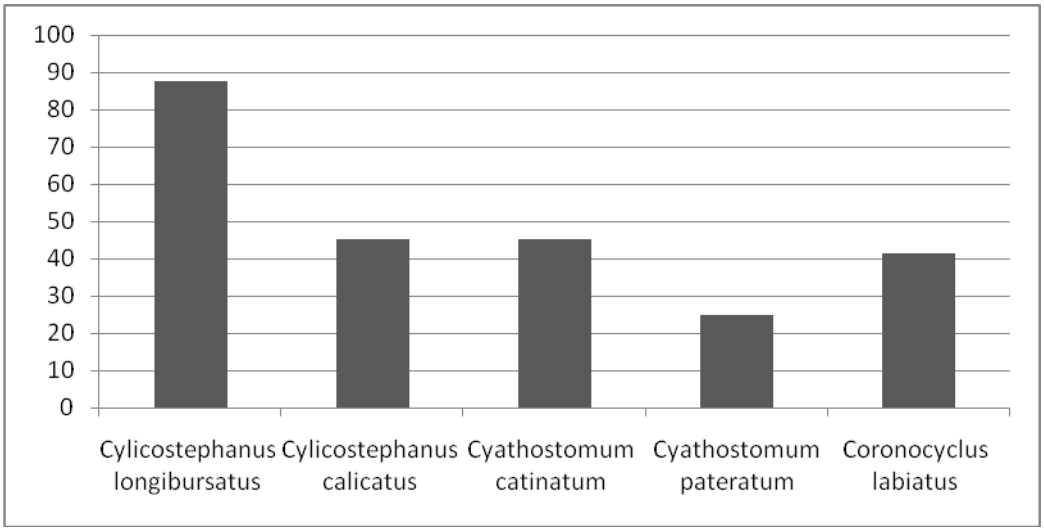

Fig. 3. Prevalence of cyathostomins species identified on horses from Stallions Deposit, Dumbrava, Neamt

Also, in examined horses belonging to the population from commons of Iassy County, we met all 5 species as follows: *Cylicostephanus longibursatus* (89.5%), *Cylicostephanus calicatus* (59%), *Cyathostomum catinatum* (52%), *Cyathostomum pateratum* (35%), *Coronocyclus labiatus* (13.5%) (*fig. 4*).

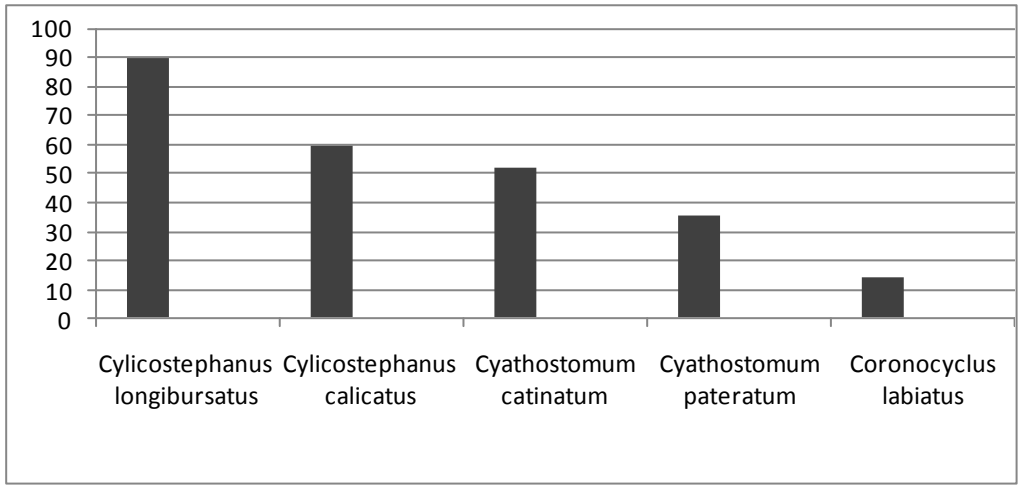

Fig. 4. Prevalence of cyathostomins species identified in horses from different communes of Iassy county

Conclusions

1. In populations of horses from these areas, the predominant cyathostomins species belong to three genera: *Cylicostephanus, Cyathostomum* and *Coronocyclus*, of which *Cylicostephanus* gender is most present.
2. Prevalence values of different species identified, were generally close to the horses of the 3 groups studied, with few significant variations.
3. The most common species is *Cylicostephanus longibursatus*, whose prevalence ranged between 87.5% and 100%, with an average of 92.5%.
4. With a similar and medium prevalence have been identified species of *Cylicostephanus calicatus* and *Cyathostomum catinatum* in horses from semi-intensive system, while in horses from extensive system was increased prevalence of *Cyathostomum calicatus* species.
5. With a low prevalence were present *Cyathostomum pateratum* species, more frequent in extensive system and *Coronocyclus labiatus* more frequent in semi-intensive system.

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THE BACTERIAL FLORA OF THE UTERUS ACCORDING TO PARTURITION TYPE IN COWS

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Abstract

The research has followed puerperal uterine bacteriology on a herd of 40 Romanian Black-Spotted breed cows of which 28 multiparous and 12 primiparous that were housed in a dairy farm located in North-Eastern Romania. The investigations were performed in the first 3 weeks after calving, both in cows with normal parturition and dystocia. The samples have been taken with sterile swabs, using the vaginal speculum in the 7th, 14th, and 21st days after calving. By the parturition type, from cows with normal parturition (in number of 28) were isolated 194 bacterial strains, while from cows with dystocia (in number of 12) were isolated 88 bacterial strains.

Key words: *bacterial strains, parturition, uterus*

Introduction

The puerperal uterine bacteriology has a special importance both in the rate and quality of uterine involution, and in the further evolution of reproductive processes as well. The quantity and quality of bacterial colonization in puerperal uterus depend on the normal or pathologic evolution of genital segments in this period. Also, the bacteriology of puerperal uterus is a very important element in the prevention of post-partum genital infections (Runceanu et al., 2001). It has a main role in establishing preventive measures, as it is known that cattle have higher natural predisposition to uterine diseases than other domestic species (Sheldon IM 2004 Sheldon IM, Dobson H. 2004). The same authors assert that after calving, the uterus is contaminated in over 90% of the cases with a variety of bacterial species both Gram-positive and Gram-negative. This contamination will occur without depending on the hygienic level in the farm (Noakes et al, 1991) and will maintain at least 2-3 weeks after calving (LeBlanc S.J. et al., 2011).

Some authors showed that the bacterial burden in the uterus increases till the 9^{th} day after parturition, respectively till the 15^{th} day and after that it decreases. At 3-4 weeks post-partum usually there is a small number of germs, and sometimes the bacteria are absent (LeBlanc S.J. et al. 2011).

The number and quality of the bacterial colonization in the puerperal uterus depend on the normal or genital pathological evolution in this period. Thus, in healthy uterus, the number of the bacteria gradually decreases along with the post-partum days (Földi et al., 2006).

Many studies have showed that the persistence of certain bacterial types in the post-partum uterus, especially after 3 weeks, will constantly lead to uterine diseases. In example, the presence of *Arcanobacterium pyogenes* in the 21^{st} day after parturition is frequently associated with severe forms of endometritis (Azawi O.I., 2008), and the presence of *Escherichia coli* concomitant with *Arcanobacterium pyogenes* is associated with various uterine diseases (Földi et al., 2006).

Material and method

In order to make the bacteriological examination, some genital secretions were collected from the cervix.

Pathological materials were subjected to the microbiological examination at the Laboratory of Microbiology of the University Centre of Medical Veterinary Research within the Faculty of Veterinary Medicine Iaşi and at the Sanitary-Veterinary Laboratory of Iaşi County.

For isolation and identification of potentially pathogenic bacterial agents were used complex culture media but also selective and differential media.

Usual culture media for growing aerobic bacteria, used in the current bacteriological practice were represented by broth (liquid form) and nutrient agar (solid form), enriched with biological products (normal horse serum and 10% ram or horse blood). For anaerobic bacteria were used VF broth and agar (Liver Meat Glucose Cysteine Broth and agar), Tarozzi broth, Veillon agar, and broth with thioglicolat.

Differential culture media contain the substrate for a certain bacterial enzyme or cytotoxin and an indicator attesting the consumption of the substrate. Of this category was used the blood agar (which differentiate the bacteria that produce hemolysis from the ones that don't), and the lactose medium with various pH indicators.

The selective culture media that were used contain substances which inhibit the growth of contaminating bacteria, possibly collected during sampling.

The used selective media and differentials that were used, are:

- the EMB environment (Levine) with inhibitor agents such as eosin Y and blue-methylene, which inhibit Gram-positive bacteria;
- Mack Conkey environment – with inhibitor agents such as bile salts and crystal violet;
- Chapman environment - contains 7.5% NaCl and mannitol used for developing coagulase-positive *Staphylococcus spp.* from biological products;
- Agar-Columbia environment with and without blood;
- Agar-heart infusion-brain environment, for pretentious anaerobic and aerobic bacteria;
- Agar-bile-esculin environment – for isolation and identification of *Bacteroides* and *Fusobacterium* genera germs;
- Agar-bile-esculin-sodium azide environment, selective for isolation and identification of the *Enterococcus spp.*;
- Brucella environment –selective agar for anaerobic microorganisms;
- CCD environment - selective agar for identification of anaerobic microorganisms;
- SPS Agar environment (Perfringens Selective Agar) – used for identification of *Clostridium perfringens.*

To examine the biochemical characteristics there were used some culture media containing substrates to which it is necessary to investigate the enzymatic activity of potentially pathogenic bacteria. This media are:

- TSI (triple sugar iron agar) containing glucose, lactose and sucrose for the study of fermentation and $FeSO_4$ for detection of hydrogen sulphide production;
- MIU (mobility, indole, urease) containing phenol red test for determining indole urease test. A tape containing Ehrlich-Kovacs reagent needs to be attached on the surface of the environment for the indole test;
- MILF (mobility, indole, lyzindecarboxilaze, fenylalanindezaminaze);

- Simmons- medium containing citrate for the study of using citrate as the only carbon source; -Agar Christensen medium, containing urea and phenol red for detecting urease activity;
- oxidase test (detection of cytochrome oxidase in the respiratory chain) using the reagent - di (tetra) methyl-parafenilen-diamine (paper strips containing reagents).

Pathological material were subjected to laboratory investigations under general conduct of bacteriological diagnosis.

Results and discussion

The bacteriological investigation was performed during the year of 2012, within a farm located in the North East of Romania, on a herd of 40 cows of which 28 were multiparous cows and 12 were primiparous cows, in the first three weeks of puerperium, showing puerperal disorders in various stages of clinical evolution.

The examination of the biological samples from the observed cows has led to isolation of numerous bacterial strains according to the type of parturition.

Of the examined cows (in number of 40), 28 presented normal parturition and 194 bacterial strains were isolated from them while 12 cows presented dystocia and 88 bacterial strains were isolated from them.

As it was observed, *Escherichia coli* is predominant in biological samples, both in case of normal parturition and dystocia.

In case of dystocia, *Staphylococcus spp.* were isolated in proportion of 10.63% of total isolated bacterial strains, *Escherichia coli* in proportion of 9.21%, *Arcanobacterium sp.* in 4.96% and *Klebsiella sp.* in 2.83%(fig.4). Other bacterial species were isolated very small proportion (*Corynbacterium spp., Listeria spp., Bacillus spp., Proteus spp.(fig.3), Actinomyces spp., Enterobacter spp. (fig.5), Bacteroides spp., Actinobacillus spp.* and *Raoultella ornithinolytic*). There was not detected any species of *Streptococcus, Enterococcus, Micrococcus, Clostridium, Pasteurella* and *Neisseria* genera (fig.1).

In case of normal parturition, the most isolated bacteria was *Escherichia coli* (19.14% of total isolated bacterial strains) (fig. 3,4,6) followed by *Staphylococcus spp.* (5.67%) (fig. 7,8), *Enterococcus spp.* (4.96%),(fig. 7), *Streptococcus spp.* (4.25%), *Pseudomonas spp.* (4.25%), *Corynebacterium spp.* (3.54%) (fig.8), *Arcanobacterium spp.*(3.54%). Other types of bacteria were isolated in a very small proportion, while *Actinobacillus spp., Raoultella ornithinolytica and Serratia spp.*were not detected (fig.2).

The usual presence on the vaginal and cervical mucosa of numerous bacterial species cumulated with parturition disorders and immunity suppression of the female can lead to occurrence of local infections which will need antimicrobial treatment. Thus, thus study also aimed to perform some antibiograms, both on pure and mixed cultures.

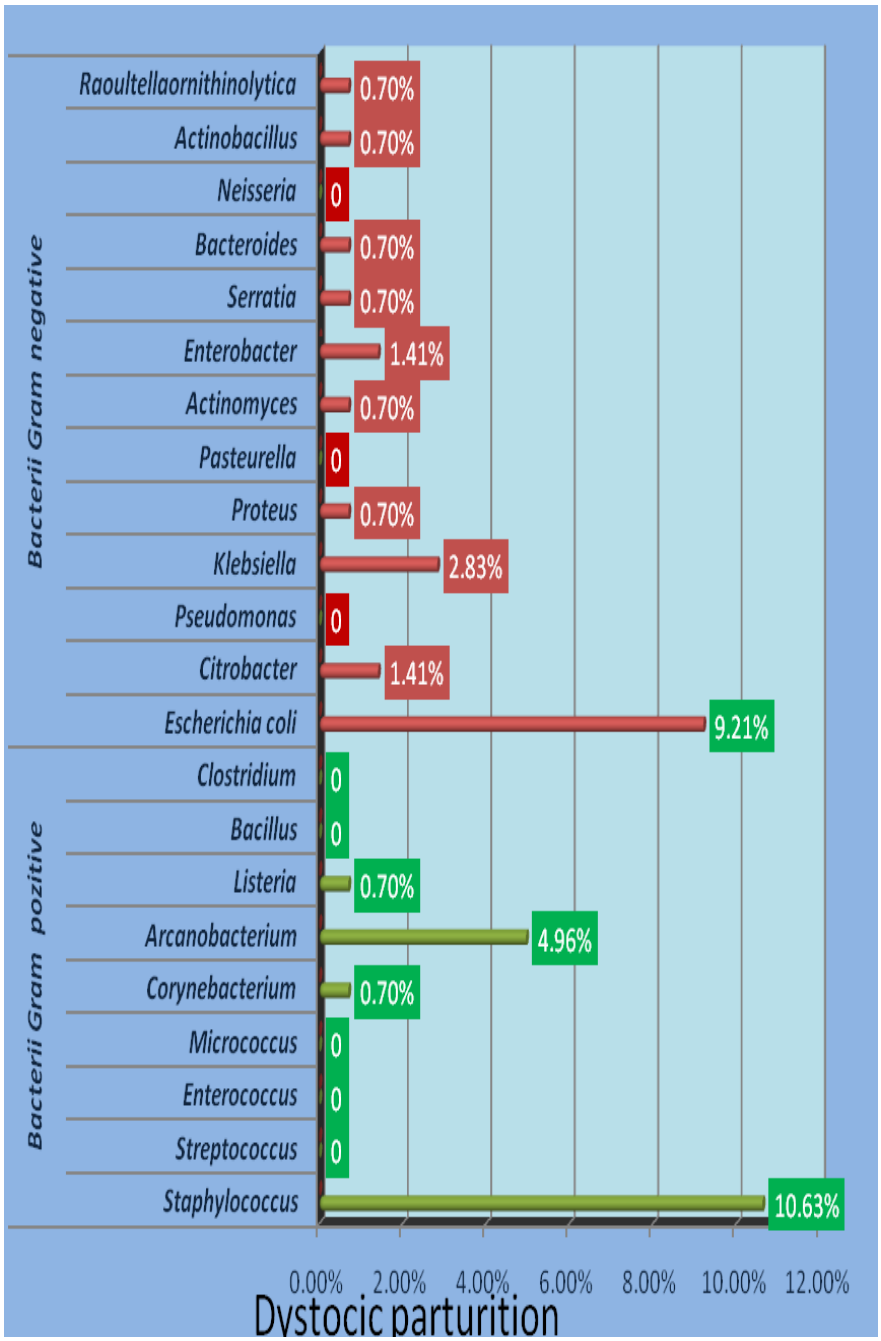

Fig. 1 Proportion of isolated bacteria in case of dystocic parturiton

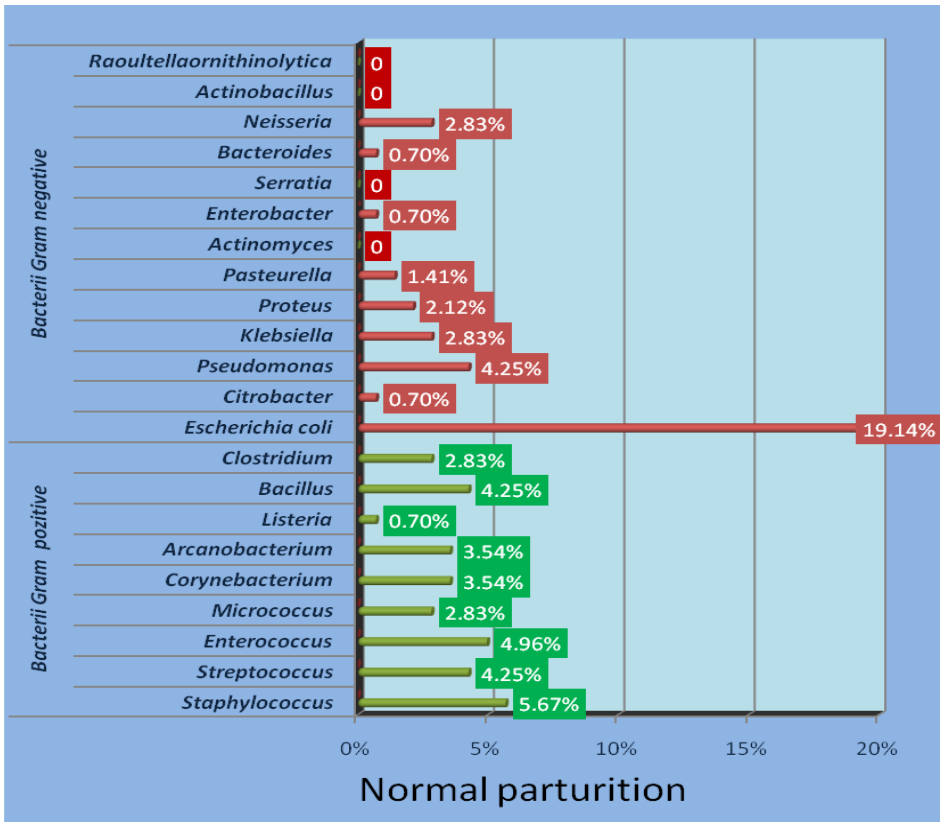

Fig.2 Proportion of isolated bacteria in case of normal parturiton

Of all the domestic animal species, the most frequent and various cases of dystocia (maternal, fetal or mixed) occur in cow. The primiparous females are more exposed to dystocia due to insufficient development of the basin.

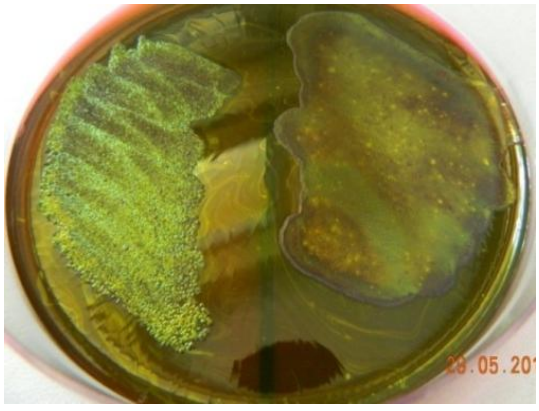

Fig.3 Mixed culture of *E.coli* and *Proteus spp.*- Levine medium

Fig.4 Mixed culture of *E.coli* and *Klebsiella spp.* – Levine medium

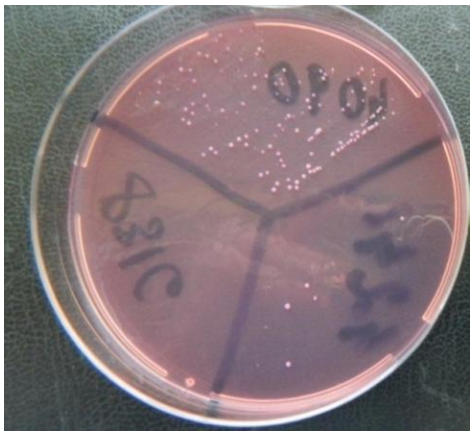

Fig.5 Mixed culture of *Citrobacter spp.* and *Enterobacter spp.*– Simmons Citrat Agar medium

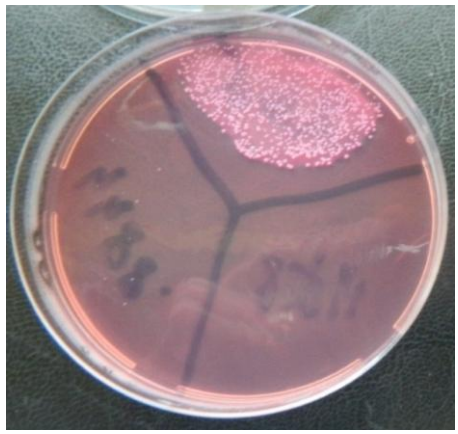

Fig.6 Mixed culture of *E.coli*,and *Enterobacter spp*.– McConkey medium

Fig.7 Mixed culture of *Staphylococcus spp.* and *Enterococcus spp*..– Agar with 10% ram blood medium

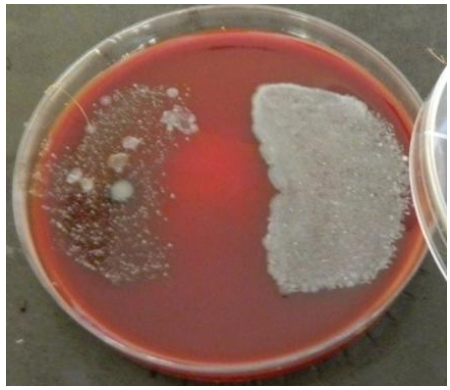

Fig.8 Mixed culture of *Staphylococcus spp.* and *Corynebacterium spp.* - Agar with 10% ram blood medium

A

B

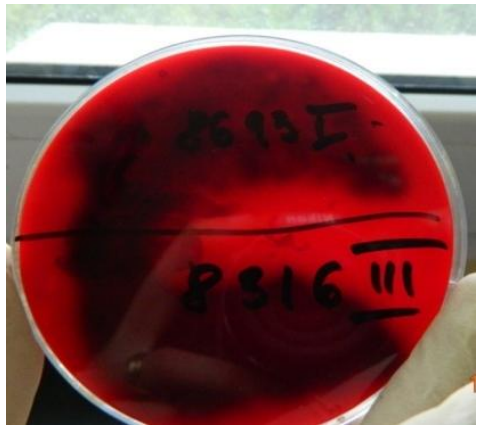

Fig. 9 Types of hemolysis present on isolated bacterial strains: alfa hemolysis (incomplete, with viridans effect) – figure A and beta hemolysis (complete) –figure B

For sure determinations of the taxonomic framing of some bacterial strains, cultural aspects were correlated with morphological and biochemical aspects.

Antimicrobial substances like enrofloxacin, tetracycline, gentamicin, amoxicillin, clavulanic acid, ampicillin, oxacilin, cloxaxcilin, florfenicol, clindamycin, lincomycin, oxytetracycline, lincomycin, spectinomycin, cloramfenicol were tested.

Of the tested substances, the most efficient antimicrobial substances were enrofloxacin, tetracycline and oxytetracycline (Fig.10,11,12).

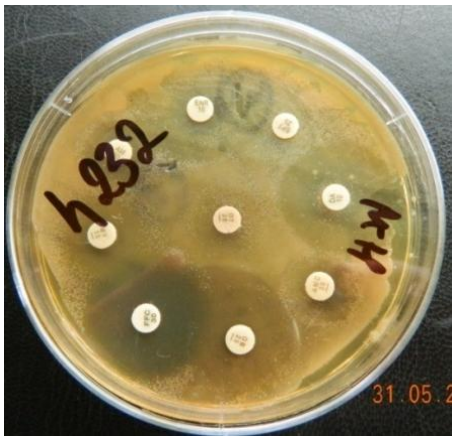

Fig.10 Antibiogram on mixed culture

Fig.11 Antibiogram on pure culture of *Klebsiella spp.*

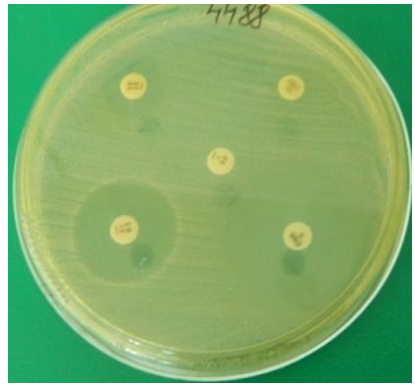

Fig.11 Antibiogram on pure culture of *Escherichia coli*

Conclusions

1. The most probable number of germs (MPN) increases progressively in the first two weeks after parturition, with higher values in cows with dystocia.

2. Of genital samples, 8 (2.76%) bacterial strains were isolated in pure cultures and 282 (97.24%) strains in mixed cultures.

3. Of the 282 bacterial cultures, 126 (44.68%) bacterial strains were Gram-positive and 156 (55.32%) were Gram-negative.

4. By respiratory type, aerobic bacteria were predominant, with 264 (93.62%) bacterial strains, of which 118 were Gram-positive and 146 were Gram-negative.

5. The anaerobic bacteria were isolated in a small number, only 18 (6.38%) strains, of which 8 Gram-positive strains and 10 Gram-negative strains.

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REMARKS CONCERNING THE PHYSIOLOGICAL CONSTANTS IN COWS DURING PUERPERIUM

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Abstract

This study was performed in months of February and March 2013, on a herd of 12 cows which were monitored trough puerperal period, in a farm from North-Eastern Romania. By inspection, which was performed at the beginning, both in groups of animals and individually, there were observed the general condition and the hindquarters of cows. The cows that showed clinical signs of puerperal disorders (placental retention, endometritis, uterine prolapse and/or uterine infections) formed the experimental group (E1). In cows that formed the E1 lot, the physiological parameters such as pulse, temperature and respiration recorded higher values than in cows that formed the control group (M), due to acute inflammatory processes with local evolution and due to local toxic reactions, which change physiological constants.

Key words: *cow, puerperium, physiological parameters*

Introduction

During gestation and parturition, maternal body is subject to major microscopic and macroscopic transformations. In the first half of gestation these transformations are slow and unapparent clinically, but they become increasingly evident as advancing in gestation, they culminate in the moment of fetal expulsion and end with the elimination of fetal annexes(Ardelean V. 2002, Bârţoiu A, Seiuciu F. 2004).

The period of time between parturition and return to the morpho-physiological status before gestation is called puerperium and is characterized by the evolution of several elements: post-partum uterine involution, elimination of fetal annexes, post-partum ovarian activity and puerperal uterine bacteriology. During this period the uterus contains a mixture of fetal fluids, destroyed endometrial tissues, fetal coverings and blood derived from rupture of the umbilical cord. (Bogdan I. et al. 2006). Thanks to these and to local temperature, is created an environment very favorable to the development of bacterial flora (King G.J. 1993).

Considering the aspects that were mentioned, it can be said that the puerperium is a very critical stage of female life because during this period can occur many diseases causing infertility. A normal puerperium ensures the reentry of the females in the breeding circuit and provide profitable husbandry (Runceanu L. et al. 2002 Puerperal affections are much more frequent in case of poor nutrition during pregnancy, lack of movement, a flawed microclimate and inappropriate conditions in maternity (Borowski O. 2003, Melendez P. Risco C. A. 2002)

In current conditions of the farms and big production units, with intense exploitation, where there are discrepancies between production, nutrition and the rest of the environmental factors, the puerperal affections produce big economic damages and create many veterinary issues(Hanzen Ch. 2009, Taverne M.A.M., Van der Weijden 2008)

This study aimed to observe the evolution of the puerperal period by monitoring the values of physiological constants in order to diagnose and treat the specific diseases of this period at an early stage (Bogdan L. et al. 2004, Rhodes R.C. 2002).

Material and method

In order to conduct the research two lots of cows of Romanian Black-Spotted breed were formed, as follows:

- M (control) group consisting of cows with normal puerperium;
- E1 group consisting of cows with puerperal disorders.

By initial inspection which was effected on animals both in group and individually, were observed the general condition and hindquarters, this way cows with clinical signs of puerperal disorders being detected. These cows formed the experimental group (E1).

The groups were composed of cows that were in puerperium, being clinical examined based on gynecological survey. This gynecological survey can be performed whenever necessary, especially when the reproduction rate of the livestock is unsatisfactory and remedial action must be taken.

The cows in the studied groups were observed in terms of general condition and gynecology. General clinical examination was performed by: history, inspection, palpation and thermometry.

History consisted in collecting previous data about the animals under study, such as: age, date of parturition and how parturition occurred (normal/dystocia), possible complications and performed treatments, the number of gestations, the emergence of sexual cycles, regularity and intensity of the heat, the number and date of previous inseminations, the appearance and type of vaginal secretions during heat, the quantity and quality of secretions during estrus, the way puerperium evolved. Because some elements apparently unimportant may be missing of gynecological statement, there was a dialogue with the farmer and farm caretaker.

The inspection was carried out at a distance and up close, individually and by group, in motion and stationary, in the standing position and in decubitus, paying special attention to the hindquarters. There were also observed the general condition, hygiene, facies, vulvar and vaginal mucosal appearance and type of discharge. Animals under study were observed in terms of: thermodynamic, heart rate and breathing.

Results and discussion

The gynecological survey was performed between February 2013 and March 2013 on a total of 12 cows that were monitored trough puerperal period by history and clinical examination in a farm from the N-E of Romania.

Each cow has been monitored in the first 7 days after parturition. The mean values for each day are shown in Table 1.

Pulse, temperature and respiration have recorded higher values in the E1 group, consisting of cows with puerperal disorders (placental retention, endometritis, uterine prolapse, uterine infections) than in the control group (M), due to acute inflammatory processes with local evolution and due to local toxic reactions, which damage the physiological constants. (Figure 1, 2, 3)

Table 1. The mean values of physiological constants

Numbers of days	Lots					
	M			E1		
	T (^{0}C)	P (nr./min)	R (nr./min)	T (^{0}C)	P (nr./min)	R (nr./min)
Day1	38,3	61	50	38,6	65	65
Day2	38,5	63	49	38,7	66	60
Day3	38,4	62	55	38,8	67	58
Day4	38,5	61	56	38,9	66	53
Day5	38,45	60	55	38,9	65	70
Day6	38,4	61	54	38,8	69	68
Day7	38,4	59	53	38,8	68	56

T – temperature; P – pulse; R - respiration

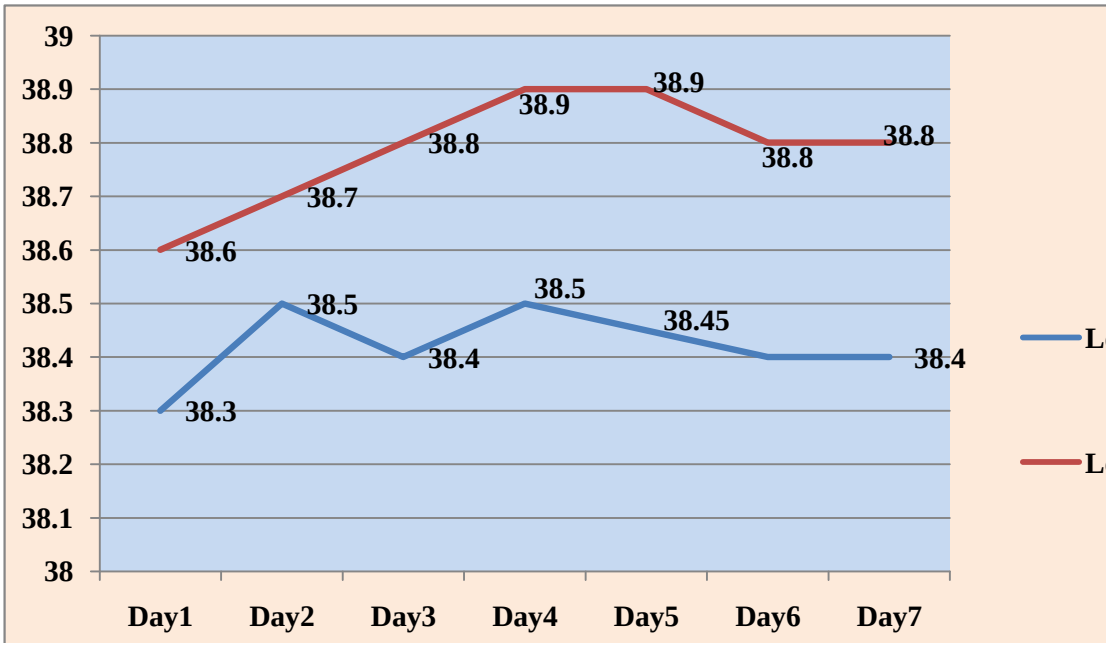

Fig. 1. Graphic representation of the rectal temperature groups of cows

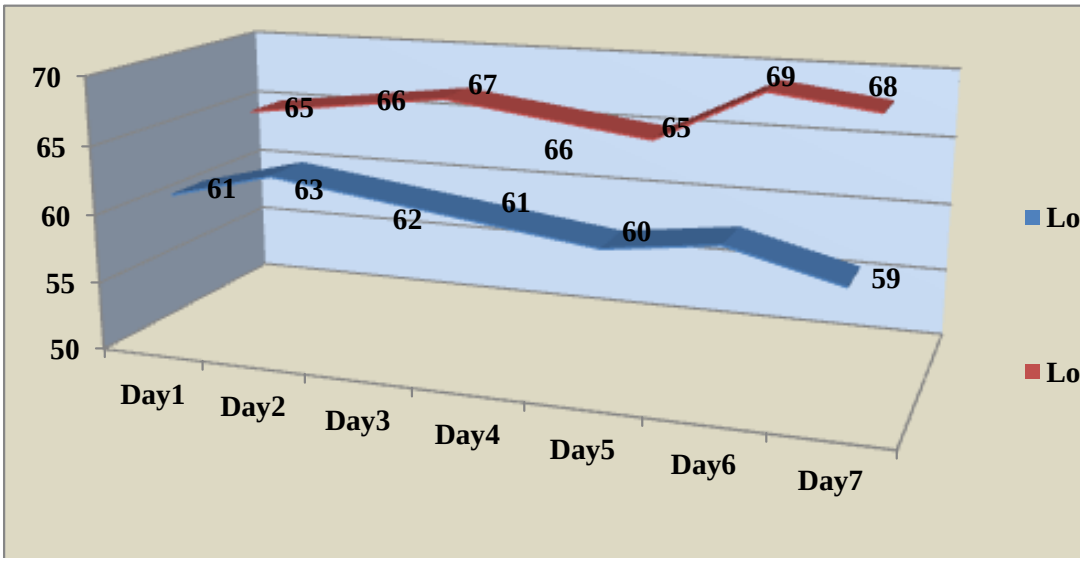

Fig. 2. Graphic representation of pulse in cow groups

By analyzing the evolution of physiological constants in E1 group compared with the one in control group, it can be noticed that the values of rectal temperature were higher in group E1, as well as pulse and respiration.

The highest values of temperature and respiration rate in E1 lot were obtained in the 5th day after parturition (temperature: 38.9 ^{o}C and respiration: 70/min.), while the highest value of the pulse was noticed in the 6th day (69/min.).

The main puerperal disorders diagnosed in cows under study are: retention of fetal annexes, uterine prolapse and puerperal infections (local and generalized).

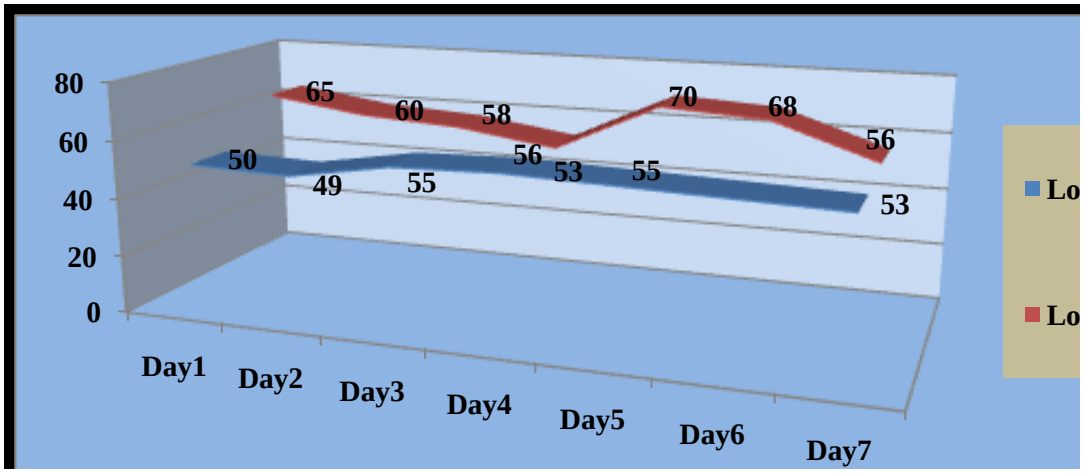

Fig. 3. Graphic representation of respiration in cow groups

Conclusions

1. Based on results it can be concluded that, in case of puerperal affections in cows, the physiological constants have higher values.
2. The end of the first week after parturition is a critical period in the evolution of the puerperal affections, being reflected by the highest values of the altered physiological constants.

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POST-TRAUMATIC SPINAL HAEMATOMA WITHOUT OSSEOSUS LESIONS IN A DOG

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Abstract

A year and 3 months old, Golden retriever female dog, weighting 28 kg, has entered in our attention when it was brought to the Medical Clinic of the Faculty of Veterinary Medicine – Iasi with paraplegia and spastic urinary incontinence installed acute progressive within 5 days. At the beginning the dog presented a deficit of movement onhind limbs, thenwithin 24hoursspastic paraplegia was installed and after 48 hours it was followed by quadriplegia with inconstant signsofSchiff-Sherrington phenomenonandurinary retention.There were no abnormalities on radiographs or computed tomography (CT) scan of the spine. The images before and after contrast administration of magnetic resonance imaging (MRI) reveal a spinal cord compression caused by fusiform intraparenchimal inthe rightT10-T11 intervertebralspace and with hypersignallesiononT2andpre-and post contrastT1image. Also, the MRI exam highlighted a slight axis displacement of the vertebrae T10-T11, near the joint. Based on this history and on the present of hyperintense lesion on MRI image was considered a traumatic event that caused the development of an intramedullary as a result of traumatic bleeding by disrupting of blood vessels.

Keywords: *spinal cord injury, hematoma, RMN, dog*

Introduction

Spinal trauma described as a form of independent spinal cord contusion as independent post traumatic result with blood tissue destructions, blood accumulation and hematoma formation and and production of secondary spinal cord compression accompanied by significant neurological dysfunction, in the absence of bone lesions, it is rarely mentioned in the medical literature. (3,4)

Hematomyelia private by post traumatic bone distruption or intervertebral disc lesions is considered a pathological exception because, normally, it develops secondary after fractures or dislocations vertebral due to a trauma. (4)

Human Medicine reports that only 10% of traumatic spinal hematomas are associated with bone lesions, according to studies of Bruny and Bosnia (1), while in veterinary medicine, there are no studies on the prevalence of this phenomenon.

Availability of advanced imaging tests for veterinary medicine in the last period and increased prevalence of cases with post traumatic spinal cord injury allowed that the spinal hematoma to be described in veterinary pathology of recent years. (10)

A pathophysiological mechanism of spinal hematoma is often unclear, but the hypothesis of a minor injury that can cause blood tissue destructions and blood collection can always be taken into account. (4, 9)

As a secondary spinal cord injury trauma, posttraumatic spinal hematoma is rapidly evolving from a few hours to several days after initial trauma and it include progressive neurological deficits, for which, identification of the spinal cord compression should be done soon. (3)

The MRI technique, through the superior inherent contrast resolution compared CT, is a priority for medullary tissue evaluation because it is able to identify both the location and severity of injury but and the cause of spinal cord compression and even, sometimes the mechanism of it. This makes the MRI technique can significantly contribute to therapy and assessment and neurological recovery of the patient. (4, 8)

Scope

Through this case study it is desired to highligh the importance of advanced imaging MRI technology for assess the patient's spinal cord tissue with medullary neurological signs and without vertebral bone lesions on radiographs and CT scan.

Selection and application of advanced diagnostic imaging techniques in case of spinal cord injury can have a major impact on patient prognosis because the acute and progressive onset of neurological signs of the first days could result in death or permanent neurologic deficits if you can not intervene promptly of medically.

Material and method

A year and 3 months old, Golden retriever female dog was brought to the Medical Clinic of the Faculty of Veterinary Medicine – Iasi with tetraplegia and spastic urinary incontinence.

The dog was underwent clinical neurological examination and imaging examination for diagnosis.

Radiological examination was performed with the device and Scan 200 and CT imaging examinations and MRI were performed under general anesthesia made from a combination of metedomidină (Domitor ® Phizer) at a dose of 0.03 mg / kg iv and ketamine at a dose of 0.3 ml / kg i.v.

CT was performed with your Toshiba 16 Slice in transverse and sagittal and dorsal reconstruction. MRI was performed with 1.5-T Toshiba device on the thoraco – lumbar spine. MRI image was captured before and after the administration of the contrast gadobenic dimeglumine dose of 1 mg / kg, iv (MultiHance ®).

Results and discussion

Medical history revealed that neurological signs appeared 5 days before, initially the owners have noticed a difficult movement on the hind legs.

The neurological signs have evolved progressively acute from ataxia to paraparesis and then tetraparesis and urinary retention phenomena. After 48 hours of onset of tetraparesis, for 3 days was given to the treatment with NSAIDs and broad-spectrum antibiotic but there has been no change.

During the clinical consultation, general physical examination of dog was normal. On palpation of the spine dog did not show hyperesthesia. The spinal reflexes of the thoracic limbs could not be evaluated because of the muscular hypertonus and the hind limb reflexes were absent. The tail was devoid of sensitivity and motility and panicular reflex was absent. Deep sensitivity of the affected limb was normal.

During the examination, the dog showed a normal mental status and cranial nerve examination was normal. The dog has undergone radiological native examination of thoracolumbar spine, in lateral and dorsum-ventral projection but did not find any vertebral abnormality such us subluxation or fracture on any of radiographic projections. (Fig.1)

Despite the absence of any radiological abnormalities the radiographic views in flexion and extension position have not been achieved because of the increased risk of exacerbation of existing spinal injury (11), reason for which was carried out a thoraco-lumbar spinal CT exam.

CT examination in transverse and sagittal did not reveal any abnormalities in bone structure that may indicate subluxation, fracture or extrusion of intervertebral disc. Also, there was no change in the spinal canal, intervertebral disc or medullar tissue. (Fig.2)

Because the CT scan was negative, the spinal injury was not exclusive and was performed immediately a native and with contrast MRI examination. The T2 image of MRI was observed a hyper intense intramedullary lesion, spindle, focused on the area between segments T10-T11 and a poor shaft displacement of T10-T11 vertebral bodies with keeping of hypo intensity of bone tissue and hyper intensity of intervertebral disc. (Fig.4)

On precontrast and post contrast T1 image, intramedullary spindle formation identified onT2 image, it has kept the hyper intense character. Also, the intervertebral disc and bone tissue maintained their normal hypointense character. (Fig. 3,5)

MRI did not show any change in the medullar cavity and no hyper signal of vertebral bone marrow was not identified thus excluding the presence of fractures or subluxation vertebral. These results have guided the initial diagnosis to a hyperintense lesion that causes myelopathy.

The differential diagnosis of MRI included edema secondary spinal cord contusion or ischemia as a result of direct trauma or other vascular disorders such as hematoma as a result of post traumatic vascular disruptions.

Based on the absence of any damage of vertebral bone on radiographs and CT scan and the presence of spindle hyperintense intramedullary lesion identified at MRI exam, the diagnosis was oriented to a traumatic spinal cord disease such as contusion type. In such situations, literature highlights the controversies that exist for the opting an effective imaging investigation, for a clearest view of lesional tissue and it recommends that the exam be done in collaboration with clinical and anamnesis data of patient. (8)

Although CT imaging provides high spatial resolution in several levels of the region of interest, and some studies support CT technique for excellent describing of tissue incriminated, in this case it was found to be limited.

For the diagnosis of acute spinal trauma, the MRI technique is very important because often neurological dysfunction can be severe and prognosis should be established as soon as possible. Also, the MRI investigation offers the possibility to assess the musculoskeletal and ligamentous injuries and it can provide important information regarding to mechanism of trauma and more importantly, it may suggest the developing therapeutic plan with correct evaluation of prognosis. (7)

The existence of hyperintense signal of intramedullary lesion on T2 image oriented the initial diagnosis to edema, infarct or other vascular pathology but maintaining hyperintense signal of lesion and on pre contrast T1 image is a clear indication of this extracellular methemoglobin, according to the literature. (5, 6)

In the spinal cord bleeding, MRI exam allows a better differentiate them from other spinal pathologies, due to the sensitivity of this imaging technique for the detection of neural bleeding and estimation of chronicity. Many studies have found and showed that hematoma MRI signal intensity changes during its degradation until it is disintegrated regardless of the location of the bleeding. (6, 10)

Signal characteristics of hematoma refer, especially, at the particular magnetic properties of hemoglobin and its degradation byproducts as well as at the protein content of the lesion. Changing these properties can elucidate the pathology age, studies have shown that the existence of a hyperintense signal of the lesion on T1 and T2 sequences corresponds to a subacute progressive developments and it is consistent with the presence of extracellular methemoglobin. (5.10)

The hyperintense signal on T1 image is the result of extracellular methemoglobin but disappearance of effects which are prone to cell lyses and increasing the dipole-dipole interaction proton-electron are likely to hyperintensity of signal on T2 image. (10)

According to studies (10), this feature occurs after 6-8 days after onset of bleeding and it can keep for up to a month. In this case, MRI was performed after 6 days of the appearance of the first neurological symptoms, MRI result thus confirming and age of lesion. Because were not identified and other musculoskeletal and/or ligament tissue abnormalities but it has been observed only slight axis displacement of vertebral bodies T10 and T11, at the level of the intervertebral joint, intramedullary vascular lesion is considered posttraumatic.

In this case, it can be taken into account trauma by hiperflexion or hyperextension of spine due to the direct application of force on an area of the spine in a manner which caused rapid axis deviation of T10-T11 vertebrae. (9)

The absence of bone fracture is related and trauma type (minimum or violence) and the degree of elasticity ligamentous may explain the rarity of bone fracture in young animals. (4)

Location of trauma on thoraco-lumbar spinal segment is a result of the high degree of mobility of the spine at this level. Approximately 50% of dogs with spinal injury have lesions in the T10-T12 segment, according to studies by Joane Parent. This phenomenon was associated with location between the rigid thoracic vertebrae and lumbar vertebrae muscular.

Evaluation of patients with thoraco-lumbar spinal trauma by MRI examination is imperative, especially, in situations when the radiological and CT scan did not reveal the presence of fractures or subluxations, because this imaging technique in addition to lesion detection has a major role in the evaluation of the extent of lesions of the level of nervous system. Also, in many cases neurological dysfunction can be severe and prognosis must be established as soon as possible.

MRI offers and the possibility of evaluating musculoskeletal and ligament injuries, can provide valuable information regarding to the mechanism of trauma and more importantly it may suggest the approach of treatment plan with correct evaluation of prognosis. (7)

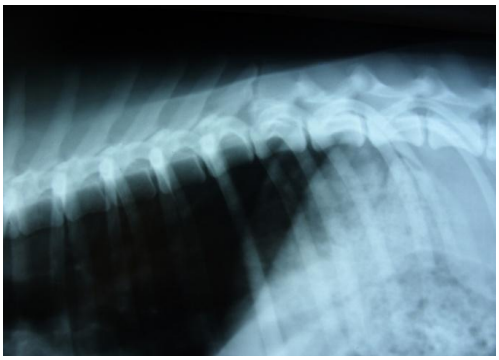

Fig. 1 Thoraco-lumbar lateral radiographic image. Negative image

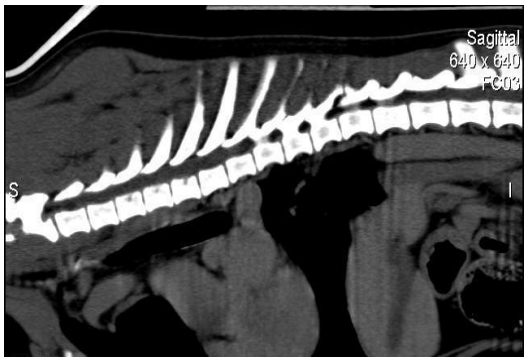

Fig. 2 Thoraco-lumbar CT image. Sagittal reconstruction. Negative image

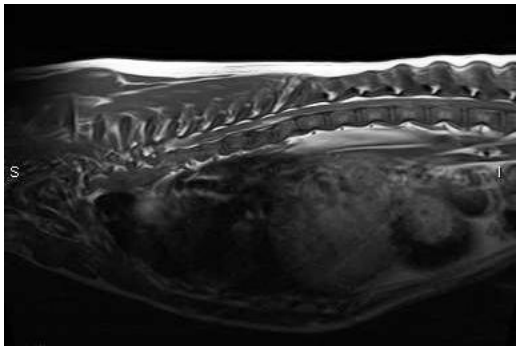

Fig. 3 Image thoraco-lumbar sagittal T1 MRI. Thoracic spinal segment. Intraparenchimal hyperintense area at the T10 vertebra

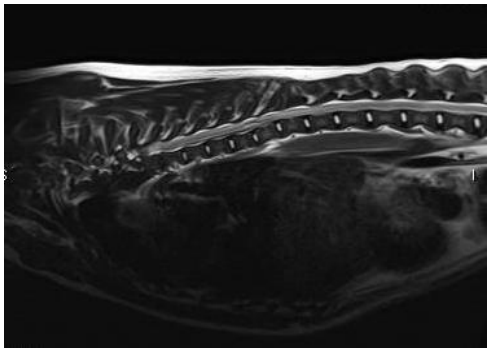

Fig. 4 Image thoraco-lumbar sagittal T2 MRI. Thoracic spinal segment. Intraparenchimal hyperintense area at the T10 vertebra

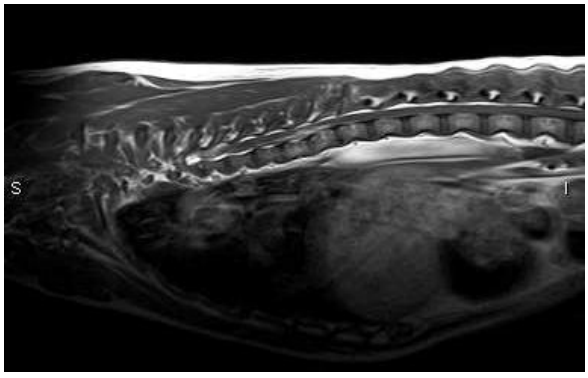

Fig. 5 Image thoraco-lumbar sagittal T1 post contrast MRI imagine. Thoracic spinal segment. Intraparenchimal hyperintense area at the T10 vertebra

Conclusions

1. The negative result of radiological and CT examination of the spinal not exclude the presence of spinal injury in a patient with medullary neurological symptoms.
2. MRI has an important role in evaluating patients with acute spinal trauma when radiographs and CT exam not detect any bone lesion.
3. MRI imaging can provide important information regarding to the mechanism of injury, extension of the lesion at the level of nervous tissue, age lesion, assumptions therapeutically and prognosis evaluation.

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USE OF ACRIDINE ORANGE AND BABES-PAPANICOLAU STAINING FOR THE DIFFERENTIATION OF VARIOUS STAGES OF THE PARASITE *BLASTOCYSTIS HOMINIS*

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Abstract:

Blastocystis hominis is an enteric protozoan with hight polimorfism describing four stages of development in the hostAcridine-Orange stainingallowed the study ofparasiticlife formsin the population, thisfluorochrome ensuring the differentcolouring of the trophozoite, youngandmaturecyst in stool sample. The central body forms have yellow cytoplasm, green vacuoles and bright yellow nuclei. When the vacuolar form passes into a young cyst, and then into a mature cyst, its colour changes from bright yellow to flaming red-orange.In this study we also usedBabes-Papanicolaustaining on stool samples because highlightscellular structures(nucleus, cytoplasm, vacuole) and assures therecognition of the parasiteinbiopsy pieces. On this staining Blastocystiscytoplasmaappearsblue or pink, and the nucleus is red and the vacuolehaveshades ofpale pinktogreenbrick.Both colorations highlight the structure and may be significant for establishing the age category inside the population (vegetative and cystic forms).

***Key Words**: Blastocystis hominis; Acridine-Orange; Babes-Papanicolau.*

Introduction:

Blastocystis sp is a common enteric protozoan in humans and animals. The species found in humans, known as *Blastocystis hominis* include several isolates which display an extraordinary genetic variability (Clark, 1997, Noel et al., 2005). Various studies have shown that many of these genotypes are similar to those isolated from animals, and it was concluded that humans are actually colonized by a number of zoonotic species of *Blastocystis* (Abe et al., 2003c, Abe, 2004, Yoshikawa et al., 2004a, 2004b, Noël et al., 2005).

Blastocystis sp is perhaps the most controversial organism that has been studied, having both polymorphism and pleomorphism within the same population, including vacuolar, granular, amoeboid, cystic, multivacuolar, and avacuolar forms (Stenzel and Boreham, 1996).

Without taking into account the systematic classification, this study refers to the population caught in stool after direct microscopic examination with Lugol as screening method, followed by acridine-orange staining (Suresh et al., 1993) and Babes-Papanicolau staining (Willmar D. Patino et al., 2007), both on wet samples.

Materials and methods

Detection and isolation of *Blastocystis hominis*

The detection of the parasite in faeces was made by Lugol direct technique (Nitzulescu V., 1976).

For the samples with over 5 elements viewed on a microscopic field at 400 we applied two different techniques to process samples before staining: Ficoll-Paque

concentration technique (Zama V., 1996; Moe et al., 1996; Snowden K. et al., 2000), and Shaudin fixation technique (DiaSys, 2008).

The Ficoll-Paque concentration technique allowed us to isolate *Blastocystis* organisms and reduced the number of bacteria in the samples, which were subsequently processed for Acridine-Orange staining. Shaudin fixation technique was used as a first step in Babes-Papanicolau staining procedure. The wet smears were placed in fixative for 30 min. - 1 hour.

Acridine-orange staining

A drop (25 µl) suspension containing the parasites isolated by Ficoll-Paque technique was mixed thoroughly on a clean glass slide with a drop (25 µl) of diluted Acridine-Orange (Brian A et al., 1981). The preparation was viewed after 3-5 minutes with a fluorescence microscope.

Babes-Papanicolau adapted staining

The staining procedure was adapted for our purpose, replacing the 95% ethyl alcohol fixation step with Shaudin fixation, specific for protozoan organisms. The next steps were the same like the standard Papanicolau staining. Such preparation is mounted in balsam for microscopy (Canada balsam) and covered with a 22x40mm coverglass.

Microscopic examination

The microscopic examination was performed using an oil immersion microscope objective for both types of preparations (Leica - LASER Confocal Scanning Microscope with DFC – Digital Film Camera).

Results and discussions

In histology, Babes-Papanicolau staining provides cell differentiation of the superficial layer, from the deeper layers. In the superficial layer, the cells cytoplasm is coloured in pink, in the same way the cytoplasm of the cells of the deeper layer becomes blue. We noticed that there were situations when *Blastocystis hominis* had blue cytoplasm (azurophilic) and other situations when the cytoplasm is pink (eosinophilic).

We believe that colour variation is related to metabolic activity. We noticed that granular or avacuolar forms have blue cytoplasm (Fig. 1 D, E) and vacuolar forms have pink cytoplasm (Fig. 1 A, B, C, F). That means that Papanicolau coloration may have the advantage of differentiating between the two forms. The central vacuole may have different shades. For example, in young forms it is pale or pink (Fig. 1 A, F), and in mature or older forms is green brick (Fig. 1 C). The colour, appearance and presence of inclusions (Fig 1. F-1) of the vacuoles can give information on the activity of the parasite. The nuclei are always red-violet.

The cystic form is smaller, with condensed cytoplasm, some with and others without central vacuole (Fig 1 F-5). This observation lead to the conclusion that cystic forms come from both the vacuolar and granular or avacuolar vegetative forms.

The coloration Babes-Papanicolau does not reveal the cellular wall, he appears in mature cystic form like a thick halo,around the compact cytoplasm ..

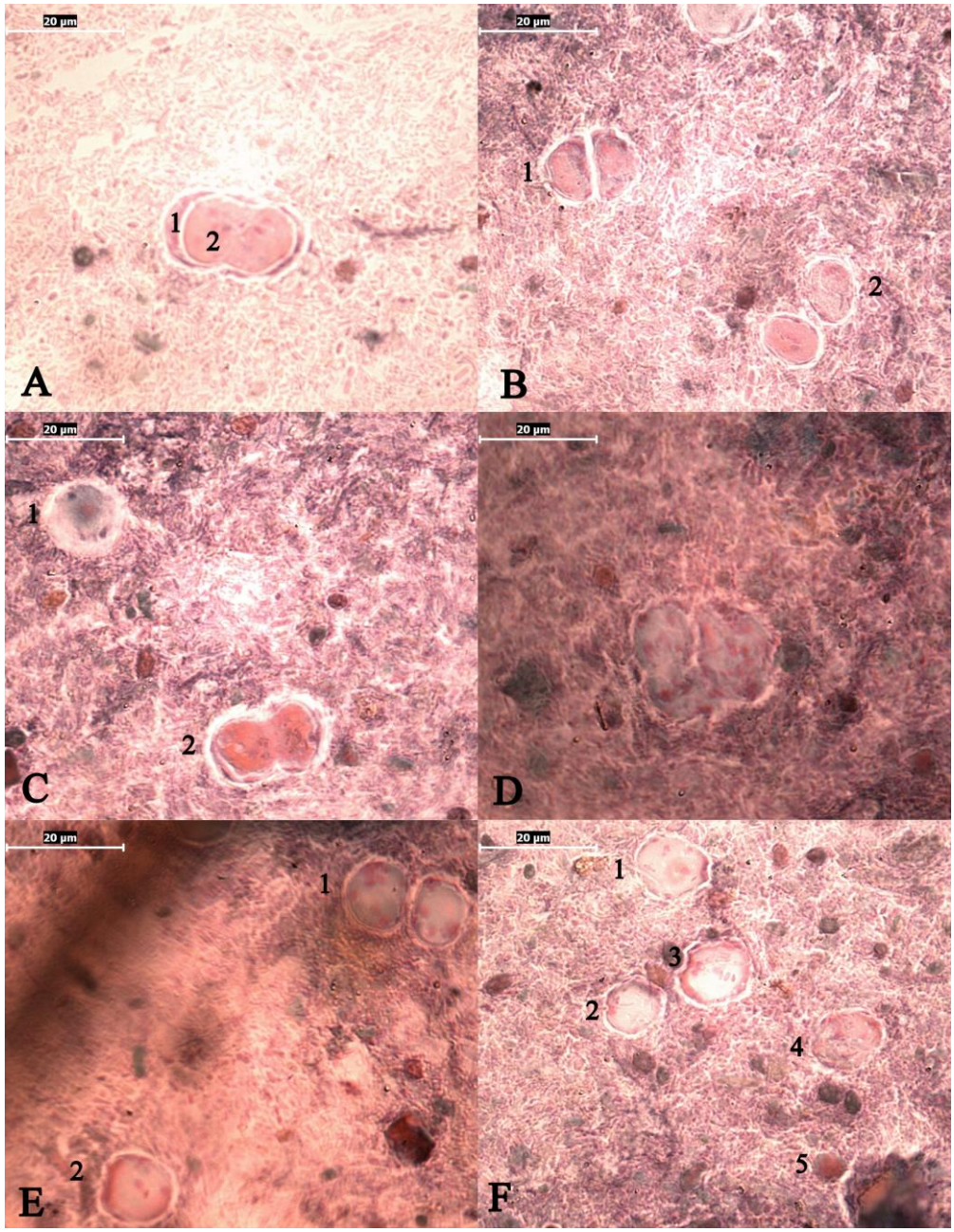

Fig 1: A) Vacuolar form with slim periferic cytoplasm (1) and pink central vacuole (2); B) Vacuolar forms after segregation (1, 2); C) Avacuolar form (1) and vacuolar form cell division (2); D) Avacuolar form cell division with blue cytoplasm; E) Avacuolar forms after segregation (1) and a intermediary form between avacuolar and vacuolar forms; F) Vacuolar forms with bacteria included in central vacuola (1,2,3,4) and young cystic form (5).

The coloration highlight also the presence of pseudopods (Fig. 2), wich have pink cytoplasm concentrate (Tan et al., 2001a).

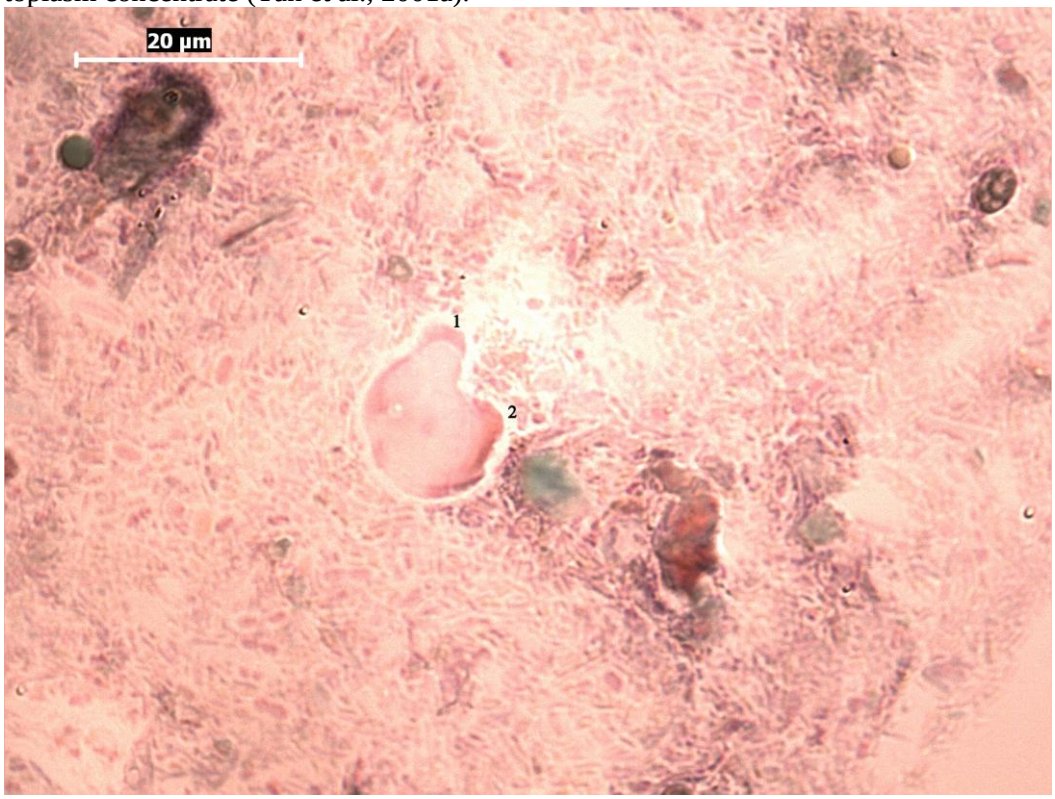

Fig. 2: Amiboidal form with pink cytoplasma, concentrated in pseudopods (1,2) and pink central vacuole

The fluorochrome Acridine Orange, has been used previously to study protozoan parasites, e.g. *Plasmodium* by Hensen, Hunter, Richards & Allred (1970), *Trichomonas vaginalis* by Ridge (1982), and *Pneutnocystis carinii* by Thomson & Smith (1982). Acridine-Orange is known to stain the DNA of nucleus, mucus and RNA as bright green, dull green, and flaming red-orange, respectively. The process of encystation seems to be accompanied by active nucleic acid and protein synthesis (Suresh et al., *Parasitology Research).*

Acridine-Orange stain differentiates vacuolar forms from cystic forms and is a complete method which we can use to easily observe the internal structure of the parasite. Nuclei appear to as yellow fluorescents spots, and central vacuole, bright green. During the encystation process the color changes from yellow to red-orange (Fig. 3).

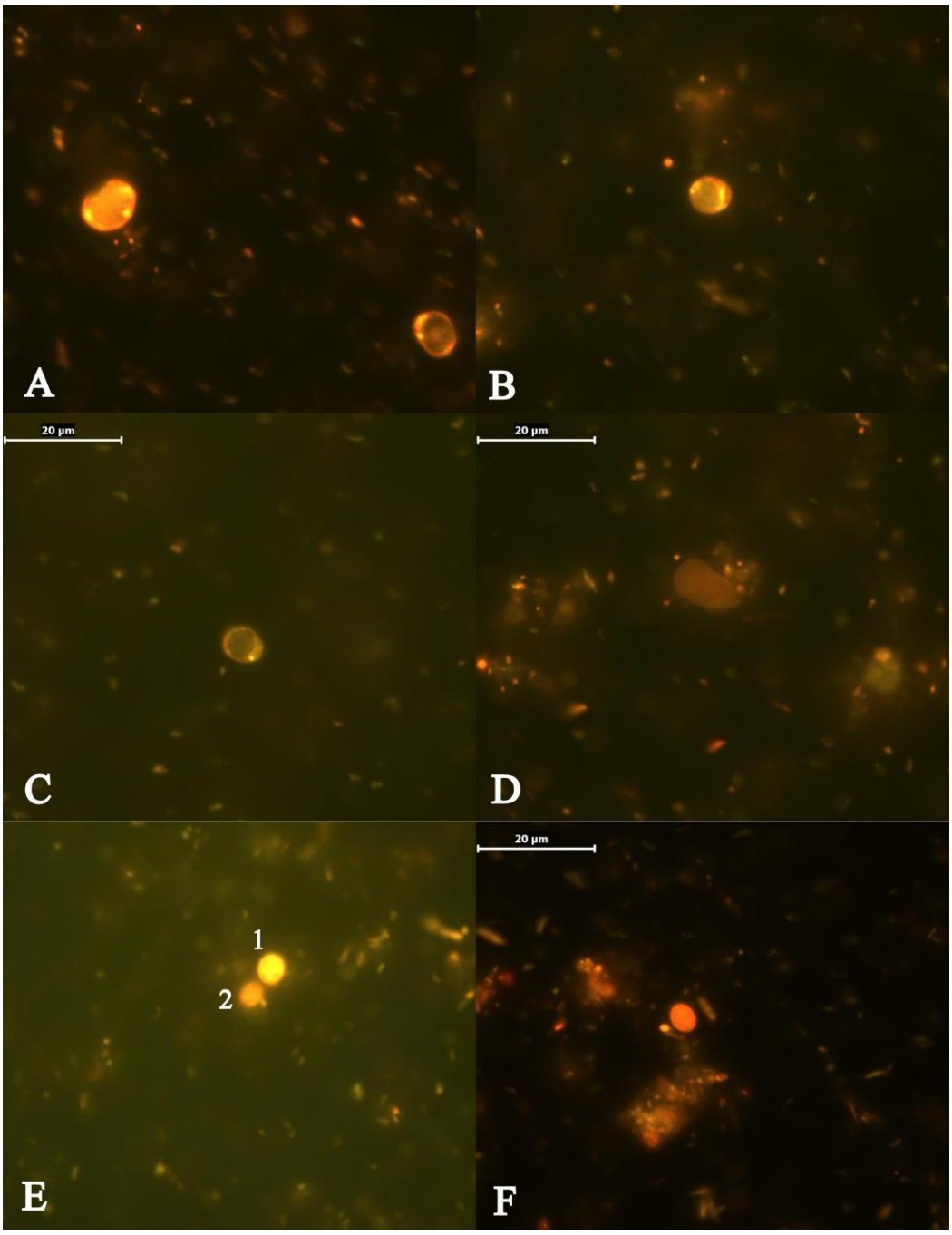

Fig. 3: A), B), C) Vacuolar forms; D) Amiboidal form; E) Young cyst (1) and mature cyst (2); F) Mature cyst

We find the Acridine-Orange staining useful in quantifying cystic stages found in stool with vegetative forms, and therefore we see the structure of the population and the infectivity of the stool

Conclusions

- Both colorations were indicated for study, because Acridine-Orange ensures a different coloration of the trophozoite precyst and cyst, and Babes-Papanicolau colours differently the cytoplasm of life forms .
- Both stains highlight the cystic form
- Both allow determining the age, and the population structure.
- They highlight very well the position and number of nuclei.
- Both ensure the differentation between morphological forms.

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THE CHARACTERIZATION OF MIDDLE LATENCY AUDITORY RESPONSES RECORDED WITH SURFACE ELECTRODES IN DOGS WITH DIFFERENT INTRACRANIAL LESIONS

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Abstract

Evoked auditory middle latency responses (MLR) offers data regarding the integrity of auditory pathways from caudal colliculi to auditory cortex and could provide information about lesions of these structures. The analysis of the recorded data is very precise in the evaluation of the functional integrity of the auditory pathways from the caudal colliculi to the auditory cortex. Even the MLR examination is not currently performed in veterinary practice, valuable information might be obtained regarding the presence, extension or evolution of different types of intracranial lesions. In this paper we describe and analyze the MLR data recorded with surface electrodes in dogs with different encephalic diseases.

Keywords: *MLR, intracranial lesions, dog*

Introduction

Comparing with human medicine, in current veterinary practice, the recording of auditory evoked potential with middle latency (MLR) for testing dogs with different intracranial lesions is not usually performed, being used only for research (Woods, Alain et al. 1995; Baez-Martin and Cabrera-Abreu 2000; Murrell, de Groot et al. 2004). Different recording methods in which subcutaneous electrodes were used were reported until now (Barth and Di 1991). Using surface electrodes in testing BAER or MLR activity in healty patients (cats or dogs) Musteata et all. (2011, 2013) had obtained different latencies than those previously described in by others (Cauzinille 1997, Myers 1985, Kawasaki 1984, Baez-Martin and Cabrera-Abreu 2000). Usually the analysis of the recorded waves is made both for latencies, amplitudes and general morphological appearance. In this study we describe MLR recording technique performed with surface electrodes in dogs with different intracranial pathologies and we will analyze the latencies and the morphology of recorded waves.

Material and methods

This study was performed on dogs presented to Internal Clinic of Faculty of Veterinary Medicine Iasi diagnosed by clinical appearance and imagistic (MRI, CT or boths) with different intracranian pathologies (e.g. hydrocephalus, traumatic brain injury, infectious encephalitis). The examination was made under general anaesthesia induced with medetomidine hydrochloride (Domitor, Pfizer), 0.05 mg/kg inj. i.m..

The MLR test was performed with the Neuropack S, MEB 9400K Electrodiagnostic System (Nihon Kohden) in the ABR program. The waves were recorded with surface electrodes placed as follows: the active electrode on the vertex, reference electrodes at the

base of each ear and the grounding electrode on the median line, retrooccipitally The area on which the electrodes were placed was trimmed, degreased with alcohol and Skin Pure (Nihon Kohden), and covered with special adhesive EEG paste (Elefix, Nihon Kohden).

An impedance check was performed before each test and it was shown to be lower than 5Ω. Alternating click stimuli of 0.1 ms were applied through earphones inserted into the auditory canal. We performed individual tests on each ear, with a stimulus with standard intensity of 80 dBSPL. Each waveform was the average of 500 stimulations, using a High-cut filter of 20 Hz and a Low-cut filter of 1000 Hz. Artifactual data were automatically rejected; when rejected waveforms have represented more than 5% of the average, the tests were repeated. The waves were manually labeled by the same examiner with N_0, P_0, N_a, P_a and when was possible with N_b.

All MLR waves were analyzed for latencies, amplitudes and general morphological appearance.

Results and discussion

The MLR morphology and latencies of waves in normal dog using surface electrodes were described previously by Musteata and Solcan (2012). They found that analyzing the MLR morphology and latencies with surface electrodes the interpretation of the bioelectric cerebral activity can be asses, giving in the same time reference ranges of latencies: N0 = 4,83±0,53 ms, P0 = 9,01 ± 0,1 ms, Na = 14,38 ± 2,52 ms and Pa = 26,2 ± 1,12.

Congenital hydrocephalus is usually characterized by a dramatic enlargement of the ventricular system with a consequent thinning of the cerebral parenchyma (Thomas 2010). Different reports (Murata 1981, Naidich 1982) mention that the cerebral white matter is firstly and dramatically affected, starting with the periventricular one followed by a centrifuge progression and gray matter involvement. In figure 1 a 3 months old male epileptic dog MLR with CT proved hydrocephalus is presented. In this case the only imagistic features consist in a middle increasing in volumes of the lateral ventricles. At this ages, the open fontanels, put difficulties in the establishment of a hydrocephalous diagnosis. A specific diagnosis implies the proving of the affected periventricular cerebral parenchyma. Moreover, MLR test was necessary to find if mesencephalon and thalamus were affected by ventricles compression.

The obtained values for either right or left ear stimulation showed an increase P_0 and lower P_a latencies. For the right ear stimulation only a decreased N_0 latency was noticed. Changing in MLR waves morphologies and latencies, in both left and right ear stimulations, demonstrated us that auditory pathway anterior to caudal colliculi was involved and a final hydrocephaly diagnosis was fixed.

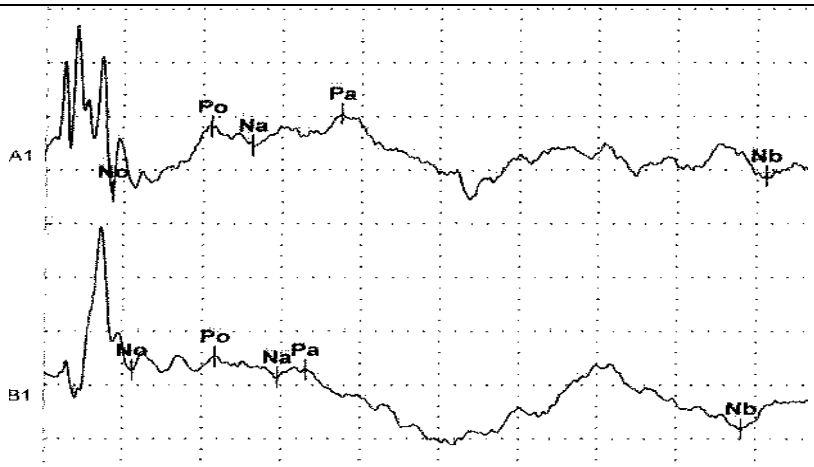

Fig. 1. Chihuahua, 3 months. Congenital hydrocephalus. MLR recorded in left and right ear stimulation at 80 dBSPL. The latencies modified compared to normal values were: in right ear stimulation - $N_{0=}$ 5.6 ms, P_0=10.85 ms, P_a=16.55 ms; in left ear stimulation - P_0=10.55 ms, P_a =18.75 ms.

Traumatic brain injuries are a common neurological presentation in veterinary practice. Probably the most difficult part (before or after the treatment is instituted) is the critical evaluation of the prognosis. As is described (Dewey 2000) immediately after trauma, a first grade of cerebral lesions occurs (involving mainly only the direct action of the traumatic event over the parenchyma). Soon after that a second parenchyma injury occurs and implies mainly the parenchyma from the neighbouring of cerebral parenchyma firstly affected. Days/weeks after the secondary injury, a third cerebral lesion occurs (an effect of the metabolic local disturbance, hypoxia, etc). Because of this evolution the clinical management of this kind of patients must be adapted and pertinent clinical or paraclinical test must be made to appreciate the patient evolution.

Traumatic brain injury was due by a car accident in a 4 months old puppy. The neurologic signs had vestibular origin: wide base stance, left head tilt, tight circle to the left and absence of hopping in both thoracic limbs. CT images could not identify a lesion in caudal fossa because of artifacts due to large amount of bone existing in this region. No IRM exam was performed (declined by the owner). In this situation BAER and MLR tests were the only possibilities for detecting lesions in caudal fossa and determining the extension of lesions. At MLR test P_0 and P_a wave latencies differed from normal limits for both left and right ear (figure 2). We must notice that even the latencies were normal on the MLR trace corresponding to the left ear stimulation the waves have losing their specific morphologies. The segment P0 - Pa tends to be isoelectric. For cases in which the brainstem is affected is difficult to assess: the vestibular signs always are clinical expressed in the detriment of the cerebral hemispheres. In this case after we had performed the MLR and BAER tests we observed that both brainstem and left cerebral hemisphere (corresponding to auditory pathway) is affected, even if on CT examination no specific lesions were observed.

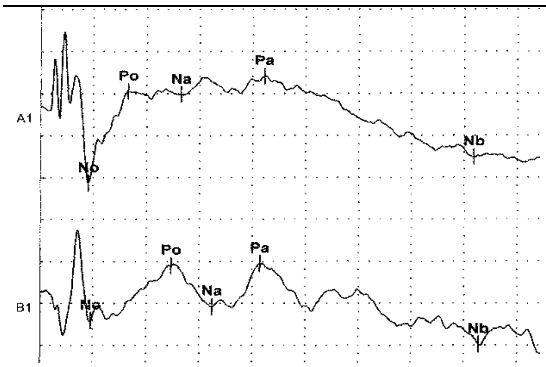

Fig. 2 Mixed breed dog, 4 months old. Traumatic brain injury. MLR recorded in left and right ear stimulation at 80 dBSPL. The latencies modified compared to normal values were: for left ear - P_0 = 8.3 ms, P_a = 21.1 ms; for right ear - P_0 = 12.35 ms; P_a = 20.7 ms)

The diagnostic of distemper encephalitis was very difficult to establish in a 2 years dog where respiratory, digestive or myoclonic jerks signs were absent. The only clinical signs in this dog were cluster seizures, proprioceptive deficits and blindness in both eyes. The possibility that the last two abnormalities to be attributed to postictal deficits were considered, the dog having 4 seizures in the day of consultation. Due to the fact that CT images did not find any abnormalities, and take into account that CT technique could not detect small lesions (inflammatory encephalitis being most of the time overlooked), differences between idiopathic epilepsy and inflammatory lesion of brain was hoped to be made using CSF examination results. In the present case, CSF nucleated cells count and protein content were in normal limits. Alteration of MLR wave latencies (fig. 3) compared to normal values, demonstrated the existence of lesions that included auditory pathways from caudal colliculi to auditory cortex. In this case, the diagnosis of inflammatory brain disease was established based on MLR results, that could identify small lesions that could not be detected in CT exam.

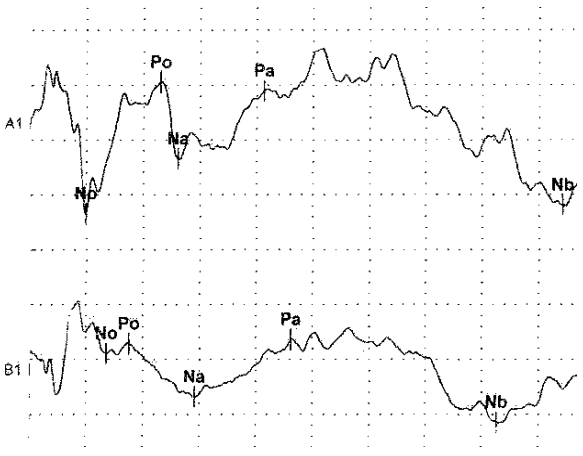

Fig. 3 Labrador, 2 years old. Distemper encephalitis. MLR recorded in left and right ear stimulation at 80 dBSPL. The latencies modified compared to normal values were: for left ear - P_0 =11.5 ms, P_a= 20.65 ms, N_b=47.1 ms; for right ear N_0=6.8 ms, P_0 =8.8 ms, P_a=23.1 ms.

Conclusions

1. The MLR test using surface electrodes offered reliable information about lesions that involve auditory pathways from caudal colliculi to auditory cortex.
2. MLR test can detect lesions that were not observed in CT images due of their small dimensions or because of CT artefact in caudal fossa.

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INFLUENCE OF MICROCLIMATE PARAMETERS IN SWINE REPRODUCTION

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Abstract

Reproductive performance of swine is influenced by many and varied natural and artificial environmental factors, their action manifesting in all "reproductive pathway." Among the most relevant factors , the microclimate of shelters is participating in the welfare, and in the units were is at a level of comfort, over a long period of the year, fades the effect of the season and leads to good results in breeding. Deficient microenvironment has a significant impact on reproduction in pigs.Improved breeds of pigs have the biological microclimate requirements more demanding.

Key words: *microclimate, breeding, swine, wellbeing, ventilation*

Introduction

Physical factors of the microclimate are important because animal body has the most intense reactions to adjust, causing their health, welfare and production.(Teusdea V.2003)

In commercial swine farms animals are maintained in shelters, where the microclimate has different values from the climate outside. The main role that it plays is to remove the influence of harmful environmental factors from the shelter animals and to ensure optimum levels of parameters in microclimate. (Dinu I. 2002)

Romania adopted parameters of microclimate for swine shelters by accepted norms in European Community countries.The microclimate elements are: temperature environment of shelter and from the rest area piglets, relative humidity, ventilation rate, air flow rate, concentration in gases (CO2, NH3, H2S). (Dinu I. 2002)

Parameters for these elements are given in optimum ,maximum and minimum limits.

Ensuring microclimate shelters pigs can be conferred in three ways, namely:

- The mechanical ventilation (conductor)
- The natural ventilation (organized)
- The combined ventilation (mechanical and natural combination)

Microclimate shelter must be within the following parameters

Category of animals	Temperature oC			Relative humidity%		Air speed m / s	
	Min	Max!	Optimum	Min	Max!	Temp.min	Temp.max
Boars	10	24	15	60	70	0.2-0.3	1.0
Pregnant sows	10	24	15 - 18	60	70	0.2-0.3	1.0
Lactating sows	15	24	18 - 22	60	70	0.2-0.3	1.0
Piglets 0-7 days	-	-	32-30	60	70	0.2-0.3	1.0
Piglets 8-14 days	-	-	30 -28	60	70	0.2-0.3	1.0
Piglets 15-21 days	-	-	28 -24	60	70	0.2-0.3	1.0
Piglets 22-28 days	-	-	24 - 22	60	70	0.2-0.3	1.0
Piglets 29-36 days	-	-	22 - 20	60	70	0.2-0.3	1.0
Other	15 to 18	24	18 - 24	55.	70	0.2-0.3	1.0

(Microclimate parameter values are provided in the *Guide for the welfare and protection of breeder pigs)*

The normal level of CO2 must be below 1000ppm.
Indicators for high demand
Reducing emissions to a level:

- Max 10.5 mg/m3 dust
- Max 700 ppm CO2

Air volume table (minimum) necessary shelter livestock is:

- Non-pregnant sows ..... 6.0 m^3 / head
- Pregnant and lactating sows ..... 21.0 m^3 / head
- Piglets ..... 3.0 m^3 / head
- Fattening pigs ..... 3.5 m^3 / head
- Boars .... 20.0 m^3 / head

Category of animals	**Necessary air m^3 / h / head**	
	Minimum winter	**Maximum summer**
Boars and sows	70-85	150
Sows with piglets	100-150	200
Piglets	10 to 20	50
30-60 kg growers	15	65
Fattening pigs over 60 kg	45	120

Materials and methods

The research was conducted in a farm,with breeding and fattening profil ,Asia Intercom, city Dragos Voda, Calarasi county.The farm is licensed by all organisms in law, including multiplication activity-selection breeding gilts. Farm meets all operating conditions for hybridization sows in closed system.

Equipping with mechanized feeding, food distribution dispensers, watering with pacifier (eliminating water loss), ventilation by depressurization, cooling (water curtain), underfloor heating, are performed each compartment according to the growth phase.

All these features are supported in operation of measuring, and control-performance computers are create optimal welfare and comfort for animals to express their superior genetic potential they have.

Organization of production process

Hall 1 - two compartments. Phase-gestation 1

Section 1 – Sectors boars - Waiting- Service.

Sows are organized into groups mount ,are housed in 8 stoll X 5 collective heads. Here is the point for semen collection from boars.

Section 2 artificial insemination.

Accommodation for sows is in individual box (2x41), which makes artificial mount.

Hall 2-two compartments.Phase-gestation 2.

For efficient accommodation, spaces were made up of two compartments, one of 16 and 14 stolls which can accommodate 180 heads.

Hall 3-Maternity-6 compartments. Phase-lactating sows.

Compartment is sized and equipped to accommodate a group of 12 sows in individual stalls maternity. In this section the sows stationary averaged 28 days.In order to create optimal conditions for product development, this section is equipped with underfloor heating for piglets, controlled by thermostat and temperature sensor, which provides temperature control from warm bed in a range between 24 º -32 º C.

Hall 4-Nursery Section -6 compartments.Growth phase-Youth

Each compartment has 6 stalls, arranged in two rows collective, designed to retrieve weaned piglets from a group of 12 sows.Thermal comfort is achieved with the same system as in the maternity.

Hall 5, 6,7 - 12 Finishing compartments. Phase - finishing (fattening)-select gilts.

In a shelter are organized 4 compartments, one compartment is equipped with 6 collective stalls.

Animal standing in this sector about 90 days, during which reach an average weight of 105 kg in living.

The factors considered in this paper are physical factors such as temperature, relative humidity and air speed.

In order to establish the physical factors of microclimate was used an electronic multifunctional device Lutron LM 8000, 4 in 1 professional measuring instrument: Anemometer, Hygrometer, Thermometer and Light meter.

Shelter	**Assesed parameters**											
	Temperature (°C)				**Relative humidity (%)**				**Air speed (m / s)**			
Hall 2	C1		C 2		C1		C 2		C 1		C2	
	18-19		18-20		63-65		63-64		0,00-0,01		0,00-0,01	
Hall 5	C1	C 2	C3	C4	C1	C2	C3	C4	C1	C2	C3	C4
	18-21	18-21	18-21	18-21	63-64	63-64	63-64	63-64	0-0,1	0-0,1	0-0,1	0-0,1
Hall 6	C1	C 2	C3	C4	C1	C2	C3	C4	C1	C2	C3	C4
	18-20	18-20	18-20	18-20	64-65	64-65	64-65	64-65	0-0,1	0-0,1	0-0,1	0-0,1
Hall 7	C1	C 2	C 3	C4	C1	C2	C3	C4	C1	C2	C3	C4
	17-21	17-21	17-21	17-21	63-68	63-68	63-68	63-68	0-0,1	0-0,1	0-0,1	0-0,1

Results and discussion

These information are part of the environmental control register of the farm ,and the parameter values checked are collected in January-April 2013.The values of these parameters are found in normal ventilation due to computer system, which ensures a constant level in relation to the physiological needs of the animals.When exceeding the optimal parameters, fans stop and reverse.The automatic system also includes concentrations of harmful gases that analyzes based switch the ventilation system.

Along with providing adequate food recipes and water at discretion, microclimate shelters is a key factor in obtaining economic output.

Parameters production in optimum microclimate in farm Dragos Voda

Piglets born alive / calving	11-11.7
Stillborn piglets / farrowing	<0.6
Mortality before weaning (%)	10
Weaned piglets / farrowing	10.2-11.2
Piglet weight at weaning (24-28zile)	6.5.
Piglets weaned / female / year	22.5-24

The good condition of microclimate in this farm lead to productive efficiency. Total number of piglets from each of the sows maintained farm as an undoubtedly key to business efficiency.Optimizing and maintaining production levels depends heavily by microclimate conditions.

Conclusions

The purpose of this paper is to demonstrate how important role has microclimate in reproduction pigs,and how involved is in production optimization.

All the microclimate recorded values falling within the normal range, due to the ventilation system automatically.

Only by creating optimal conditions of wealth and comfort ,animals can express their superior genetic potential .

The microclimate of shelters is a key factor in obtaining economic output.

Physical factors of the microclimate influence the health,welfare and animal breeding.

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HEPATIC PROFILE OF DAIRY COWS IN RESPONSE TO A TREATMENT OF LIVER DISEASES WITH A PROPYLENE GLYCOL BASED PRODUCT

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Abstract
The effect of administration of a propylene glycol based product on blood biochemical parameters was studied on 40 dairy cows as prevention and treatment of liver diseases. Investigations were conducted on the Bălţată Neagră Românească (BNR) breed dairy cows from a farm in Iaşi County. These cows was divided in 3 groups: Control(C)) treatment (T), prevention (P); each cow from the last 2 groups was drenched with 500 ml product twice per day, 3 days. Blood was collected and analyzed for a s series of metabolites: glucose, proteins, cholesterol and liver enzymes: *alanineaminotransferase (ALT), aspartateaminotransferase (AST), gammaglutamyltransferase (GGT), alkaline phosphatases (PAL) and lactate dehydrogenase (LDH). Urine samples were tested with a urinary strip*.

Keywords: *ketosis, liver enzymes, dairy cows*

Introduction

Liver diseases are a series of disorders frequently observed in dairy cows during first period of lactation witch causes important economic loss in dairy herds. Blood parameters that may reflect nutrient status of the cow, such as glucose, cholesterol, proteins and also enzymes are direct indicators and reveal liver status. These major disorders frequently seen in after parturition are ketosis with liver damage and fatty liver disease. Both of these conditions have been shown to be associated with other diseases in the periparturient period (Bobe, 2004).

The main objective of the study was to asses the efficacy of a propylene based product on hepatic biochemical parameters in the treatment of liver diseases in dairy cows in the postpartum period.

Material and methods

Thirty Bălţată cu Negru Romanească dairy cows were selected from a farm in Iasi County. They were divided in two groups: control group, 15 cows received an oral drench of 500 ml product/ cow per day for three, and a treated group 15 cows received an oral drench of 200 ml product/ cow twice a day for three days. The composition of the used product is: propyleneglycole 82%, water 17, 6%, sorbitole 0, 24%, magnesium 0, 06%, vitamins 0, 09%, flavour 0, 009%.

Blood samples were collected from the coccygeal vein, in tubes containing clot activator a 1, 3, 7 and 30 days following the last administration. The serum was harvest after centrifugation al 3000 rpm for 10 minute and used to determine a series of metabolites. AST, ALT, GGT; LDH, total protein, glucose concentrations were determined by enzimatic spectrophotometry using an automatic chemistry analyzer (Accent 200, Cormay).

The measurement of ketone bodies in the urine samples was determined on a semi quantitative scale by rapid urinary strips (Uripath, Plasmatec laboratory, UK).

The date between the control and treated group were compared using student's t-test, Windows Excel.

Results and discussions

Average concentrations of blood biochemical parameters are presented in the tables 1 and 2.

Table 1. The average concentrations of biochemical parameters in control group

Nr	Parameter	D0 M ± SD	D1 M ± SD	D7 M ± SD	D30 M ± SD	Normal values
1.	GLU(mg/dL)	79,22±18,4	67,39±18,97	67,4±15,08	71,05±10,30	**42-100***
2.	CHO(mg/dL)	50,54±8,9	48,01±11,65	56,18±16,58	61,20±15,88	**62-193***
3.	PT(g/l)	5,59±0,51	6,18±0,73	6,6±1,07	6,04±0,65	**6,7-7,5***
3.	ALT(IU/L)	20,66±2,73	18,41±3,8	20,82±5,05	21,18 ±12,95	**6,9-35***
4.	AST(IU/L)	101,87±24,16	114±19,29	111,57±25,43	95,84 ±12,95	**60-125***
5.	PAL(IU/L)	143,12±39,55	128,75±51,88	25,37±4,78	112,81±32,4	**18-153***
6.	GGT(IU/L)	19,66±4,14	22,55±1,93	25,37±4,78	24,81±3,77	**4,9-26***
7.	**LDH(IU/L)**	**328,78±3,54**	**328,85±0,86**	**327,75±2,87**	**328, 84±6,25**	**309-938***

* references guide (Merck Veterinary Manual, on line edition 2013)

Table 2. The average concentrations of biochemical parameters in Treated group

Nr	Parameter	D0 M ± SD	D1 M ± SD	D7 M ± SD	D30 M ± SD	Normal values
1.	GLU(mg/dL)	54,21±9,32	61,28±13,18	67,16±10,72	74,11±11,73	**42-100***
2.	CHO(mg/dL)	72,31±24,81	76±24,81	98,88±61,57	83,98±35,46	**62-193***
3.	PT(g/l)	6,04±0,71	5,88±0,71	6,23±0,52	6,41±0,34	**6,7-7,5***
3.	ALT(IU/L)	24,13±3,35	25,03±9,84	24,51±5,72	25,45±7,34	**6,9-35***
4.	AST(IU/L)	134,26±24,45	152,76±34,28	128,58±31,41	100,13±16,89	**60-125***
5.	PAL(IU/L)	65,66±24,76	46,55±18,01	74,72±21,31	99,86±26,46	**18-153***
6.	GGT(IU/L)	25,2±3,73	21,9±7,90	20,96±5,30	23,55±3,57	**4,9-26***
7.	**LDH(IU/L)**	**366±30,38**	**363,76±41,44**	**324,63±22,70**	**340,01±13,11**	**309-938***

* references guide (Merck Veterinary Manual, on line edition 2013)

Compared with the concentration of glucose the control group had higher values that the treated group, with statistically significant differences (p<0, 05).

Average concentration of glucose was at the lower limit in the treated group, as consequence of ketosis with liver damage. Hypoglycemia is it correlated with a series of conditions like weigh loss, underproduction, infertility, liver and kidney steatosis and ketosis.

Ketosis with hypoglycemia regards dairy cows it is always observed in cows with an energy deficit in the peak of lactation, 3 to 4 weeks after parturition. These cows had the

lowest body condition score. However, after the drench in treated group at day 1,7,30 following the last administration we observed an increased concentration of glucose in the blood circulation, but the differences were not were not statistically significant ($p > 0.05$). These results were in agreement with the results observed by Grummer, 1997, Rukkwamsuk 2004. In the control group this parameter remained in the normal reference value.

Average serum cholesterol concentrations for the control group were below the inferior limit. In case of liver damage we can observe an increase concentration, in acute inflammatory or degenerative diseases, and a decrease concentration in biliary obstruction, acetonemia. The oral administration of the product increased the concentrations for the treated group but with no statistical significance.

Serum total proteins were situated below the inferior physiological limit in both groups. The majority of proteins are synthesized in the liver, so a decrease can be the result of a liver damage like necrosis. Hypoproteinemia it is observed in the following situations: liver chronic diseases, malabsorption, cahexia.

AST, GGT, and ALT are considered to be liver associated enzymes which are released into the bloodstream when hepatocytes destruction takes place, although ALT activity is not considered a specific marker for cattle (Russel, 2007).

Regarding this three parameters the determined concentrations showed statistical significance between the two groups at day 0. Increased concentrations of AST are seen in cows suffering from different degrees of liver failure and values above 100 IU / L are consistent with the existence of fatty liver syndrome (Gerolff, 1999).

The determination of GGT activity is the most sensible test for hepatobiliary diseases in cows. This is due to hepatic lesions and swelling of hepatocites that compress the biliary ducts leading to cholestasis (Duncan, 1994). Our determinations showed normal concentrations of GGT in both groups, but in the treated group the average was closer to the upper pshysiological range suggesting a disposition to cholestasis close related to a liver damage in ketotic cows.

LDH is found in large amounts in the liver, kidney, muscle and myocardium. The activity of this enzyme is frequently elevated in acute hepatocellular damage, may be slightly decreased to normal values in animals with chronic liver failure (Oglive, 1998). LDH concentrations presented a difference with statistical significance between the two groups at day 0; the average concentrations were in the physiological range.

On a semiquantitative scale the average urine ketone concentration was 2, 33 ±1, 29 mmol/L. BHB is the predominant ketone body found in the circulation of cows. Although the presence of ketone bodies is normal as an adaptative response to parturition, the excess amount passing 1200mmol/L of BHB is considered to be the threshold of subclinical ketosis (Nielen et al., 1994).

For all cows in the treated group urine strip test was negative for ketone after second administration of the product; in the control group the test was negative from day 0.

Conclusions

In this study administration of propylene glycol had a positive effect on blood glucose concentration by providing a precursor for the hepatic gluconeogenesis with the result of increased energy balance of cows in early lactation.

Oral administration after parturition generally improved the energy status and maintained a normal concentration of glucose.

Administration of the propylene glycol product decreased until disappearance urine concentrations of ketone bodies by reducing the mobilization of adipose tissue, having anti-ketogenic properties.

In conclusion the supplementation of the ratio cows in early lactation reduced the risk of subclinical and clinical ketosis by s a tendency to increase the concentrations of serum glucose and decrease the concentrations of some liver enzymes: AST, GGT.

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INTERNATIONAL TRAVEL INCREASE AND MALARIA IMPORTATION IN ROMANIA, 2007-2012

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Abstract

Malaria is a rare diagnosis in Europe, but it is a medical emergency. A travel history is the key to suspecting malaria and is mandatory in patients with fever. Malaria continues to have a high morbidity rate associated among European travelers. This report aims to assess the epidemiological characteristics of imported malaria in Romania, in the context of international travel increase, and to compare them with the data reported by other European countries. The number of Romania citizens who traveled to endemic areas in 2007-2012 increased as compared to the previous years. During the years 2007–2012, 142 cases of imported malaria were registered in Romania, with 2 deaths. Most patients were male (137) and most of them acquired the infection in Africa. Plasmodium falciparum was involved in most cases.

Key Words: *malaria, Plsamodium falciparum*

Introduction

Malaria is the most commonly spread disease in the world. Millions of people are annually infected in Africa, India, South-Eastern Asia, Middle East, Southern and Central America, and continuously, over 41% of the world population is under the risk of malaria infection.

Thousands of sick people or people infected with malaria travel annually in malaria free countries, thus reintroducing the risk of malaria reemerging. Malaria is currently responsible for twice as many deaths as AIDS is. Malaria constitutes a serious obstacle in the social and economic evolution of many countries in the world.

In the 2003 report, UNICEF and OMS declared that malaria is the main cause for infants death and the biggests threat for pregnant women and new-born babies. However, the strategies adopted în 1998, that stipulate the use of insecticide nets and easy access to therapeutic drugs reported good results. In spite of thiese measures, no country in Africa registered a substantial decrease of cases of malaria infections. (Walker K,2007).

Snow et al. made a mapping of malaria distribution within the world in 2002, the year when 515 millions of clinic episodes of Pl. falciparum have been registered. These global estimations are over 50% larger than the ones reported by The World Health Organization OMS and 200% bigger for areas outside Africa.

Malaria is rarely diagnosed in Europe, but still represents a medical emergency. In Europe, malaria was eradicated except in most countries except Azerbaidjan, Georgia, Kârgâzstan, Tadjikistan and Turkey. It is estimated that 25-30 millions of people travel annually from Europe to areas where malaria can be transmitted. The most up-to-date information from Great Britain and Europe reveal an increase in malaria imported cases, the

same is also proven by the data for SUA, where a 14% increase has been registered by comparison with 2010 **(**Helena H Askling et al.2012).

More than 5 million immigrants from Africa live in Europe, a third of them coming from below the Sahara area in Africa. Following the eruption of malaria in Greece in 2011, ECDC organized a conference in Europe in January 2012 in order to establish the risk of transmitting the parasite. From the available information, it has been concluded that malaria transmission in Greece seems to have been produced following the anual introduction of the parasite by means of immigrants.

The increase in native cases signaled in 2011 indicates that conditions can be favourable for local transmission in affected areas.

In the past 10 years sporadic native malaria transmissions were signaled in many European countries: Bulgaria, France, Germany, Greece, Italy and Spain. 63 cases of native malaria have been registered in Greece between 21st of May and 5th of December 2011.

For 1999, 32 malaria cases have been reported in Romania. In proportion of 78%, the ethiologic agent Pl.falciparum, 5 cases imported from Turkey had Pl.vivax as ethiologic agent. The most affected age group was between ages 21-50, 65,65% of the cases were registered with sailors (Nicolaiciuc D, et.al 1999).

In 2010, 47300000 people of foreign origin have been present in the European Union, who correspond to 9,4% out of the total population. Most of them, 31,4 millions were born in countries outside the European Union, while 16 millioans were born in another EU state. The data describing people who come from malaria endemic countries are insufficient. Estimations indicate that more than 5 millioan African immigrants could live in Europe. Out of them, almost 2 thirds are from Northern Africa (Algeria, Maroco and Tunisia), nd the rest are from areas below Sahara in Africa, most part of Western Africa (Ghana, Nigeria and Senegal). Aproximately 4 millioans come from South-Eastern Asia and almost 2,2 millioans come from Latin America.

Most cases were imported in Western Europe, France, UK, Germany and Italy representing more than 70% out of all cases. (Begooa Monge-Maillo et.al.2012).

Material and method

The hereby paper undertakes the establishment of the risk of malaria reemerging in Romania, following the increase of travels within malaria endemic areas.

For ths paper there have been taken into consideration the malaria cases diagnosed in Romania between 2007-2012.

There has been made a distribution of cases according to age, sex, profession, purpose of travel in the endemic areas, continent and country where the infestation has been produced. Also, it has been taken into consideration the Plasmodium species that produced the infection and also the establishment of profilactic measures before and during the period of stay in the endemic area that produced the infection.

Results and discussions

Malaria cases reported in 2007

24 malaria cases have been reported in 2007 (all of them having been imported), incidence of 0,11%000, comparable to the one in 2006, which was smaller.

For most cases of registered malaria, the endemic area of infestation was the continent of Africa (87,5%).

In most cases 15 (62,5%) the aim of the trip to malaria endemic areas was professional.

Out of the total malaria cases, 5 of them (20%) were caused by Pl.vivax, 16 cases (66%) Pl.falciparum, 1 case(4%) Pl.ovalae, and 2 cases associated Pl.falciparum and Pl.malariae.

Table 1. Diagnosed Plasmodium species

Species	No. Of cases	Native	Imported
Pl.vivax	5		5
Pl. Falciparum	16		16
Pl.malariae			
Pl.ovale	1		1
Pl.f/Pl.v			
Pl.f/Pl.m	2		2
Pl. f/Pl .o			
Total	24		24

Table 2. Age group (sex of the patients diagnosed with malarie)

Age group (years)	Imported males	Imported females
0-4		
5-10		
11-20		
21-30	4	
31-40	5	
41-50	6	1
50+	8	
Unknown		
TOTAL	24	

The biggest incidence of malaria cases was reported at age group over 50 years (33%), 96% of the cases being males. In 2007 a death was also registered (4,16%) (male, 40, country of infestation Uganda), whish presented a toxic form of malaria with Pl.falciparum, without profilactic medication.

Malaria cases reported în 2008

In 2008 13 cases of malaria were reported in the whole country (all of them having been imported)

Table 3. Malaria cases diagnosed in Romania in 2008

Species	No. Of cases	Native	Imported
Pl.vivax	3		3
Pl. Falciparum	6		6
Pl.malariae	2		2
Pl.ovale			
Pl.f/Pl.v	2		2
Pl.f/Pl.m			
Total	13		13

Out of the total 13 reported cases, 78% were caused by Pl.falciparum, followed by Pl.vivax 39%.

Table 4. Age groupe (sex of the patients diagnosed with malaria).

Age group (years)	Imported males	Imported Females
0-4		
5-10		
11-20		
21-30	2	
31-40	7	
41-50	1	
50+	3	
Unknown		
TOTAL	13	

Out of the total cases of diagnosed malaria, 91% were males aged between 31-40.

The majority of diagnbosed cases had the African continent as a correspondent endemic area, 11 (84,6%), followed by Asia having 1 case (7,69%). The import countries were: China (1 case), The Ivory Coast (1 case), RD Congo (2 cases), Camerun (1 case), India (1 caz), Nigeria (4 cases), Senegal (1 case), Zambia (1 case), Uganda (1 case).

Out of the 13 patioents diagnosed with maalria, 7 (53,84%) of them did not take any profilactic medication, 5 (38,46%) of them took incomplete or inconsistent profilactic medication and 1 case (7.69%) followed a complete and correct scheme of profilactic medication.

Table 5. The purpose of the trip/staying in the country where malaria infestation was produced

Population group	TOTAL
Immigrants/refugees	**1**
Workers/professional	**9**
Students	**1**
Military personnel	**2**
Turists	
Crue traveling by air or sea	
Unknown	
TOTAL	**13**

For the year 2008 the cases of diagnosed malaria reached 13, they have been mostly represented by males aged between 31-40 (91%), who travelled in endemic countries on business purposes. Out of the total presented, 53,84% did not take any profilactoc medication, which describes a poor informational program of the population over the risk of traveling to these areas.

Malaria cases diagnosed in 2009 in Romania

12 malaria cases were diagnosed in Romania in 2009, all of them were imported, no death was registered. The general incidence by malaria was 0.05%0000 in 2009, compared to 0,6%0000 in Europe in 2008, which placed Romania on the 18th place within the european countries.

Table 6. Plasmodium species registered in malaria cases diagnosed in 2009

Species	No. Of cases	Native	Imported
Pl.vivax	**2**		**2**
Pl.falciparum	**8**		**8**
Pl.ovale			
Pl.f./Pl.v.			
Pl.f./Pl.m.	**1**		**1**
Pl.f./Pl.o.			
Pl.v./Pl.o.			
Pl.v./Pl.m.			
Pl.m./Pl.o.			
Pl. Speciae	**1**		**1**
TOTAL	**12**		**12**

The majority of diagnosed cases were caused by Pl. falciparum (66%), followed by Pl. vivax (16,6%),Pl.f./Pl.m. (8,33%), Pl.speciae (8,33%).

All registered diagnosed cases were males, more frequently aged 31-40 and over 50+ (33,3%).

Table 7. Age group registered with diagnosed cases

Age group (years)	Males	Females
0-4	**0**	**0**
5-10	**0**	**0**
11-20	**0**	**0**
21-30	**1**	**0**
31-40	**4**	**0**
41-50	**3**	**0**
50+	**4**	**0**
Unknown	**0**	**0**
TOTAL	**12**	**0**

83,3% of the cases had the African continent as endemic area (83,3%), followed by Asia (16,6%).

The countries involced in import cases were: The Ivory Coast (2 cases), Congo (2 cases), India (1 case), Liberia (2 cases), Nigeria (1 case), Philipines(1 case), Siera Leone (1 case).

Table 10.The purpose of trip/stay in the coubntry where the infestation was produced

Population Group	TOTAL
Immigrants/refugees	**0**
Workers/professional	**9**
Students	**0**
Millitary personnel	**1**
Turists	**0**
Crue travelling by air and sea	**2**
Unknown	**0**
TOTAL	**12**

Out of the 12 malaria cases, 8 cases (66,67%) did not follow any profilactic medication, 3(25%) took an incomplete or inconsistent profilactic medication and only 1 (8,33%) took a complete profilactic medication with doxicyline.

Malaria cases reported in 2010

19 cases of malaria were diagnosed in 2010, all from import, no death was registered. Malaria incidence in 2010 was 0,08%0000, compared to 0,05%0000 in 2009.

Table 11. Plasmodium species present in diagnosed cases

Species	No. of cases	Native	Imported
Pl.falciparum	**16**		**16**
Pl.vivax	**3**		**3**
TOTAL	**19**		**19**

Pl.falciparum represented the cause for 84,2% of the cases, followed by Pl.vivax (15,7%).

Table 12. Malaria cases distribution by age and sex

Age group (years)	Males	Females
20-24	**3**	**1**
30-34	**3**	**0**
35-39	**2**	**0**
40-44	**4**	**1**
45-49	**0**	**0**
55-59	**3**	**1**
60+	**1**	**0**
TOTAL	**16**	**3**

Malaria diagnosed cases in 2010 were regstered at various age groups 20-60+, 89,4% travelling in endemic areas for business purposes, corelated with the world crises and the migration of the work force abroad, in various areas.

Table 15. Puirpose of trip/stay in the country where infestation was produced

Population group	Total
Immigrants/refugees	**1**
Workers/professional	**17**
Students	**1**
Military poersonnel	**0**
Turists	**0**
Crue travelling by air, sea	**0**
Unknown	**0**
Total	**19**

Ourt of the 19 diagnosed malaria cases, 16 cases (84,2%) did not take any profilactic medication, 3 cases (16%) took an incmplete or inconsistent profilactic medication.

Africa continued to be the continent registering the most cases of all (78,9%), followed by Asia (21%). The origin countries where infestation was produced were : Birmania (1 case), Camerun (1 case), Ecuatorial Guinee (7 cases), Ghana (2 cases), India (2 cases), Madagascar (1 case), Nigeria (4 cases), Pakistan (1 case).

Malaria cases reported in 2011

42 cases were reported in 2011, out of which 40 were confirmed by the laboratory, all of them from import. The general incidence through malaria is 0,19%0000 compared to 0,08%0000 in 2010. Most cases come from the African contnent (36 cases), mainly from Ecuatorial Guinee (19 cases). The predominant species is Pl.Falciparum (28 cases). The age of the persons infested was, as in the precdent year, very diverse, between 20-59, which shows the continuous migration of the work force, 78,5% travelling with business purposes.

Table 18. The purpose of travel/stay in the country where the infestation was produced

Population group	Total
Immigrants/refugees	**1**
Workers/professional	**33**
Students	**1**
Military personnel	**0**
Turists	**4**
Crue travelling by air, sea	**1**
Unknown	**0**
Total	**40**

As to what the cases in Greece are concerned (Peloponezian area), considered as having natoive transmission, there were 2 Romanian persons who lived several months in this area where a lot of citizens from Afganistan, India and Pakistan live and work legally/illegally, who are possible carriers of Plasmodium vivax (diagnosed species). In the international reports there are 4 signaled cases with the Romanain citizens working in Greece, 2 being diagnosed in Romania.

2 death cases were also registered. One of them was a female aged 60 de ani, with rheumathoid poliarthritis, under treatment with Metotrexat for 2 years. The death was due to the complications caused by hepatioc and renal insufficiency. The diagnosed species was Pl.falciparum, the infestation country was Ghana.

The second case of death was a male aged 24 ani, who was late diagnosed with malaria Pl.falciparum species, infestation country Nigeria. Death came after hyperparasitemis, hepatic and renal insufficiency and cardio-vascular colaps.

Out of the 40 cases, 12 of them took specific profilactic medication, but not the dose recmmended most of the times, and neither for the whole period of staying in the endemic area.

Cases reported in 2012

In 2012 32 cases were reported in the whole country, however detailed information i9s not yet offered by CPCBT.

In 2013 3 cases were diagnosed in Iasi, out of which 1 case came from Ecuador (infested with Pl.vivax), it was a fall after 6 months from returning into ther country. The case re4ceived treatment at the Hospital for Infectious Diseases in Iasi,a nd after 1 month from leaving the hospital the person came back causing high fever. At the moment the person is in the hospital in Bucharest, under treatment.

Conclusions

Having a media of 84%, the malaria diagnosed cases in Romania during 2007-2012, coame from the African continent, followed by a much lower prevalence by Asian continent. Plasmodium falciparum represented the species diagnosed in a proportion of 72%.

In 74,88% of the cases the purpose of the trip/stay in the endemic areas was the business purpose, majority males aged between 20-50+. By comparison to the precedent years, the incidence of malaia in Romania increased from 0,11%000 in 2006 to 0,19%0000, which can be explained by the economic crises that has been trigerred, that lead the

population to having to look for jobs in areas as variate as possible. In most cases the profilactic medication was completely missing or was inadequately or inconsistenly administered. Thus, for preventing malaria infestation in the context of intesifying trips with any purposes in the endemic areas it is rcommended a better proces of informing all persons concerning measures of prevention (phisical and medication).

The necessity of mapping global malaria distribution is large because this undergoes a continuous change, and the expenses involving malaria control could produce a huge economic imbalance. Malaria is seasonal in the areas where climatic conditions differ from a season to another, allowing periodic development of malaria parasite and vector. The indigenuous population does not have time to develop a suficient immunity, thus the groups of persons that present a risk of contracting the disease are hard to identify and the mortality rate can be higher. In most cases, the plagues involve the absence, decrease, or loss of immunity, which imposes as an alarm for our country, where our population completely lacks immunity.

Thus, climatic factors play a very important role whithin the risk of reemergence of malaria in several parts of the world, where vectors are present. Parasites develop in the mosquito at a speed dependend on the background temperature.

The cases diagnosed in two Romanian persons who worked in Greece (country where malaria was eradicated) prove that the climatic factors which are in a continuous change and the presence of vectors are sufficient for triggering malaria plague.

Now all malaria cases in Romania come from import from endemic areas of a large number of persons (on business or turistic purposes).

In Romania, the risk of malaria reemergence constitutes the presence of anofel vector(I have shown in precedent papers the presence of vectors in Anopheles maculipennis: Anopheles messae, atroparvus, maculipennis and a new species for Romania, Anopheles labranchiae, which is the main vector of malaria in Italy). IN Romania malaria does not represent a public medical emergency up to the present.

Africa remains the main endemic area where the Romanian cases are imported.

A major problem in trying to control the avoidance of import malaria represents the lack of chemioprofilaxy, due to lack of medication (especialy for the resistant forms of Pl.falciparum). Cloroquine is usually used in Romania as a chemioprofilactic agent, and in our country 30-60 cases of import malaria are registred annualy.

In temperate and subtropical areas, the development of malaria varies from one year to another. In many parts of the world, malaria presents a seasonal periodicity due to relapses, toghether with an increase of transmision frequency.

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LOCAL OZONE MONOTHERAPY IN SKIN INFECTION IN A DOG – CASE REPORT

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Abstract
A case report, based on ozone monotherapy in a dog with a localised skin infection, is being detailed, considering the role of septic skin wounds in generating local, regional or systemic infections, the growth of bacterial resistance to antibiotics and the relatively few treatment options.A Bozon N Econika ozone generator was used, powered by a Bozon N Econika oxygen/ argon generator. The combination of oxygen and ozone was administered locally by daily injections, for 5 days in a row. During the course of treatment, neither local or general therapy, nor any form of wound dressing was applied.5 days after starting the treatment, the septic infection was 100% healed (suppuration disappeared by day 3), and a 95% in local healing was observed, which finished by eliminating the crust. The effects of ozone were evaluated clinically and photo statically, at intervals of 12 – 24 hours, using a Nikon D3X DSLR professional camera.This case report shows the therapeutic effects of ozone (antiinfective, antiedematous, analgesic, regenerative) in localised skin infections, when used in monotherapy.

Key words*: dog, ozone, monotherapy, skin infection*

Introduction

The cutaneous tissue forms an outside barrier for the body, having multiple roles: protection (mechanical protection, against direct light, water proof), in biochemical homeostasis, metabolic, immunologic, sensorial role and also in communication between individuals (1, 4). Because it is constantly exposed to the external factors, it can suffer many traumatic injuries, followed by septic inflammatory conditions (1, 2).

Because of the discomfort created by the up mentioned conditions and also due to the possibilities of generalization, surgical approach is constantly changing, trying to shorten the time of healing, making it possible for the animal to faster recuperate from such affliction. In the past years, ozone therapy has presented itself as an alternative to classic surgery (excision) combined with local or general antibiotic medication (3).

Ozone (or tri-oxygen, a tri-atomic molecule, consisting of three oxygen atoms) is a pale blue gas, slightly soluble in water and it is formed from di-oxygen, by the action of ultraviolet light. Ozone is a powerful oxidant, and has a characteristic smell, which can be detected at about 0.02 μl/l in the air. It can be obtained from oxygen in electric generators, at discharges of 4.000 – 13.000 V (4, 5, 6, 7).

Also known, in medicine, as the "antibiotic which doesn't induce resistance", ozone act directly or indirectly, through the reaction compounds (5, 6, 7, 8, 9) as it follows:
- it destroys the cellular wall of bacteria, viruses or fungi by H_2O_2 and lipid oxidation;
- it increases the capacity of red blood cells to resist to deformitation of the membrane, and it releases oxygen at a tissular level;
- it plays an antiinflammatory role by modulating the activity of prostaglandins, an analgesic one (it raises the threshold of pain perception), it reduces local edema, by dilating the blood vessels and by stimulating angiogenesis, it relaxes the muscles, etc.

Knowing these benefic roles of ozone, the present paper is detailing the results obtained after giving a mixture of oxygen and ozone, in a chronic, localized skin infection in a dog.

Material and method

The study has been undertaken at the Centrovet Clinic, Bucharest, in february 2013. The patient selected was a 6 year-old female crossed breed dog, in good shape. Behavioral, the dog oftenly adopts the sitting position, noticing the hypertrophic scar regarding the ischial tuberosities (fig. 1).

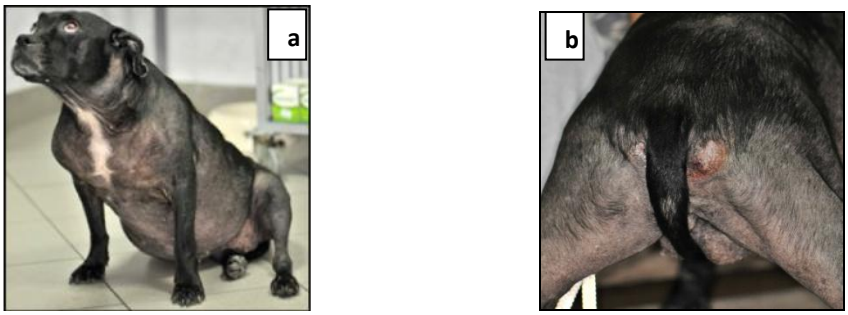

Fig. 1. a) ischial decubitus, b) hypertrophy of the skin regarding the ischial tuberosities

Gradually, the patient, due to a chronic affliction of the left hip joint, prefers to lean more on the right side, leading to trauma of the hypertrophic skin, with local deformity, hyperemia and hyperthermia. In the center of the region, a fistula is noticed, with loss of the skin tissue, from which a muco-purulent secretion is draining (fig. 2).

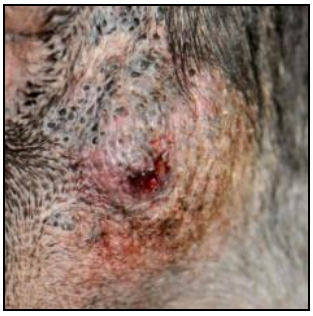

Fig. 2. Detailed image of the infected area surrounding the right ischial tuberosity

The medical mixture of ozone and oxygen was obtained using a Bozon N-SO Econika system, formed from: a medical ozone generator (Bozon Oxy Econika) – 96% oxygen, 4% argon, 0-5 l/minute adjustable flow; a N Bozon medical ozone generator, with pre-established medical technical characteristics (ozone concentration: 0,5 – 100 mg/l, with a +/- 10% variation, flow: 1 – 1000 ml/minute, procedure duration: 1 – 7200 seconds).

Non specific supplies that were used during the procedure were 30 ml sterile syringes and 22 gauge needles.

The mixture of ozone and oxygen was used for treatment purposes at a concentration of 15 µg/ml and 500 ml/min flow, for 2 seconds; 0,5 – 3 ml/ injection site were administered all around the area in 6-8 points, at 2-4 mm apart, hypodermally and intradermally (fig. 3). This procedure was preceded by local antisepsis.

The injections were administered once a day for 5 days, with clinical examination every 12 hours.

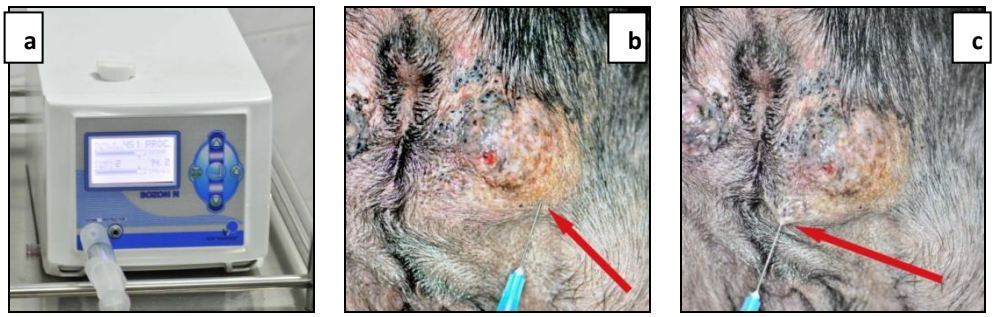

Fig 3 – Obtaining the mixture of ozone in oxygen (15 µg/ml) and injecting it
a) producing the mixture and filling the syringe; b) hipodermal injection
c) intradermal injection

Results and discussions

The liquid state of the ozone-oxygen mixture makes it easier to be administered in the pre established injection sites, on the animal well confined in a position that isn't discomforting. After inoculation, slight pressure is applied for 5 seconds, and the transition to the other point must not be detected by the animal.

Clinical evaluation points out that this procedure is safe for the animal and it doesn't induce discomfort, as seen in the unchanged behavior. Therapeutic effects are rapidly noticed, therefore, ozone therapy is clearly superior to other classic treatment options.

Clinical parameters and their evolution are detailed in table 1.

The data presented in this table point out the favorable evolution, even at 12 hours after the first injection, therefore, 36 hours after the treatment was initiated, several clinical parameters had values close to zero. Because some aspects, such as erythema and edema, persisted even at 72 hours, ozone therapy was continued until complete resolution, in order to avoid relapses.

Clinical visual inspection follows local erythema, behavior (adopting normal recumbence), suppuration (quantitative, qualitative), appearance of crust, and normal healing. Palpation gives us information about the presence of pain and local edema.

Erythema diminished at 12 hours, and by 24 hours it was 60% less obvious, indicating local vascular debit regulation. Consistently, edema disappeared, indicating the opportunity of favorable healing.

24 hours after the treatment has started, we noticed definite improvement in pain perception, at palpation or in locomotion. The dog could easily walk or stand, for long periods at a time. Local suppuration has reduced in quantity, transforming itself, by 12 hours, from muco-purrulent to a high consistency secretion, and disappearing by 24 hours. Because the secretion had no longer a septic character, it dried to the surface of the wound and it formed, in 24 hours, a crust, which helped the healing process.

Table 1. Evolution of clinical parameters of a skin infection in local ozone mono therapy

Clinical parameter	Time (hours)									
	0	***12***	***24***	***36***	***48***	***60***	***72***	***96***	***120***	***132***
Erythema	100%	60%	40%	20%	-	-	10%	-	-	-
Edema	100%	50%	30%	10%	-	-	50%	-	-	-
Pain	100%	50%	10%	-	-	-	-	-	-	-
Behavior	100%	50%	10%	-	-	-	-	-		-
Local suppuration	100%	60%	10%	-	-	-	-	-	-	-
Crust	0%	20%	50%	50%	-	-	-	-	-	-
Granulation tissue	0%	0%	0%	20%	-	-	-	-	-	-
Healing	0%	10%	30%	50%	50%	70%	70%	80%	90%	100%

The analysis of the upper mentioned parameters shows that ozone monotherapy has benefic effects on skin healing after localized infections.

Conclusions

1. The mixture of ozone and oxygen is administered once a day for 5 days, 0,5 – 3 ml/ injection site were administered all around the area in 6-8 points, at 2-4 mm apart, hypodermally and intradermally.

2. The benefic effects of ozone monotherapy can be rapidly seen, even at 12 hours after the first administration, with improvement of local signs.

3. Ozone therapy of skin infections reduced the time of healing, especially when regarding erythema, edema, local suppuration.

4. Ozone therapy is especially indicated for patients with medical problems, which cannot undertake surgery, or for those with resistant skin infections.

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THE APPLICABILITY OF SEMEN COLLECTION IN DRONES OF *APIS MELIFERA CARPATICA*

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Abstract

Forbeekeeping farms, the bee reproduction is one of the most important things. If it is well known and controlled, it leads to high productive performances. Therefore, it is required that bee growing and reproduction to be assisted in all of the apiaries. The biotechnologies of reproduction can be and are applied in bees also, and they involve semen collection from drones, semen dilution and preservation, and artificial insemination of queen bees.This study aimed to test the applicability of semen collection in drones, according to their age and collection technique. Thus, 200 drones were used, of 2 age categories (L1 - 15 days old drones and L2 – 30 days old drones). Of studied drones, in 55.5% (111/200) the ejaculation occurred, and this way the semen collection was possible. In the rest of the drones, (89/200), the endophallus was not revealed and the ejaculation did not occur. Of drones that served to collection, in 75.7% (84/111) the ejaculation was total, while in 24.3% (27/111) the ejaculation was partial. Based on age categories, in L1 group the collection succeeded in 52% of the drones (69.2% with total ejaculation and 30.7% with partial ejaculation) while in L2 group the results were better, semen being collected from 59% of the drones (81.4% with total ejaculation and 18.6% with partial ejaculation.The applicability of semen collection in drones depends on genetics, age, and physiological condition.

***Key words**: drone, semen, collection*

Introduction

By applying the biotechnologies in assisted reproduction in beekeeping it is aimed to raise the number of the individuals in the bee family so the family will have a higher capacity of picking when the environment offers high quantities of nectar and pollen. One of the main aspects of the bee growing and reproduction through which is realized maximum development of the families and consequently the increase of production is represented by assisted breeding of the getters originated from high biological value parents (5, 8).

As it's well known, the bee reproduction is sexual and parto-genetic. By sexual way, worker bees and queen bee are born of fertilized eggs. The drones develop from non-fertilized eggs.

In order to achieve the mentioned goals (strong families and high yields), it is necessary to know very well the essential element soft the complex process of reproduction, namely: game to genesis, mating or artificial insemination, fertilization and metamorphosis of the three classes of bees (queen bee, worker bees and drone).

Oogenesis occurs in ovaries, primary ovogonies develop and transform in to oocytes, which by multiple divisions become mature egg capable of fertilization.

Spermatogenesis occurs in testicles. In drones, the spermatozoa are produced starting with the 6^{th} day of the larval stage. Three days after hatching they are completely mature and suitable for fertilization, and they migrate in seminal vesicles.

The phenomena of polyandry metin mating process, have quite high importance in term so the physiology of reproduction. This represents the best and the most complete hybridization, being known that drones are free to move from one family to another and from an apiary to another. They fly on a distance of 8-10kmand the mating flight occurs only out

side the hive. Polyandry provides increased prolificacy and high vitality of the descendants (1, 4).

Hetero sperm mating activates the fertilization of the eggs. 5-10 spermatozoa participate in fertilization of an egg, but only the most vigorous one fuse with femalegamete.

These natural characteristics specific to the beesex plain the preservation of the fertilization capacity of the sperm stored in spermateca over a long period (several years), and prevent degeneration of the species through in breeding (2,7).

The key to success in conservation of be biological materialis likely tobe in spermateca. By study ing the biochemical and physiological environment that ensures sperm survivalfor many years, the best way oflong-term preservation of semen could be found.

Until now, most of the literature says that the drones eject two substances during revealing of endophallus: semen and mucus (9, 10).

Material and method

Forsemen collection were used a number of 200 drones of Apis Melifera Carpatica. Drones originated froman apiary that comply with existing veterinary standards and is accredited as organic apiary after EU criteria. Sexual maturity occurs in 13 to 15 days after hatching, so there were formed two groups, by age (L1- 15 day's old drones, and L2-30 days days old drones).

Semen collection was performed using the following technique: the collecting syringe was prepared by fillingit's cuspwith saline solution. As saline solution, it was used Hyes physiological solution. After extraction of one drop of saline solution, as mallair bubble was aspirated and then the collection started. The drone used for collection was positioned at the cusp of the syringe, under microscope. Semen was aspirated separate from annexes glands secretion and the collection of the mucus was avoided. Between two crops an air gap was kept and some saline solution was aspirated to avoid drying.

The sperm is ejaculated along with homogeneous white mucus, of which his easy to differentiate by it sye llowish colorand different structure. Hig her content of spermatozoa is reflected in more intensecolor and viscosity

Results and discussion

Of studied drones, in 55.5% (111/200) the ejaculation occurred, and this way the semen collection was possible. In the rest of the drones, (89/200), the endophallus was not revealed and the ejaculation did not occur. Of drones that served to collection, in 75.7% (84/111) the ejaculation was total, while in 24.3% (27/111) the ejaculation was partial. Based on age categories, in L1 group the collection succeeded in 52% of the drones (69.2% with total ejaculation and 30.7% with partial ejaculation) while in L2 group the results were better, semen being collected from 59% of the drones (81.4% with total ejaculation and 18.6% with partial ejaculation.

There are currently few data regarding the proportion of drones from which semen can be collected manually by revealing then dophallus (2).Unli keour results (55.5% successful collecting) Collins and Pettis (2001) collected semen from 60% of 12-day-old drones, and Anderson (2004) reports a successful collection from 90% of 20 days-old drones.

Manual labor for collecting semen from drone involves contention and stimulation of abdominal contraction this way producing a partial erection (partial revealing of end

ophallus) in the first time and afterwards total. (Figure no 3, 4). Initially is revealed the bulb, which is empty and push ed out, and then fill ed with semen and content of mucous glands. Chitinous plates of the bulb appear to the left ofthe vestibular hole. Constant pressure in the abdomen determines the bulb to becom pletely revealed and leads togradual and total end ophallus revealing. As a result a drop of creamy sperm appears at the end ofit. This stage of endophallus eversi on is essential in semen collection process. (figure no 1, 2).

Sperm collecting process is extremely important, because the success of semen preservation and artificial in semination may depend on it. Collection of the sperm must be under aseptic conditions, to avoid its infection with different pathogens. The equipment of collecting must be made of a material that does not affect the viability of spermatozoa.The used methods should be practical and easy to apply in the field or laboratory, and they should lead to obtaining alarge, sperm-rich quantity of ejaculate.

During the operation of collecting sperm from drones, usually there are large differences between in dividuals in terms of ease of collection, quantity and quality of the ejaculate. Some of them ejaculate very easy, but in other individuals this operation turns to be extremely difficult oreven impossible. Semen quantity is also very different, especially in drones originated from in bred lines. Some drones have no sperm at all, in others ejaculation occurs with out full eversion when the process of artificial collecting was initiated; in some drones, thee version with ejaculation is so violent that the penis"explodes" and the spermis lost.

Triggering of the artificial sperm ejaculation can be obtained by massaging the chestand abdomen. The collection technique should be designed to stimulate abdominal contractions and not tosqueeze the spermout. A collecting process started correctly releases the spermof the drone completely and not mixed with mucus, which appears entirely separated.

Ejaculations can occurin two modes: partial ejaculation and total ejaculation. Partial ejaculation is obtained by holding drone’s head and thoraxand pushing its abdomen. Stimulation is required and it is done by holding the head and thorax of the drone and turning the drone slightly. During partial ejaculation, the abdomen contracts and forms the antennas. Pushing the anterior part of the abdomen must not be powerful; other wise the abdomen could be crushed.

Sometimes it is necessary toins ist, compressing the abdomen to stimulate total ejaculation, which occurs with total revealing of the endophallus. This pressure forces end ophallus glands which produce semen. The sperm is ejaculated along with homogeneous white mucus of which differs by yellow is colorand by structure.

Emptying the genital tract occurs through contractions of the muscles from seminal vesicle wall and mucous glands, starting from the top, and the contentis ejected with considerable pressure. In mature drones, this processis triggered after as light pressureon the abdomen. A collecting process started correctly releasesthe sperm of the drone completely and not mixed with mucus, which appears entirely separated. It is important ocollect only semen, not mucus.

Table 1. Success in semen collection processaccording to age of the drones

	Drones used to collection		**Successful collection**		**Drones that did not ejaculate**	
	No.	%	No.	%	nr	%
L1 (15 days)	100	50	52	52	48	48
L2 (30 days)	100	50	59	59	41	41
Total	**200**	**100**	**111**	**55,5**	**89**	**44,5**

Table 2. Types of ejaculation in drones according to their age

	Drones that ejaculated		**Partial ejaculation**		**Total ejaculation**	
	No.	%	No.	%	No.	%
L1 (15 days)	52	46,8	16	30,7	36	69,3
L2 (30 days)	59	53,2	11	18,6	48	81,4
Total	**111**	**100%**	**27**	**24,3%**	**84**	**75,7%**

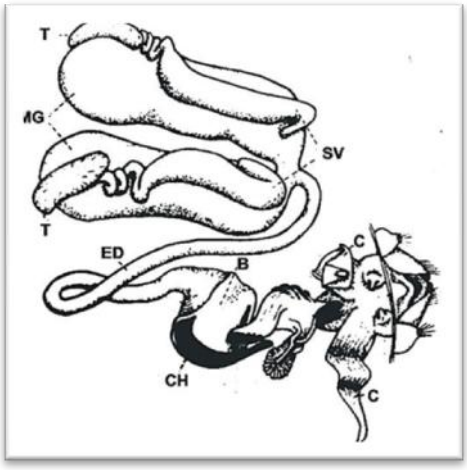

Fig. 1. Scheme of the genital apparatus in drone–after Woyke J. et al. 2001
T – Testicle, MG – Mucous glands, SV –Seminal vesicles, ED – Endophallus, B- Bulb, CH- Chitinous plates.

Fig. 2. Revealing of the genital apparatus (endophallus) with annexes glands

Fig. 3. Partial revealing of endophallus with partial ejaculation

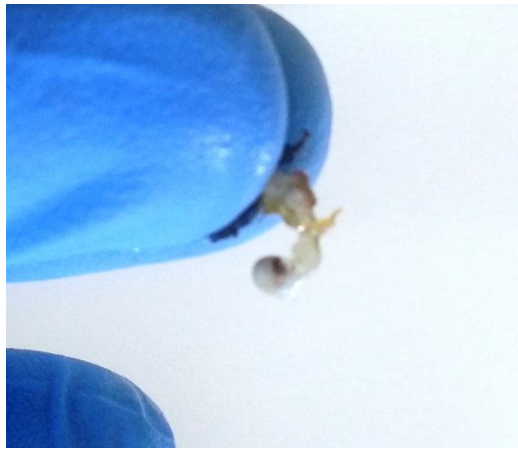

Fig. 4. Total revealing of endophallus with total ejaculation

Acknowledgement

This study was effected on biological material originated from an apiary that comply with existing veterinary standards and is accredited as organic apiary after EU criteria 834/ 2007 and Reg. C.E. 889/2008, PFA CIORNEI STEFAN GREGORE. Laboratory methodology was performed in Laboratoryof ReproductionofUSAMV- FMV, Iasi.

Conclusions

1. Manual labor of collecting semen in drones involves contention of their head and chest, pressing roundly and moderate, in order to stimulate contraction ofthe abdomen, thus producing a full erection (revealing the entire endophallus) which will assure the ejaculation. We do not recommend abdominal pressing, to avoid crushing.
2. In 55.5% (111/200) of subjects ejaculation occurred, and thussemen was collected. Of these, 75.7% (84/111) had total ejaculation.
3. By age, the best results were obtained in group L2, in which collection was successful in 59% (81.4% total ejaculation and 18.6% partial ejaculation). We recommend usage of drones older than 30 days for collecting semen.

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ANALYSIS OF SOME HEMATOLOGICAL AND METABOLIC PARAMETERS IN THE CONDITIONS PROVIDED BY TESTING OF ANTIDEZENTER ON HEALTHY SUCKLING PIGLETS

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Abstract

The purpose of this research was to analyze the development of some erythrocyte, leukocyte and biochemical parameters, relevant for evaluating safety and tolerance of a soluble powder containing neomycin sulphate and oxytetracycline hydrochloride administered orally to 2 groups of healthy piglets. According to the investigation protocol, the tests were performed on 21 clinically healthy piglets of 10-15 days, divided in 3 equal groups, with the following variables: group 1 dose (1 g product/kg/day for 4 days), group 2 - double dose (2 g product/kg/day for 4 days), group 3 -untreated control. The product was administered orally, following the procedure suggested by the manufacturer: 100 g powder suspended in 200 ml of drinkable water, thoroughly mixed and administrated orally using an appropriate syringe; in doses of 3 ml / kg of body weight for the piglets treated with the single dose, or a 6 ml/kg body weight for double dose. Evaluation of safety, tolerance and resistance required in the initial phase clinical and laboratory examinations for the selection of healthy animals and also during the course of testing (pre-and post-treatment). Clinical examinations were based on the usual techniques and the laboratory investigations included evaluation of red blood cell parameters (PCV, hemoglobin, total RBC, MCV, MCH, MCHC), leukocyte (white blood cell count and leucocyte subpopulation count), and metabolic profile (ALP-alkaline phosphatase, ALT-alanine aminotransferase, CHOL-cholesterol, TBIL-total bilirubin, ALB-albumin, U-urea). Hematological and biochemical tests were performed using automated analyzers (Abacus Junior Vet, respectively VetScan VS2); the recorded individual and mean data were analyzed by usual methods of biostatistics and interpreted according to the European and national guidelines (EMEA and Order 186 ANSVSA). The development of red blood cell parameters and mean erythrocyte constants corresponded to a normal hematological profile, indicating a good tolerance to therapeutic doses without affecting electrolyte balance, erythrocytes homeostasis or other erythrocyte functions. Analysis of the total number of leucocytes and leukocyte subpopulations revealed that the tested doses did not exert negative influences on non-specific defense and the leukocyte subpopulations showed a high level of tolerance for this product in piglets indicating no sensitization reactions, or adverse effects on leucopoiesis and leukocyte functions in general. The blood chemistry analysis did not show significant differences between the investigated groups, the obtained values corresponding to a normal metabolic profile without any indicators of toxicity or other metabolic disorders. In support of these findings the following mean values recorded pre-and post-treatment in the group of piglets treated with double dose are considered relevant: 6.6-7.13 T/L for total RBCs, 12.3-12.4 g/dL for hemoglobin, 38.14-37.70% for PCV, 200-500 G/L for platelets; 20.54-20.26 G/L for total leukocytes. Also in this context it is relevant that the percentage values of neutrophils, eosinophils and lymphocytes was situated between physiological limits. Regarding the monocyte subpopulation, the recorded values exceeded the normal limits. The observed monocytosis (26.57 to 30.00%) was considered a leukocyte reaction to the environmental microbial population associated with an increased susceptibility due to age. In conclusion, based on the results obtained in the clinical, hematological and biochemical investigations it can be stated that the investigated product was well tolerated and can be given even the most sensitive categories of piglets, which can lead to an increase in its market availability and potential effectiveness.

***Keywords**: neomycin, oxytetracycline, safety, tolerance, healthy piglets*

Introduction

The available information regarding the tolerance and safety of some soluble therapeutic medicinal products based on neomycin and oxytetracycline in piglets is still sufficiently limited because the pharmacokinetics and bioavailability of various active substances is significantly influenced by age and especially on suckling of the young of different species of animals (Ognean et al., 2006). In this context, specific morphological and physiological aspects of the piglets are actually basic guidelines in performing therapeutic and toxicological studies in pigs. Starting from the consideration that age is a basic factor of variability in the organism's response to active substances some aspects of pharmacokinetics in pigs should be presented.

The processes of absorption, distribution, metabolism and excretion, in piglets, undergo a continuous change, during the development of the organism to adulthood (Sarandan et al., 2004). In consequence it is necessary to establish dosing regimens for this age and a miniaturization of drug therapy, in order to make adequate adjustments to individual variations. The newborn can rapidly adapt to the environment through a series of visceral and physiological changes that trigger modifications in active substances pharmacokinetics (Ghergariu et al., 2000). It is a known fact that the infant has relatively less fatty tissue, in the first month of life; the liver being the largest organ compared to bodyweight. The liver in the fetus represents 4% of the body weight, while only 2% in the adult (Ognean et al., 2004).

In newborns and infants, glomerular filtration is reduced by 30-50% compared to the adult, tubular reabsorption and secretion being also decreased (Ognean et al., 2004). Enteral absorption is influenced by various factors that change with age, gastric pH, speed of stomach contents evacuation, intestinal motility and blood flow (Reece, 2005). The elimination rate of gastric contents into the intestine is lower in the newborn. In general peristalsis is very irregular in infants, thus making them absorb active substances at a lower rate compared to adults. This is due mainly to the prolonged evacuation of stomach contents (absorption taking place mainly in the intestine), irregular peristalsis, and some particular features of the intestinal mucosa (Leucuța, 1989).

In this paper we propose an analysis of the main erythrocyte, leukocyte and biochemical parameters, relevant in assessing the safety and tolerance a oral antidiareeic product containing a soluble powder based neomycin sulfate and oxytetracycline hydrochloride, in piglets.

Materials and methods

The research was carried out on 21 clinically healthy Large White piglets, aged 10-15 days, selected and marked with identification numbers for Antidezenter soluble powder consisting of neomycin sulfate (0.01 g/g) and oxytetracycline hydrochloride (0.01 g/g) product testing.

Experimental groups. The study began with the preliminary investigation, consisting of clinical and laboratory investigations (complete blood counts) for the selection of clinically healthy pigs and formation of the experimental groups. The selected animals were divided into the following 3 groups, with dose as the main variable: group 1 (n = 7) containing pigs given the therapeutic dose (1 g product/ kg body weight/day single dose) for 4 consecutive days, group 2 (n = 7), consisting of pigs subjected to administration of a double dose (2 g

product/ kg body weight/day, double the therapeutic dose) for 4 days, group 3 consisting of untreated controls that were kept in identical conditions to those in the experimental groups. The product was administered orally, according to the following procedure suggested by the manufacturer: dissolving 100 g powder in 200 ml water, mixing and oral administration using an appropriate syringe, 3 ml/kg of body weight in single dose, or 6 ml/kg body weight for the double dose.During the experimental period a strict schedule, established by the working protocol, was kept, schedule differentiated into days and hours.

Experimental protocol. In summary, the study protocol consisted of blood collection on day zero for hematological and biochemical tests, and subsequently the administration of the products once a day for 4 days, in the doses described above. Daily clinical examinations were made and water and food consumption was observed. After the last administration, at 10-24 hours the final tests were carried out, consisting of a repetition of clinical, hematological and biochemical investigations. All groups were kept under clinical observation for 15 days after completion of the experiment.

Performed investigations. In conducting the clinical examinations, the usual semiotic techniques were employed and for the hematological and biochemical investigations automated analyzers, Abacus Junior Vet, respectively VetScan VS2 – Abaxis were used. The investigated hematological parameters included: quantification of basic erythrocyte and leukocyte parameters: packed cell volume (PCV), hemoglobin (Hb), total number of erythrocytes (E), mean erythrocyte constants (MCV, MCH, MCHC), total number of leukocytes (L) and differential leukocyte counts. Biochemical tests included determining the following metabolic profile parameters of relevance for determining the liver and kidney functions: alkaline phosphatase (ALP), alanine aminotransferase (ALT), cholesterol (CHOL), total bilirubin (TBIL), albumin (ALB) and Urea (U).

Individual and mean data were processed and statistically analyzed by the usual methods of biostatistics, and for the interpretation of the results we used bibliographical references regarding this field (Merck Veterinary Manual, 2011; Reece, 1996; Ghergariu et al., 2000) respectively the European regulations (Official Journal of the European Communities Official Journal of the European Union).

Results and discussion

The initial clinical examinations performed at the beginning, during and post-treatment, there were no reported changes in general condition, appetite and general function, making punctual presentation of recorded data not necessarily. The situation was different in the evolution of hematological and biochemical parameters, which were characterized by large fluctuations, usually within physiological limits, or with insignificant variations from them. In the following are presented the dominant hematological and biochemical profiles outlined in suckling piglets in correlation with dose, based on changes in pre-and post-treatment mean and individual values of the main investigated indices.

As shown in the analysis of data regarding the evolution of red blood cell parameters, the mean values obtained from individual data processing, were within the physiological ranges, assessment based on data from the control group correlated with those recorded in bibliographical references (Merck Veterinary Manual, 2011). The recorded values obtained after the administration of therapeutic doses of Antidezenter in piglets showed an increasing trend of red blood cell parameters, more or less obvious in case of PCV (38.89 to 39.77%),

Hb (12.17 to 12.67 g/dl) and total number of erythrocytes (6.80 to 6.90 T/L) associated with less significant oscillations situated within the physiological ranges of the mean erythrocyte constants (Table 1). The administration of the double dose did not produced significant decreases in the erythrocyte indices compared to those found in the groups treated with a single dose or in untreated controls. The individuals from this group even recorded post-treatment increases if the total number of erythrocytes from 6.60 to 7.13 T/L (Table 1).

Table 1. Mean values recorded in erythrocyte and platelet parameters in piglets treated with therapeutic doses (group 1) and double (group 2) of Antidezenter compared with the untreated controls (group 3)

Parameter	Group 1	Group 2	Group 3	*Reference values*
	Mean±Standard deviation	*Mean±Standard deviation*	*Mean±Standard deviation.*	
Pre treatment/initial				
PCV (%)	38.89±1.98	38.14±3.05	36.05±1.92	*36-43*
Hb (g/dl)	12.17±1.03	12.03±1.02	10.51±0.89	*9-13*
Erythrocytes (T/L)	6.80±0.69	6.60±0.42	6.09 ±0.57	*5-8*
MCV (fl)	58.00±4.32	56.29±3.64	58.71±3.30	*52-62*
MCH (pg)	18.53±1.15	17.99±0.95	18.06±0.75	*17-24*
MCHC (g/dl)	31.96±1.16	31.54±1.03	30.34±0.99	*29-34*
Platelets (G/L)	293.71±105.59	320.71±183.84	578.71±71.62	*200-500*
Post treatment/final				
PCV (%)	39.77±1.92	37.70±1.66	39.59±1.85	*36-43*
Hb (g/dl)	12.67±0.76	12.04±0.79	11.83±1.22	*9-13*
Erythrocytes (T/L)	6.90±0.47	7.13±0.38	6.99±0.74	*5-8*
MCV (fl)	57.71±3.82	58.71±3.30	56.71±3.64	*52-62*
MCH (pg)	18.41±1.20	18.06±0.75	19.27±2.33	*17-24*
MCHC (g/dl)	31.87±1.00	30.34±0.99	30.96±1.13	*29-34*
Platelets (G/L)	312.57±61.10	459.43±165.93	368.71±47.68	*200-500*

Unimportant fluctuations were recorded in the values of total number of platelets (293-578 G/L), which were predominantly situated within the physiological limits of the species (200-500 G/L). Exceptions from the normal ranges were found in some piglets from the control group; however the recorded increases were not significant. The overall analysis of the evolution regarding this parameter highlights a good functioning of primary hemostasis mechanisms both pre-and post-treatment (Table 1).

Analysis of the developments regarding erythrocyte indices revealed a high welfare level of the piglets according to their increased weight gain and adequate adaptation to their new living conditions. The variation found in the values of erythrocyte parameters, mostly showing insignificant fluctuations circumscribed by the physiological limits of the species and category reported for the 3 groups, showed that therapeutic and double doses of neomycin and oxytetracycline from the product Antidezenter, had no adverse effects on hematopoiesis and erythrocyte functions in suckling piglets.

The configuration leukocyte counts presented in table 2, revealed the predominant development of individual and mean values within physiological ranges, except for some cases that showed a trend towards an increase in the total number of leukocytes and monocytes subpopulation. Thus, the total number of leukocytes showed higher values compared to the physiological limits (11 to 22 g/l), in a few isolated cases, as well as in the initial values of the control group, when a mean of 25.94 G/L was recorded (Table 2). The prevalence of piglets with total number of leucocytes situated near, or even exceeding the upper normal limits, is due to physiological leukocytosis characteristic to age, commonly found in the offspring of mammals in general (Ognean et al., 2004; Reece, 2005; Pârvu et al., 1984). Furthermore, it was observed that this slight leukocytosis regressed towards the end of the experimental period, when for the control group ranged between 11 and 20 G/L. After administration of a double dose of the product, it was found that the total leukocyte count was situated within the normal range, with mean values of 20.54 G/L, respectively 20.26 G/L (Table 2).

The correlative analysis of the development of leukocyte subpopulations revealed normal cytological configurations and values situated within physiological ranges, indicating a normal dynamic of the proportion of neutrophils, eosinophils and lymphocytes. An exception was found in the monocyte subpopulation, which was characterized by increased individual and mean values, reaching initial proportions of 27.14% and a final of 30% (Table 2). This situation can not be attributed to the influence of the or to the variable administered dose because it was observed both pre-and post-treatment including in the control group. In this context, the increased proportion of circulating monocytes (monocytosis) in piglets within the first weeks of life, confirms the predominance of cellular nonspecific defense processes, during the newborn and infant stages of life (in general found in the offspring of mammals) while the humoral defense mechanisms are still ineffective.

Table 2. Mean values of leukocyte parameters in the piglets from groups 1, 2 and 3, treated with therapeutic (1g/kg body weight / day), double (2g/kg body weight / day) doses of Antidezenter respectively untreated controls

Parameter		**Group 1**	**Group 2**	**Group 3**	*Reference values*
		Mean±Standard deviation	*Mean±Standard deviation*	*Mean±Standard deviation.*	
Pre-treatment/initial					
Leukocytes (G/L)		20.71±1.93	20.54±2.01	25.94±4.71	*11-22*
Leukocyte pulation (%)	Neutrophile	45.86±7.17	45.71±11.80	64.86±9.17	*20-70*
	Eosinophils	5.29±2.69	4.29±2.06	3.00±1.83	*0-15*
	Basophiles	0.00±0.00	0.00±0.00	0.29±0.49	*0-3*
	Lymphocytes	21.71±6.55	23.43±10.26	13.86±5.18	*35-75*
	Monocytes	27.14±9.67	26.57±3.78	18.00±8.08	*0-10*
Post-treatment/final					
Leukocyte (G/l)		19.10±1.62	20.26±2.38	21.48±1.16	*11-22*
Leukocyte pulation (%)	Eosinophils	45.43±8.16	44.86±14.78	49.14±14.19	*20-70*
	Basophiles	4.14±2.19	2.86±2.27	4.86±2.79	*0-15*
	Lymphocytes	0.14±0.38	0.00±0.00	0.14±0.38	*0-3*
	Monocytes	26.14±7.13	22.29±12.62	41.43±14.43	*35-75*
	Eosinophils	24.14±5.24	30.00±5.16	4.43±1.90	*0-10*

The development of the biochemical parameters according to the individual and mean data presented in table 3, showed characteristic aspects of a normal piglet metabolic profile, including more or less important deviations in the main indicators specific to hepatic and renal functions. General analysis of the individual and mean values of the biochemical determined parameters (ALP, ALT, CHOL, TBIL, ALB and U) revealed their fluctuation, according to experimental variables. The mean values ranged between 98.57 and 126.43 U/L for ALP, 32.43 and 35.00 U/L for ALT, 110.57 and 113.29 for CHOL, 0.17 and 0.23 for TBIL, 25.14 and 25.86 for ALB, 16.07 and 18.44 respectively for U. These developments show that double and therapeutic doses of neomycin and oxytetracycline as Antidezenter product did not affect metabolic status and functional mechanisms at the hepatic and renal level (Table 3).

The synthesis of data from animal monitoring in the selection phase and during the course of the experiment shows that none of the piglets investigated reported clinical, hematological or biochemical changes because those with uncertain developments were excluded from the experiment, as required and inclusion-exclusion criteria. Also it is important to mention that the piglets have achieved normal weight gain during the investigations.

Table 3. Mean metabolic profile parameters in piglets of groups 1, 2 and 3, treated with therapeutic (1g/kg bodyweight/day) double (2g/kg bodyweight/day) doses of Antidezenter and untreated control

Parameter	**Group 1**	**Group 2**	**Group 3**	*Reference values*
	Mean±Standard deviation	*Mean±Standard deviation*	*Mean±Standard deviation.*	
Pre-treatment/initial				
ALP (U/L)	118.86± 44.08	123.57± 33.13	126.43± 39.94	*41-176*
ALT(U/L)	33.43± 8.36	32.43± 9.32	31.43± 8.83	*22-47*
CHOL (mg/dl)	113.29±17.13	111.86±15.74	110.71±18.87	*81-134*
TBIL (mg/dl)	0.23± 0.21	0.19± 0.15	0.21± 0.20	*0-0.5*
ALB(g/l)	25.86± 4.78	25.86± 5.34	25.14± 5.40	*19-32*
Urea (mg/dl)	18.44± 4.20	18.73± 4.86	16.07 ± 5.46	*8.2-25*
Post-treatment/final				
ALP (U/L)	110.43±37.58	119.86± 29.84	98.57± 35.46	*41-176*
ALT(U/L)	32.57± 7.52	31.29± 8.28	35.00± 5.63	*22-47*
CHOL (mg/dl)	110.57± 16.13	111.00± 15.26	112.29± 13.62	*81-134*
TBIL (mg/dl)	0.17± 0.15	0.21± 0.18	0.17±0.17	*0-0.5*
ALB(g/l)	26.29± 4.82	25.57± 3.99	24.57± 5.19	*19-32*
Urea (mg/dl)	17.04± 5.96	16.66± 5.90	18.07± 4.73	*8.2-25*

Pre- and post-treatment fluctuations as a whole were insignificant and within the physiological limits of the species respectively age group for most of the investigated parameters confirming that testing was performed exclusively on healthy animals whose health indicators have remained constant throughout the period under study. Also important is the fact that the results of monitoring clinical, hematological and biochemical parameters showed no digestive or liver manifestations, indicating intolerance or other side events.

In the following, it is important to consider these findings in relation to some of the basic principles of pharmacology. Thus, regarding the distribution of the active substances, it is known that it is influenced by the volume of tissue compartments, respectively, water content, higher in of the newly-born, with a higher ratio of extracellular to intracellular water, while the fatty tissue is poorer (Reece, 2005). The volume of distribution in newly-born comparative adults determines an increases the initial dosage in order to achieve the desired plasma concentrations. The different speeds and in different pathways of the metabolism of drug substances, make their biotransformation in newborns differ qualitatively and quantitatively from that seen in adults (Leucuță,1989).

Insufficiency in metabolizing enzyme of some active substances in newborns and low renal clearance speed causes a lengthening of the biological half-life. Moreover, the absorption after intramuscular administration depends on regional blood flow, which is variable during the first weeks of life. On the other hand, the availability given by the length of systemic absorption time of the active substance is influenced by nature, the dosage form and its interaction with the absorption medium (Rani and Pargal, 2004). As is well known, the effectiveness of active substances administered to animals depends on the amount in which they arrive at the site of pharmacological action (respectively in the immediate vicinity of the biological receptor) and the length of time that the adequate concentration is maintained at that particular level. Thus, the biological action level of an active substance is based on its reactivity to affinity towards the receptor (Leucuța, 1989).

According to the data from the prospect, the antimicrobial product in the study is highly active against gram-negative bacteria through the neomycin sulphate content, which is an aminoglycoside with good bacteriostatic effect at the intestinal level. The bacteriostatic action against a broad spectrum of germs bacteria and even protozoa is given by its content in oxytetracycline hydrochloride; a synthesis tetracycline capable ofpenetrating the microbial cell through mediation by a carrier protein.

Conclusions

Correlative analysis of the results from clinical, hematological and biochemical investigations performed on the 3 groups of piglets showed a very good tolerance of the suckling piglets to therapeutic and double doses of neomycin and oxytetracycline, which gives a high level of therapeutic safety and provides good potential of the tested product on the market.

Following administration of therapeutic and double doses of the investigated product, no systemic adverse reactions with or without humoral component, respectively secondary manifestations such as: digestive, temperatures, general staus, appetite, hematological and biochemical parameters were maintained within the normal limits of the species and category in all piglets used in the experiment.

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FAUNE ECTOPARASITAIREET INDICES ÉPIDÉMIOLOGIQUES DU TILAPIA DU NIL *OREOCHROMISNILOTICUS* ÉLEVÉ EN ZONE DES LAGUNES (CÔTE D'IVOIRE)

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Résumé

En Côte d'Ivoire, depuis une décennie, élevages de tilapia du Nil Oreochromisniloticus sont sujettes à de nombreux cas sporadiques de mortalité inexpliquée et à des comportements anormaux principalement observés dans la région des Lagunes. Ces symptômes sont plus accentués en phase de reproduction et de pré-grossissement. Face à ces problèmes, il a paru impérieux de trouver des éléments de réponse. C'est dans cette optique que la présente étude, indispensable à la compréhension de ce phénomène, a été menée afin d'identifier en premier lieu la faune ectoparasitairedu tilapia du Nil d'élevage. Pour ce faire, des analyses parasitologiques ont été réalisées sur 50 poissons échantillonnés tous les mois et de façon aléatoire dans les fermes piscicoles durant 12 mois (Soit 600 poissons). Comme résultats, il a été identifié cinq (5) espèces de monogènes (Cichlidogyrusrognoni, Cichlidogyrushalli, Cichlidogyrussp., Suctogyruslongicornis et Gyrodactylussp), deux (2) espèces de protozoaires (Trichodinasp. et Ichthyophthiriusmultifiliis), deux (2) espèces de copépodes (Argulus ambloplites et Argulus sp), une (1) espèce de champignon (Saprolegniaparasitica), trois (3) espèces d'annélides (Hirudomichaelseni, Hirudosp. et Limnatissp.) et deux (2) espèces d'insectes (Chironomuspulmosus et Naucorissp.). Les indices épidémiologiques (Prévalences et intensités moyennes d'infestation) de ces parasites ont variés les saisons et le poids des poissons. Les espèces Cichlidogyrus, Suctogyrus, Gyrodactylus, Trichodina, Ichthyophthirius, Argulus, Saprolegnia, Naucoris, Chironomus, Hirudo et Limnatis ont été récoltées sur le même hôte de poisson au Brésil par d'autres recherches.

Mots clés *:Oreochromisniloticus, élevage, ectoparasites, indices épidémiologiques*

Introduction

Dans la plupart des fermes piscicoles de la Côte d'Ivoire, le tilapia du Nil *Oreochromisniloticus* reste la principale espèce de poisson élevée en étang et en cage flottante à l'image de nombreux pays africains (Legendre &Pauguy, 2006). Localement appelé « Carpe où tilapia», *Oreochromisniloticus*est beaucoup prisé par les consommateurs. Les nombreux atouts qu'offre ce poisson (Reproduction facile en captivité, croissance rapide des mâles, alimentation facile, résistance aux conditions environnementales défavorables…) amènent les populations africaines vivant au sud du Sahara à préconiser son l'élevage ; souvent en association avec la riziculture. Mais depuis une décennie, les pisciculturesdu tilapia du Nil *Oreochromisniloticus* sont sujettes à de nombreux cas sporadiques de mortalité inexpliquée et à des comportements anormaux principalement observés dans les zones de Lagune (5.25'.00'' latitude nord et - 4.20'00'' longitude ouest). Ces symptômes sont plus

accentués en phase de reproduction et de pré-grossissement. Face à ces problèmes, il paraît impérieux de trouver des éléments de réponse. C'est dans cette optique que la présente étude, importante à tout début de recherche de causes de pathogénicité, a été menée afin d'identifier les ectoparasites ou parasites externes de ce poisson. En effet, selon Euzet et Pariselle (1996), les nombreuses pathologies signalées en élevage de poissons ont principalement été causées par des ectoparasites holoxènes ou monoxènes : Protozoaires, Monogènes et Copépodes, dont les formes de dispersion (kystes, murs ou larves) libérées dans les bassins n'ont aucune peine à trouver un nouvel hôte chez qui leur accumulation prend une allure asymptotique conduisant à la pathogénie et à la mort de l'hôte. Aussi, les connaissances en matière de pathologies et de parasitologie des poissons des eaux tropicales et subtropicales sont-elles loin d'être satisfaisantes (N'douba, 2000). Ainsi, le but de ce travail est d'identifier la faune ectoparasitaire et les indices épidémiologiques du tilapia du Nil *Oreochromisniloticus* élevé en région des Lagunes en Côte d'Ivoire.

Matériel et méthodes

Echantillonnage, examens externes et dissection des poissons

L'échantillonnage a été réalisé tous les 30 jours dans des structures d'élevage des fermes piscicoles rurales de la région des Lagunes. A chaque échantillonnage 50 poissons ont été prélevé de façon aléatoire (Mouchiroud, 2002) car selon Palm (2004), 35 individus sont nécessaires pour une bonne étude épidémiologique. Les spécimens ont été mis dans une glacière et / ou des aquaria aérés, puis transportés au laboratoire pour les différentes analyses parasitologiques pour lesquelles les poissons vivants ont été anesthésiés et euthanasiés par immersion dans une solution d'acide bicarbonate. Après l'euthanasie, les poisons ont été immédiatement examinés sous une loupe binoculaire pour la recherche d'ectoparasites au niveau de la peau, de la bouche, des nageoires et des opercules. Chaque arc branchial a été déposé dans une boîte de pétri contenant une solution de NaCl à 0.9 % (Cissé&Belghyti, 2005 ; Farhaduzzaman*et al.*, 2010). Chaque organe a ensuite été observé à la loupe binoculaire et au microscope optique pour la récolte des parasites. Les parasites récoltés ont été fixés avec de l'acide formol alcool (AFA) pendant 1 à 2 h puis conservés dans l'éthanol 70 % et stockés dans des piluliers (Lyndon, 1994). Des frottis frais ont également été réalisés sur les organes, puis gardés pour la technique de coloration au nitrate d'argent afin de rechercher les protozoaires (Buchmann, 2007). Après quoi, chaque poisson a été pesé au milligramme près et les longueurs standards (Ls) et totales (Lt) ont été prises au millimètre près. Les poissons ont ensuite été regroupés en classes de poids selon Douellou*etal*. (1985), Klimpel*et al*. (2004) et Sabri*etal*. (2009) afin de voir l'évolution des indices épidémiologiques en fonction de ces critères. Ainsi, sur la base des trois (3) différents stades de croissance de *Oreochromisniloticus* d'élevage, les classes de poids de 1-5 g, 6-50 g et 51-500 g ont respectivement été formées pour les poissons d'alevinage, de pré-grossissement et de grossissement.

Identification des parasites

Des observations morphologiques et biométriques ont été réalisées sur les parasites afin d'en ressortir les caractères génériques et ou spécifiques. Les différents genres et espèces de parasites ont été identifiés à l'aide des clés proposées par :Rushton-Mellor (1994) pour l'identification des copépodes, Pariselle&Euzet (2009) pour identifier les Monogènes ,

Klemm (1972), Utevsky&Trontelj (2005) et Oosthuizen (1991) pour l'identification des annélides ou sangsues, Lom (1970), Lom &Dykova (1992) et Dobberstein& Palm (2000) pour l'identification des protozoaires, Cheng*et al*. (2001) et WaterbugCompany (2012) pour l'identification des larves d'insecte. La prévalence (P) et l'intensité moyenne d'infestation (I) du parasite telles que définies par Bush *etal*. (1997) ont permis d'estimer les indices épidémiologiques des parasites chez l'hôte.Des analyses de variation de ces paramètres épidémiologiques ont été faites en fonction des saisons et du poids des poissons (Douellou*et al*., 1985 ;Sabri*etal*., 2009).

Résultats

Identification et sites de fixation des parasites

Le tableau 1 indique l'espèce, la famille, le groupe et le site de fixation des parasites récoltés sur le tilapia du Nil *Oreochromisniloticus* en pisciculture dans les trois régions de la Côte d'Ivoire.

Tableau 1 : Inventaire des parasites et organes cibles de *Oreochromisniloticus*

Espèces de parasite	Familles	Groupes	Organes ciblés
Cichlidogyrusdageti	Ancyrocephalidae	Monogènes	Branchies
Cichlidogyrushalli			
Cichlidogyrussp.			
Suctogyruslongicornis			
Gyrodactylussp.			Branchies et nageoires
Ichthyophthiriusmultifiliis	Hymenostomatidae	Protozoaires ciliés	Peau, nageoires et branchies
Trichodinasp.	Peritrichidae	Protozoaires ciliés	Peau, nageoires et branchies
Argulus ambloplites	Argulidae	Copépodes	Peau, nageoires, opercules, branchies et bouche
Argulus sp.			
Saprolegniaparasitica	Saprolegniaceae	Champignons	Peau, nageoires et opercules
Hirudomichaelseni	Hirudinidae	Annélides	nageoires et opercules
Limnatissp.			
Hirudosp.			
Chironomuspulmosus (Larve)	Chinoromidae	Insectes	Peau et nageoires
Naucorissp.	Naucoridae		

Au total quinze (15) espèces d'ectoparsites reparties en onze (11) genres ont été identifiées. Parmi ces genres, il a été identifiés deux (2) genres de monogène appartenant à la famille des Ancyrocephalidae, un (1) genre de monogène de la famille des Gyrodactylidae deux (2) genres de protozoaires ciliés de la famille des Ichthyophthiriidae et des Trichodinidae, un (1) genre de champignon de la famille des Saprolegniaceae, un (1) genre

de copépode de la famille des Argulidae, deux (2) genres d'annélide issus de la famille des Hirudinidae et deux (2) genres de larve d'insecte de la famille des Chinoromidae et des Naucoridae. Les espèces de monogènes identifiées ont été *Cichlidogyrusdageti*, *Cichlidogyrushalli*, *Cichlidogyrussp.*,*Suctogyruslongicornis* et *Gyrodactylussp*. Elles ont tous été récoltées au niveau des branchies à l'exception de *Gyrodactylussp.*qui, en plus de cet organe, a été retrouvé sur les nageoires du poisson. Les espèces *Trichodinasp*. et*Ichthyophthiriusmultifiliis* ont été les seules protozoaires ciliés récoltés sur la peau, les nageoires et les branchies du tilapia du Nil. Les espèces de copépode *Argulus ambloplites* et *Argulus sp*. ont été localisées au niveau de la peau, des nageoires, des branchies, des opercules et de la bouche de *Oreochromisniloticus*. L'espèce *Saprolegniaparasitica* a été observée au niveau de la peau, des nageoires, des opercules de l'hôte. Les espèces *Hirudomichaelseni*, *Hirudosp*. et*Limnatissp.* ont été identifiées au niveau des nageoires et des opercules du poisson. La larve d'insecte du genre *Chironomus* a été observée sur la peau, les nageoires, les branchies et les opercules des poissons morbides. L'insecte *Naucorissp*. a été identifié au niveau de la peau et des nageoires du tilapia du Nil *Oreochromisniloticus*.

Variation mensuelle des indices épidémiologiques

Les prévalences du tilapia du Nil vis-à-vis des différentes espèces de parasite, ont variées en dent de scie d'un mois à l'autre sur toute l'année. Pour les espèces de parasite ayant les valeurs de prévalence plus élevées comme *Argulus ambloplites*, *Argulus sp*. et *Cichlidogyrusspp*., les pics de 77.14, 77.14 et 80 % de prévalence ont été respectivement atteints en juin, février et septembre (figure 1).

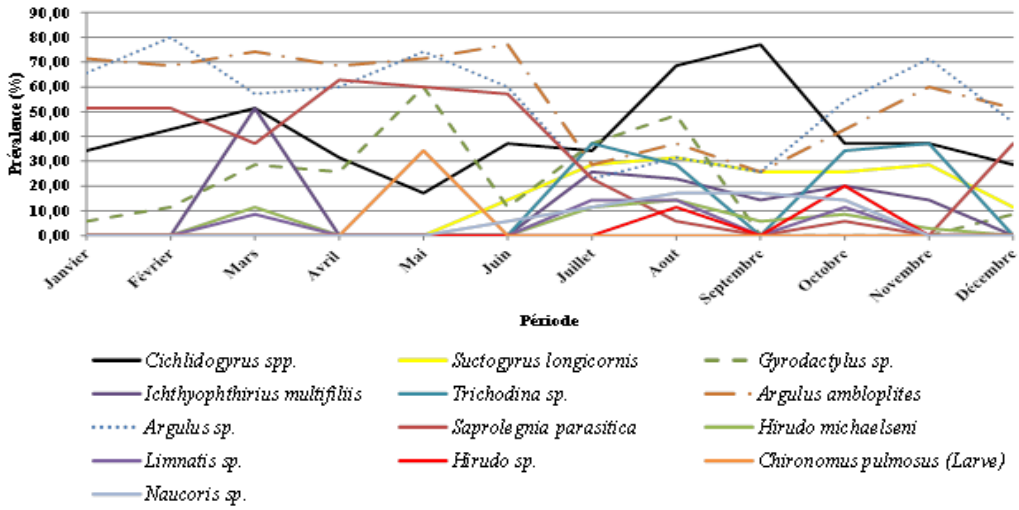

Fig. 1. Variation mensuelle des prévalences ectoparasitaires chez *Oreochromisniloticus* d'élevage en région des Lagunes en Côte d'Ivoire

Pour ce qui concerne les intensités moyennes d'infestation, elles ont également évolués en dent de scie durant toute la période de l'étude. En région des Lagunes, le nombre de parasites par poisson a été plus élevé pendant le mois de février pour *Argulus*

*ambloplites*et *Argulus sp*. avec des valeurs respectives de 71.43 et 65.71 et le mois d'août pour *Cichlidogyrusspp.*avec un pic de 78.17 (figure 2).

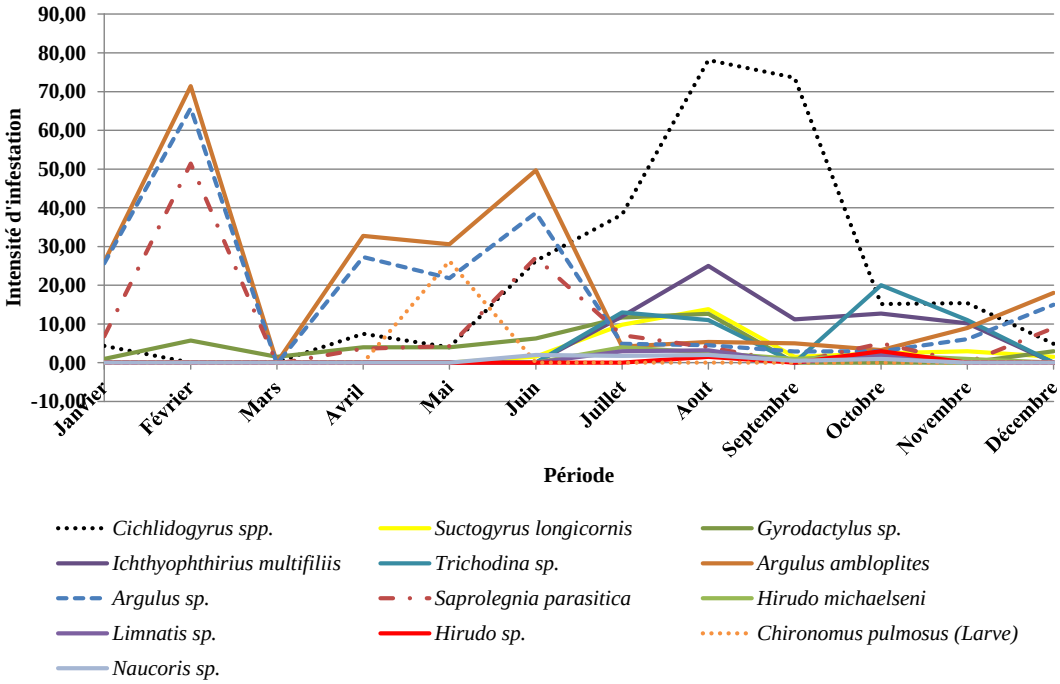

Fig. 2 . Variation mensuelle des intensités d'infestation ectoparasitaires chez *Oreochromisniloticus* d'élevage en région des Lagunes en Côte d'Ivoire

Variation des indices épidémiologiques en fonction du poids des poissons

Le tableau 2 illustre les différentes valeurs de prévalences en fonction des classes de poids. Dans l'ensemble, les différentes classes de poids de tilapias issus des fermes d'élevage ont été infestées par toutes les espèces de parasites identifiées sauf ceux de la classe de poids de 1 à 5 g (Les alevins). Les poissons de cette classe de poids n'ont pas été infestés par les parasites *Hirudomichaelseni, Limnatissp.,Hirudosp.* et les larves de*Chironomuspulmosus.* Les valeurs de prévalence du tilapia du Nil *Oreochromisniloticus* envers les espèces *Cichlidogyrusspp.,Gyrodactylussp., Hirudomichaelseni, Hirudosp.* et les larves de*Chironomuspulmosus* ont été positivement corrélées aux classes de poids avec un coefficient de corrélation ajusté $R^2 \geq 0.98$ et une probabilité d'erreur $P \leq 0.05$. Quant aux autres espèces de parasites n'ayant pas eu de forts coefficients de corrélation ajustés R^2 et de probabilités d'erreur $P \leq 0.05$, telles que *Suctogyruslongicornis, Ichthyophthiriusmultifiliis, Trichodinasp., Argulus ambloplites, Argulus sp.1, Saprolegniaparasitica*et*Limnatissp.*, il a été observé de fortes valeurs de prévalence pour les classes de poids élevées. Seul l'insecte *Naucorissp.* a plus infesté les poissons de faibles poids. Les tilapias de la classe de 1 à 5 g ont eu des taux de prévalence de 12.92 % pour le parasite *Cichlidogyrusspp*. et 11.98 % pour *Ichthyophthiriusmultifiliis.* Ceux ayant des poids compris entre 6 et 50 g et entre 51 et 500 g ont été les plus infestés par les copépodes *Argulus ambloplites* et *Argulus sp*. avec des valeurs

respectives de 20.96 % et 19.44 % pour la classe de 6 à 50 g et 51.25 % et 39.73 % pour celle de 51 à 500 g.

Tableau 2. Variation des prévalences ectoparasitaires en fonction du poids de *Oreochromisniloticus* d'élevage en région des Lagunes en Côte d'Ivoire

	Prévalences en fonction des classes de poids (g)		
Espèces de parasites	[1 - 5 [	[6 - 50 [	[51 - 500 [
Cichlidogyrusspp.	$12.92^{a} \pm 3$	$18.19^{a} \pm 4$	$25.45^{b} \pm 5$
Suctogyruslongicornis	$2.62^{a} \pm 1$	$2.58^{a} \pm 1$	$8.70^{b} \pm 2$
Gyrodactylussp.	$7.00^{a} \pm 2$	$15.85^{b} \pm 3$	$19.66^{b} \pm 4$
Ichthyophthiriusmultifiliis	$11.98^{a} \pm 3$	$17.75^{a} \pm 5$	$30.25^{b} \pm 5$
Trichodinasp.	$5.14^{a} \pm 1$	$5.48^{a} \pm 0.5$	$20.75^{b} \pm 3$
Argulus ambloplites	$1.51^{a} \pm 2$	$20.96^{b} \pm 3$	$51.25^{c} \pm 5.45$
Argulus sp.1	$1.35^{a} \pm 0.9$	$19.44^{b} \pm 3.1$	$39.73^{c} \pm 4$
Saprolegniaparasitica	$1.35^{a} \pm 0.6$	$6.00^{b} \pm 2$	$19.78^{c} \pm 3.4$
Hirudomichaelseni	0.00^{a}	$1.47^{b} \pm 2$	$6.45^{b} \pm 3$
Limnatissp	0.00^{a}	$1.28^{b} \pm 0.09$	$12.50^{c} \pm 3$
Hirudosp	0.00^{a}	$1.32^{b} \pm 0.089$	$6.97^{c} \pm 1.4$
Chironomuspulmosus (Larve)	0.00^{a}	$7.99^{b} \pm 2.5$	$11.68^{b} \pm 2$
Naucorissp	$11.79^{a} \pm 3$	$9.63^{a} \pm 3.1$	$4.60^{b} \pm 1$

Les valeurs d'une même ligne ayant en exposant des lettres alphabétiques identiques ne diffèrent pas statistiquement ($p \geq 0.05$). Celles ayant des lettres alphabétiques différentes possèdent une variance significative ($p \leq 0.05$).

Concernant l'intensité moyenne d'infestation, les valeurs sont indiquées dans le tableau 3. Les parasites *Gyrodactylussp.,Trichodinasp., Hirudomichaelseni, Hirudosp.* et les larves de*Chironomuspulmosus* ont possédé des intensités moyennes d'infestation positivement corrélées aux aux classe de poids avec un coefficient de corrélation ajusté $R^2 \geq 0.97$ et une probabilité d'erreur $P \leq 0.05$.

Les poisons de la classe de 1 à 5 g ont enregistrés les plus fortes intensités d'infestation de 17.15 ± 3.12 spécimens de *Ichthyophthiriusmultifiliis* et de 5.72 ± 1 individus de *Cichlidogyrusspp.*. Par contre, aucun tilapia de cette classe de poids n'a été parasité par les espèces *Hirudomichaelseni, Limnatissp.,Hirudosp.*et les larves de *Chironomuspulmosus*. Les espèces de copépode *Argulus ambloplites* et *Argulus sp*. ont été les plus nombreuses sur les tilapias de poids de 6 à 50 g et de 51 à 500 g. En moyennes 28.51 ± 4 et 65.22 ± 9 individus de *Argulusambloplites* ont respectivement été récoltés par tilapia de poids appartenant à la classe de 6 à 50 g et 51 à 500 g. Quant à *Argulus sp.*, environ 22.06 ± 1 et 42.71 ± 2 individus ont respectivement été identifiés par poisson de classe de poids allant de 6 à 50 g et 51 à 500g.

Tableau 3. Variation intensités d'infestation ectoparasitaires en fonction du poids de *Oreochromisniloticus* d'élevage en région des Lagunes en Côte d'Ivoire

	Intensités en fonction des classes de poids (g)		
Espèces de parasites	[1 - 5 [	[6 - 50 [	[51 - 500 [
Cichlidogyrusspp.	$5.72^{a} \pm 2$	$7.29^{a} \pm 1$	$15.3^{b} \pm 2$
Suctogyruslongicornis	$3.03^{a} \pm 2$	$4.8^{a} \pm 1$	$8.18^{b} \pm 1$
Gyrodactylussp.	$4.16^{a} \pm 2$	$10.2^{b} \pm 2$	$15.81^{c} \pm 3.4$
Ichthyophthiriusmultifiliis	$17.15^{a} \pm 3.12$	$21.71^{b} \pm 3$	$28.08^{c} \pm 4$
Trichodinasp.	$4.41^{a} \pm 1$	$12.78^{b} \pm 3.8$	$23.55^{c} \pm 3$
Argulus ambloplites	$1.42^{a} \pm 0.06$	$28.51^{b} \pm 4$	$65.22^{c} \pm 9$
Argulus sp.1	$1.39^{a} \pm 0.9$	$22.06^{b} \pm 1$	$42.71^{c} \pm 2$
Saprolegniaparasitica	$3.92^{a} \pm 0.4$	$6.63^{a} \pm 1$	$20.51^{b} \pm 4$
Hirudomichaelseni	0^{a}	$2.63^{b} \pm 1.04$	$5.48^{c} \pm 1$
Limnatissp	0^{a}	$1.42^{b} \pm 0.09$	$4.19^{c} \pm 1$
Hirudosp	0^{a}	$1.92^{b} \pm 0.14$	$3.87^{c} \pm 0.75$
Chironomuspulmosus (Larve)	0^{a}	$12.1^{b} \pm 2$	$125.69^{c} \pm 15$
Naucorissp	$3.09^{a} \pm 0.8$	$3.13^{a} \pm 0.11$	$1.43^{b} \pm 0.09$

Les valeurs d'une même ligne ayant en exposant des lettres alphabétiques identiques ne diffèrent pas statistiquement ($p \geq 0.05$). Celles ayant des lettres alphabétiques différentes possèdent une variance significative ($p \leq 0.05$).

Discussion

Les onze (11) genres de parasite récoltés sur le tilapia du Nil *Oreochromisniloticus* d'élevage, à savoir : *Cichlidogyrus, Suctogyrus, Gyrodactylus, Trichodina, Ichthyophthirius, Argulus, Saprolegnia, Naucoris, Chironomus, Hirudo et Limnatis*, ont été pour la plupart reconnus comme étant naturellement ectotoparasites de cette espèce hôte. Maurício*et al*. (2010), dans leurs études sur la faune parasitaire de cette même espèce de poisson au Brésil, n'ont obtenu que trois (3) genres dont deux (2) espèces du genre *Cichlidogyrus*, deux (2) espèces du genre *Trichodina* et une (1) espèce du genre *Lamproglena* (Copépodes). Les espèces du genre *Naucoris*, et plus particulièrement ceux du genre *Chironomus*,ont toujours été signaleés comme des proies potentielles pour certains poisson comme *Cyprinuscarpio* (Adámek et *al*., 2004). Du fait que ces insectes ou larves d'insectes aient été retrouvés bien accrochés à la peau et aux nageoires des poissons étudiés, alors il pourrait s'agir d'une nouvelle forme d'interaction ou stratégie pour échapper à la prédation. Ou encore, s'agirait-il d'une sorte d'interaction entre deux espèces différentes appelée « phorésie » au cours de laquelle un être vivant sert de support à l'autre comme c'est le cas pour l'acarien *Parasitusfucorum* qui est transporté par la Tipule *Tipula maxima* (Bastien, 2011) ? Quand bien même que cela serait le cas, il faut dire que Bichi et Dawaki (2010), Maurício*et al*. (2010) et Akoll*etal*. (2011), dans leurs études récentes sur la faune parasitaire de la même espèce hôte d'élevage, n'ont signalé aucune de ces deux espèces d'insecte. S'agirait-il alors d'un simple coup de hasard ; quand bien même on sait que ces espèces ont été récoltées dans plusieurs fermes piscicoles soumises à l'étude ? Ces différentes interrogations pourraient avoir des réponses avec Adler et Wheeler (1984) et Constant (2007) qui ont découvert que certaines espèces d'insectes de l'ordre des Heteroptères étaient capables d'avoir occasionnellement des comportements coprophagiques et nécrophagiques afin de survivre. N'douba et *al*. (1997) ont signalé que, la famille des Cichlidae dont fait partie l'espèce *Oreochromisniloticus*, est généralement parasitée par des monogènes ectoparasites

appartenant à quatre genres à savoir : *Cichlidogyrus, Onchobdella, Scutogyrus* et *Gyrodactylus*. Selon Mousavi*et al*. (2012), les espèces du genre *Gyrodactylus* font partie des ectoparasites qui sont capables de parasiter une large gamme d'espèce de poisson en aquarium. Les parasites appartenant à ces genres ont également été récoltés sur les arcs branchiaux de leurs hôtes. Quant aux protozoaires des genres *Trichodina*et*Ichthyophthirius,* localisés sur les branchies, la peau et les nageoires des tilapias d'élevage objets de cette étude, leur présence sur la même espèce hôte *Oreochromisniloticus* a été notifiée par Akoll*etal*. (2011) et Bichi et Dawaki (2010) respectivement dans des fermes piscicoles de l'Uganda et du Nigéria. La présence des parasites des genres *Argulus* et *Saprolegnia* sur la peau, les branchies et les nageoires de *Oreochromisniloticus* a été également indiquée par Bucur*et al*. (2011) dans une ferme d'élevage en Roumanie. Les espèces du genre *Saprolegnia* sont connues pour leurs attitudes opportunistes car elles apparaitraient généralement au niveau des lésions corporelles occasionnées par les ectoparasites tels que *Argulus spp*.,*Ergasilusspp*. *Lernatropusspp, Hirudospp*. ou*Hirudinariaspp*. Les espèces de sangsue de la famille des Hirudinidae dont sont issus les genres *Hirudo* et *Limnatis* sont reconnues comme étant des parasites hématophages temporaires qui s'attaquent aux mammifères, aux oiseaux, aux invertébrés, aux mollusques et aux poissons juste pour prendre leur repas sanguin (Malcolm et Kutschera, 2011). Klinger et Floyd (2009) ont observé des espèces de sangsue sur des poissons élevés en étangs. Selon Subasinghe*et al.* (2001), la compréhension de la fréquence, de la distribution et de la composition des communautés de parasites en aquaculture est très importante car elle permet de planifier les stratégies de gestion des pathologies.

Conclusion

1. La faune ecto-parasitaire de tilapia du Nil *Oreochromisniloticus* élevé dans la région des Lagunes a été composée de cinq espèces de monogènes (*Cichlidogyrusrognoni, Cichlidogyrushalli, Cichlidogyrussp., Suctogyruslongicornis* et *Gyrodactylussp*), deux espèces de protozoaires (*Trichodinasp.* et *Ichthyophthiriusmultifiliis*), deux espèces de copépodes (*Argulus ambloplites* et *Argulus sp*), une espèce de champignon (*Saprolegniaparasitica*), trois espèces d'annélides (*Hirudomichaelseni*, *Hirudosp.* et *Limnatissp.*) et deux espèces d'insectes (*Chironomuspulmosus* et *Naucorissp*.).

2. Les espèces *Chironomuspulmosus* et *Naucorissp*. ont été récoltées pour la première fois comme étant des parasites de poisson. Les indices épidémiologiques de ces parasites ont variés selon les saisons et le poids des poissons.

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EPIDEMIOLOGICAL STUDY OF SPONDYLOPATHIES IN DOGS

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Abstract

Different spinal injuries are described at all levels of the vertebral column in dogs and can irreversibly affect the welfare of the animal. The aim of the study was to describe the epidemiological characteristics of spondylopathies in dogs. From January 2010 to July 2012, a total of 327 dogs (14.49%) out of 2257 of patients presented to the Faculty of Veterinary Medicine of Iasi were diagnosed with different spondylopathies. Various dog breeds were included in this study. Mixed-breed dogs were the most affected (76/327), followed by the German shepherd (40/327), Rottweiler (20/327), Bulldog (13/327) and Dachshund (10/327). The male: female ratio was 1.84:1. The age ranged from 2 months to 18 years. Thoracolumbar spine was the dominant spinal level affected (43.12 %), while coccygeal spine was less affected (0.61%). Spine injuries are a common cause of neurological disorders in dogs and the employment of epidemiological, clinical and paraclinical investigations can lead to a certain diagnosis.

Keywords*: epidemiological, spondylopathies, dog*

Objectives

Different spinal injuries are described at all levels of the vertebral column in dogs and can irreversibly affect the welfare of the animal (1).

In Romania there are few studies regarding the epidemiologic data of canine spondylopathies (2).

The purpose of the study was to describe the epidemiological characteristics of spondylopathies in dogs.

Materials and methods

From January 2010 to July 2012, a total of 327 dogs (14.49%) out of 2257 of patients presented to the Faculty of Veterinary Medicine of Iasi were diagnosed with different spondylopathies (table 1).

Various dog breeds were included in this study (table 2).

Table 1. Annual distribution of cases

Year	Total no. of cases	Dogs	Dogs with spondylopathies
2010	1182	973	132
2011	1055	897	137
2012 (02.07.2012)	468	387	58
Total	2705	2257	327

Table 2. Prevalence of spondylopathies at different spinal level

Breed	Year						Total	
	2010		2011		2012			
	Spinal level affected							
	T-L	L-S	T-L	L-S	T-L	L-S	T-L	L-S
Beagle	-	-	1	1	-	1	1	2
Bichon	1	-	4	1	2	-	7	1
Boxer	-	-	1	4	-	2	1	6
Bulldog	4	2	4	-	3	-	**11**	2
Bull terrier	-	-	-	-	-	1	-	1
Cane corso	-	-	-	-	1	1	1	1
Caniche	2	-	1	1	1	-	4	1
Cavalier king charles spaniel	-	-	1	-	-	-	1	-
Ciobanesc german	9	10	12	5	4	-	**25**	**15**
Cocker	1	1	2	-	-	-	3	1
Dalmatian	3	1	-	-	-	-	3	1
Doberman	-	-	-	1	-	-	-	1
Dog argentinian	-	-	-	-	1	-	1	-
Golden retriever	-	2	-	-	-	-	-	2
Husky siberian	1	1	-	-	-	-	1	1
Jack russel terrier	1	-	-	-	-	-	1	-
Labrador	1	-	1	2	-	2	2	4
Lagotto romagnolo	-	-	-	-	1	-	1	-
Mastiff	1	-	-	-	-	-	1	-
Mixed-breed	16	11	24	9	5	11	**45**	**31**
Mops	-	-	-	-	1	-	1	-
Pekingese	4	-	1	1	-	-	5	1
Pinscher	-	-	1	-	1	-	2	-
Pitbull	1	2	2	-	-	-	3	2
Rottweiler	3	11	1	4	-	1	4	**16**
San Bernard	-	-	-	2	-	-	-	2
Schnautzer	-	1	-	-	1	-	1	1
Setter englez	-	-	1	3	-	-	1	3
Shi-tzu	-	-	-	1	-	-	-	1
Spinone	-	1	-	-	-	-	-	1
Spitz	1	-	-	-	-	-	1	-
Teckel	6	-	4	-	-	-	**10**	-
Terranova	1	-	-	-	-	-	1	-
Volpino italiano	-	-	-	-	-	2	-	2
Yorkshire terrier	1	1	1	-	1	-	3	1
Total	**57**	**44**	**62**	**35**	**22**	**20**	**141**	**100**

Results

Mixed-breed dogs were the most affected (76/327), followed by the German shepherd (40/327), Rottweiler (20/327), Bulldog (13/327) and Dachshund (10/327).

The male:female ratio was 1.84:1 (table 3). Most of the dogs affected by different lesion on the spine were intact males (67%), and less affected were castrated males (2%) (fig. 2). The age ranged from 2 months to 18 years (fig. 1). Thoracolumbar spine was the dominant spinal level affected (43.12 %), while coccygeal spine was less affected (0.61%).

Table 3. Distribution of spondylopathies by the sex of the dogs

Year	Males		Females	
	No.	%	No.	%
2010	91	68,94	41	31,06
2011	90	65,69	47	34,31
2012 (02.07.2012)	31	53,45	27	46,55
Total	212	64,83	115	35,17

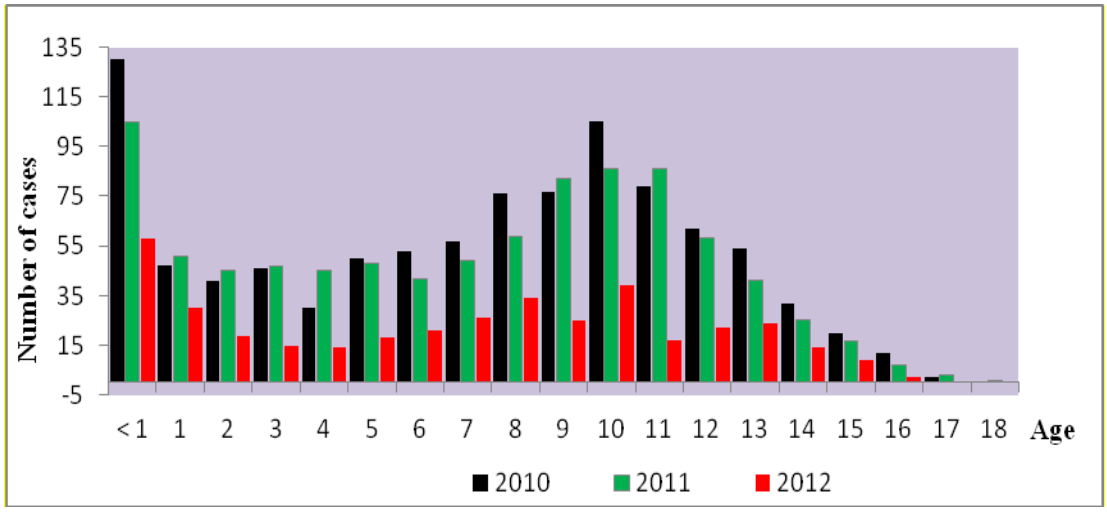

Fig. 1 Annual distribution by dog's age

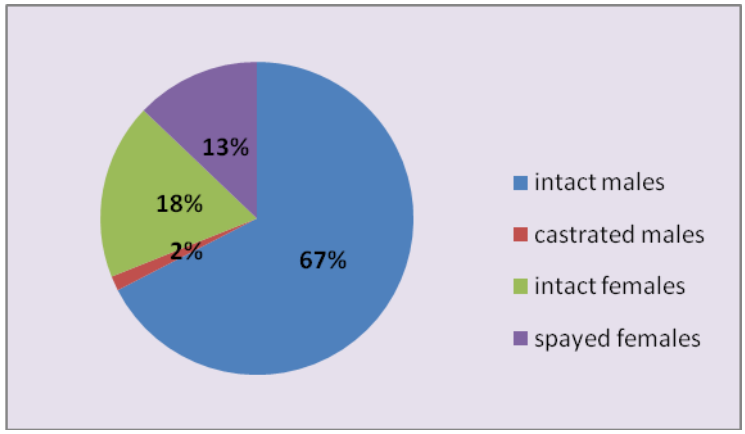

Fig. 2 Prevalence of spondylopathies by integrity of the reproductive system

Conclusions

Spine injuries are a common cause of neurological disorders in dogs and the employment of epidemiological, clinical and paraclinical investigations can lead to a certain diagnosis.

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NORMAL ANATOMY OF DOG POPLITEAL LYMPH CENTER, ANATOMICAL VARIANTS AND NON - INVASIVE ASSESSMENT USING ULTRASOUND TECHNIQUES

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Abstract

An important issue of the clinical examination of carnivores is the superficial lymphatic system evaluation. The main explored lymph node is the popliteal lymph node, due to its easy palpation. Nineteen clinical healthy canines of different breeds and ages were enrolled in this study. The research methodology was developed in two directions and two stages. Forty-eight popliteal lymph nodes were identified using grey scale ultrasound and evaluate by Doppler technique. The ultrasonographic pattern, shape, ecostructure, echogenicity, borders, appearance of hillus, presence and distribution of vascular flow, was described and the length and diameter were determined. In morphological research for describing normal anatomy and anatomical variants of popliteal lymph node, we used the injection of coloring solution. Dye solution identified in all subjects popliteal lymph nodes, ranging in size from 0.3-3.5 cm, oval shape in fifteen subjects and round shape in four subjects. Anatomical variants were represented by the presence of two popliteal lymph nodes on each side in three subjects and three popliteal lymph nodes on each side in one subject. There was a total concordance between ultrasonographic findings and coloring results. We conclude that ultrasound techniques are valuables tools for the identification and non-invasive evaluation of popliteal lymph nodes.

Keywords: *lymph node, polpiteal fossa, ultrasound, dye solution*

Introduction

Evaluation of the lymphatic system is an important part of the clinical examination. Currently, the most evaluated lymph center is the popliteal lymph center due to its easy plapatory approach on one hand, and on the other hand the fact that it drains important structures like the distal part of pelvic limb, mammary glands or genitals is very important (1, 2). Because efferent lymphatic vessels, which accompanying popliteal and femoral artery, are afferent vessels to deep inguinal lymph, popliteal lymph center examination, may guide the diagnostic methodology to a target system. Both in human and animals, in addition to clinical examination in assessing popliteal lymph center, ultrasound may increase the sensitivity and specificity of lymph nodes examination by proving information on size, shape, internal structure, nodal border and hilar pattern (3, 4). Doppler sonography can be used to evaluate the presence and distribution of intranodal vessels (5). In the literature there are few data describing the popliteal lymph center ultrasound. Based on these considerations, the aim of the present study is to describe the ultrasonographic anatomy of the popliteal lymph center in addition to the morphological description and to determine the correlation between the two methods.

Materials and methods

Research was conducted on a total of nineteen subjects, for a period of three years. Inclusion criteria were: clinically healthy individuals, aged between three and fourteen years old.

Breed and weight were not exclusion criteria. The research group was composed of: two Pekingese, one Pincher, two Labrador Retrievers, two Rottweiler, one Doberman, two Dachshund and eight common breed specimens. In this way the weight was between 3 and 35 kg. The study was approved by the Ethics Committee of the University. Informed consent for inclusion and participation in the study was signed by the animal owners. The animals were brought to the University for Euthanasia due to excessive aggressiveness and inability to place in a kennel or in a family. The study was performed in two steps: the first step was the ultrasound assessment of popliteal lymph center using grey-scale ultrasound and Doppler techniques. The lymph nodes were imaged using a Logiq 7 device or a Logiq 9 (General Electric) device and a linear transducer at 7.5-12 MHz, using a standard small part-imaging mode. The ultrasonographic examination was performed by one and the same observer who scanned the popliteal fossa in all subjects. The size of lymph node was measured at its shortest and longer axis, and the short/long axis ratio was calculated to assess the lymph nodes shape. A ratio<0.6 was assessed to oval shape, while a ratio>0.6 was assessed to rounded lymph nodes. Echogenicity, echostructure, nodal margins characteristics, presence or absence of a nodal hilum were recorded. Doppler method using color flow mapping were used to evaluate the vascular supply to and within the lymph node. The presence and pattern of vascular distribution was recorded. Initially, each subject underwent to a general physical examination. A rigorous mechanical preparation was made before the ultrasound examination. Ultrasound examination was performed under stunning, using Butorphanol (0.5 mg/bw). Popliteal lymph center were imaged in supine position from posterior approach (Fig. 1).

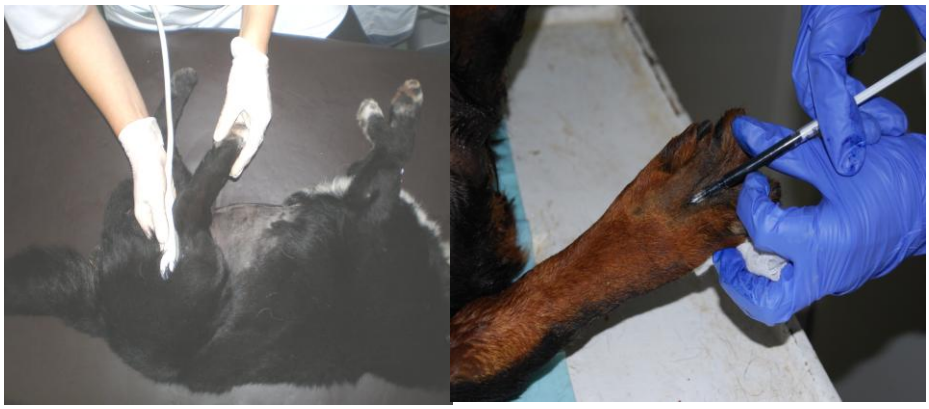

Fig. 1 The posterior approach of popliteus lymph center (left) and Interdigital injection of blue dye solution (right)

The second step of present research was performed by the injection of dye solution in order to identify the popliteus lymph node. 2.5% Blue Dye Evans was injected in the hind paw into the web spaces between second and third digits. Subjects were maintained under careful supervision till the next day. At 24 hours after dye injection, euthanasia was made by administration of Euthasol ®(pentobarbital sodium phenytoin sodium 390mg + 50mg/ml, Virbach AH, Inc.) 0.22ml/kgbw. Stratigraphic and regional dissection was performed through an incision in the popliteal fossa, followed by careful widening, in cranial and caudal direction and careful skin removal of the entire length of the lower limb.

Statistical analysis was performed dependent on variable studied. The quantitative variables were expressed in mean + /-SD using Descriptive statistics program. Qualitative variables were expressed in ratio. Comparison of quantitative variables between groups was performed using Student t test and / or Wilcoxon test, where $p < 0.05$ was considered statistically significant.

Results

Nineteen healthy dog subjects were included in the study. Dog had a mean age of 8.5 years + / - 2.9 years old and mean weight of 18.7 kg + / - 10.5 kg. Different breeds were more or equal to two in five cases, and only two breeds appearing once. Forty-eight lymph nodes were identified using both, ultrasound and blue dye injection in all nineteen subjects. In fifteen subjects were identified thirty popliteal lymph nodes, one on each side, three subjects had the popliteal lymph center compound by two lymph nodes on each side (twelve lymph nodes), and one subject has three lymph nodes on each side (six lymph nodes). Data are summarized in Table 1.

Table 1 Ultrasound and blue dye detection of popliteal lymph center

Nr of subjects(n=19)	15	3	1
Nr of ln (n=48)	30	12	6

Ultrasound examination of popliteal fossa was made by posterior approach, subjects being placed in the supine position. The same position was maintained in all subjects and the cross-sectional images were obtained. Ultrasound detection of popliteal lymph center was easy by posterior transverse approach compared to distal third to the front of the thigh approach, when unclear longitudinal sections were obtained, or even with the longitudinal posterior approach with the transducer positioned on long axis of the lower limb. There was no difference in detecting the popliteal lymph center in the left or right side.

Regarding the shape of popliteal lymph center, we found that fifteen subjects present oval shape of popliteal lymph center, while in four subjects in whom the popliteal lymph center was composed only by a single lymph node, the shape was rounded. SA/LA ratio was between 0.38 and 0.89 (mean 0.58) in right popliteal center and between 0.35 and 0.87 (mean 0.56), in left popliteal lymph center. In subjects who had a popliteal lymph center compound of more than one lymph node, the shape was oval in all cases. We found no significant difference between longitudinal axis dimensions from right or left size (p=0.44), or short axis dimensions between right and left size (p=0.46). On the forty-eight ultrasound evaluated lymph nodes 36(75%) had a homogenous echostructure, slightly heterogenous in 12 lymph nodes. All lymph nodes were mildly hypoechoic relative to the perinodal fat, even isoechoic

in six lymph nodes. Nodal border were regular in all cases with well-defined capsule. Lymph nodes hillum was detected in 32(66%) of lymph nodes, as a hyperechoic structure that is continuous with adjacent tissue. In 16 lymph nodes we did not identify the hillum. Doppler examination revealed the presence of vascular signal in 38 (78%) lymph nodes, starting from the hillus toward to the lymph node capsule (Fig. 2).

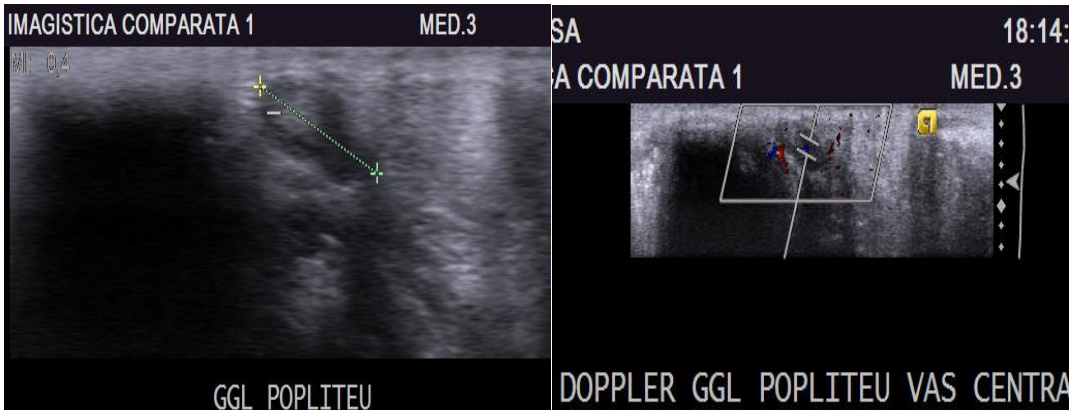

Fig. 2 Grey scale and Doppler ultrasound of popliteal lymph center

We found no differences between the ultrasound features of right and left popliteal lymph center in the same subject.

Interdigital injection of blue dye Evans identified in all subjects the popliteal lymph center. Lymphatic superficial vessels were visualized as a fine network at the injection site, at the dorsum of the paw, and later, pair's vessels run on each side of dorsal metatarsal vein. Then cross to the lateral side of the limb, and around to the third middle of the calf they become postero-lateral (Fig.3).

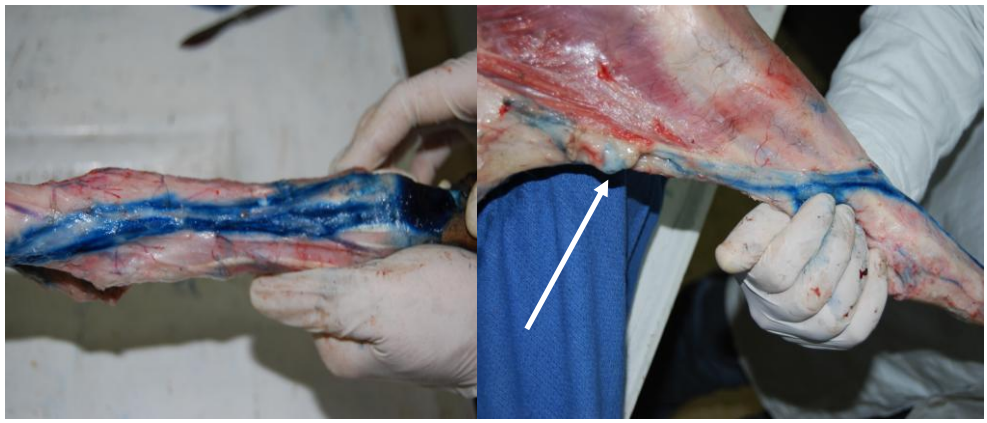

Fig.3 Lymphatic vessels on the dorsum of the paw (left) and passage of lymphatic vessels to the popliteal lymph center (right)

We found the existence of anastomoses between the two lymphatic vessels and their crossing in front of or behind the vein. In the middle third of the calf, we found the presence of a collateral lymphatic network, the posterior one making anastomoses to the superficial medial lymphatic vessel by crossing the Gastrocnemian muscle. In the upper third of the calf, these vessels are located deeper and covered by a thin fascia and subcutaneous tissue, and stands slightly postero-medial. Here, these vessels are placed on each side of safenous vein on the lateral side of gastrocnemian muscle. Making an incision on the popliteal fossa, we visualized well colored the lymphatic vessels placed on the medial side of semitendinosus muscle, between it and semimembranosus muscle. Before entering the popliteus lymph node, the two main vessels separate in smaller lymph vessels which entering into the lymph node by crossing the capsule.

In specimens that had more than one lymph node compound the lymph center, the blue dye staining was very obvious in the distal lymph node, the second appeared colorful too, but not so strong as the first draining lymph node. The specimen that had three lymph nodes in popliteal lymph center, the third lymph node was not impregnated with dye on the right side, but was colored in the left side. Regarding the shape of the colored lymph nodes, it was kept the same features as those obtained at ultrasound examination. The shape was oval in fifteen subjects and four subjects have a round shape. The capsule appeared like a smooth layer, being visible perinodal adipose tissue. The efferent lymphatic vessel leaves the hilum of lymph node in upward direction toward to the deep inguinal lymph nodes. In two subjects it was colored the medial iliac lymph nodes too. Anatomical size measured on the long axis of the right popliteal lymph center was between 0.9-3.5cm (mean 2.0 + / - 0. / 7), and the diameter was between 0.4-2.9 cm (mean 1.1 + / - 0.5). On the left side the anatomical length was between 0.9-3.2 cm (mean 2.1 + / -0.6) and the diameter ranged from 0.5-2.9 cm (mean 1.1 + / -0.5). The results are shown in table 2.

Table 2. Comparison on US features and Blue dye Evans pattern

Parameter	**US features (n=19)**	**Blue dye pattern (n=19)**
Shape	Oval (n=15)/rounded (n=4)	Elongated (n=15)/round(n=4)
Margins	Well-defined, midly hipercogenous	Continuous, unsharp, well coloured
Ecogenicity/Uniformity	Midly hipoecogenous, isoechoic with surrounding tissue / Homogenously	Uniform
Hilar pattern	Hillar tisuue definition	Well coloured, with clear eferrent lymphatics
Vascular flow	Hillar	Not aplicable
Perinodal fat	Present, midly hiperecogenous	Visible, above coloured capsule

Comparing dimensions obtained at ultrasound examination with those obtained after injection of dye solution, we found that in the right popliteal lymph center it was a perfect match on eleven subjects, six of them showing the anatomical dimensions above the dimensions registered on the ultrasound, and two subjects had anatomical dimensions below the ultrasound measurements. Also there were differences between measurements obtained in left popliteal lymph center. In twelve subjects the ultrasound dimensions matched with those

obtained after blue dye injection, in five subjects ultrasound dimensions were below the anatomic range and in two subjects the ultrasound measurements were above the blue dye value. But, statistically analyzing the obtained values, there were no statistically differences between the ultrasound dimensions and anatomical range.

Discussion

Popliteal lymph center is, due to its location the most explored lymph center in physical examination. Paraclinic investigation methods of the popliteal lymph center are relatively rarely documented in specialized literature, especially for animals. In the present study, it has been achieved to identify using ultrasound techniques all the popliteal lymph centers in all the subjects. Nevertheless, the most explored lymph centers in carnivores are the lymph centers which drain the mammary gland due to the similarities with the lymphatic drainage of the mammary glands in woman (2, 6) and the lymph nodes of the abdominal cavity. Normal lymph nodes of the abdominal cavity are hard to examine due to their reduced size or because of similar ecogenicity with the adjacent tissues, both in human and animals (7, 8). Studies focusing on abdominal cavity lymph nodes in dogs, sometimes report certain constraints of ultrasound examination, related to it's in depth location, or to the presence of digestive tract gases, which affect the image quality, or even leads to the impossibility in analyzing the lymph node.

The lymph nodes that are identified most often, besides the superficial lymph nodes, are the iliac medial ones and the jejunal ones (8, 9). In the present study ultrasound has proved to be a simple and easy way to evaluate the popliteal lymph center. This was realized from the posterior of the popliteal fossa, compared to the lateral approach or even the posterior one in longitudinal section. The approach of the popliteal fossa is missed by these constraints: on one hand due to the relatively superficial sitting of the popliteal lymph node in the popliteal fossa triangle, and on the other hand, the lack of gasses which can affect the image quality. However we can say that more attention was needed for identifying all the popliteal lymph nodes because in six subjects the popliteal lymph centers were izoecogenous with adjacent perinodal tissue.

Another issue concerns the possibility to locate the popliteal lymph center using ultrasound with the help of adjacent anatomical structures as landmarks (10). In assessing abdominal cavity lymph nodes this can be done if we are to consider the perivisceral or juxtavascular location of the lymph nodes, and if possible the combination of the two, it is quite easy to identify. In our study, as anatomic landmarks we used the ultrasonographic aspect of the caudal femoral artery (hipoecogenous, with vascular signal) and the ultrasonographic characteristics of the distal portion of the semitendinosus muscles, the semimembranous muscles, and the proximal features of the gastrocnemius muscle (hipoecogenous structures with smooth hiperecogenous septums).

Regarding the ultrasound dimensions of explored lymph center from the study, they are in accordance with the anatomical descriptions (1, 2, 11) and in direct relation with the animal's weight.

There are studies which take into consideration lots of other ultrasound parameters for the lymph node assessment: shape, lymph node capsule pattern, presence of nodal hillus and aspects of lymph node vascularization both in human and animals (11, 12). We have followed the same parameters in our research. In healthy subjects, the normal lymph node shape is oval

while a rounded shape signifies the presence of illness as is mentioned in numerous studies (13).

Nevertheless, we found in four subjects rounded shape of popliteal lymph center both using ultrasound examination methods and blue dye injection, healthy specimens being considered. This fact empowers us to state that rounded shape or less elongated shape of popliteal lymph nodes does not indicate the presence of pathology. The same results were obtained in another study concerning submandibular lymph center (11, 13). Moreover, normal lymph nodes from different anatomical regions have different conformations and shapes. As an example, mesenteric lymph nodes are more elongated compared to other visceral lymph nodes (8, 12, 13).

All lymph nodes in the present research have a well defined, smooth capsule which delineates the lymph node from adjacent tissues, this being verified after dye injection too. Sometimes this aspect is hard to detect using ultrasound, especially in the absence of a pathological process which would modify the acoustic impedance between the two areas: normal and pathological.

As for ecogenity, our results show the mildly hipoecogenous, even izoecogenous pattern for all the examined lymph nodes. Many researches state that hiperecogenicity is associated with the presence of metastatic pathology, alongside the heterogenous feature (7), which does not apply to the current research. Also, another contradictory aspect regarding the possibility of interpreting a lymph node being hiperecogenous, is the apparition of motion artifacts generated by respiratory movements in mandibular lymph nodes examination or axillary or abdominal cavity lymph nodes, which, again, does not apply in the evaluation of popliteal lymph center.

In our study, the appearance of hyperechogenic hilum was detected in 66% of the examined lymph nodes. This pattern is characteristic to the normal lymph nodes, and it is given by both, blood and lymphatic vessels which leave the node, together with the supporting connective sheath. It is worthy to remember this, because in case of tumoral pathology this characteristic is lost, due to its infiltration or disruption by metastatic cells.

Regarding vascular pattern, in our study the presence of vascular signal was detected in most lymph nodes, especially in those greater than 0.5 cm. If we take into consideration that the hillum is the entry point of blood vessels, this pattern is natural, on one hand, and on the other hand, the subjects of our study were all healthy. It was easier detecting the vascular signal in bigger lymph nodes, in which the distribution of blood vessels was towards the capsule. This fact is also reported in humans, in which the presence of vascular signals is detected in over 90% of normal lymph nodes with a short axis greater than 0.5 cm.

Most studies have as golden standard the histological or even cytological lymph node examination. Indeed, in the studies which compare pathological and normal lymph nodes histological assessment is needed (14). Our research uses as golden standard identification of lymph nodes using blue dye injection based o fact that all subjects were clinical healthy.

Furthermore, we can state that ultrasound results are deeply in concordance with morphological identification results.

We can conclude that ultrasonography is a very useful technique in non-invasive assessment of popliteal lymph center in dogs. Therefore lymph center identification should be part of ultrasound routine examination, contributing with concrete data for diagnostically orientation or towards other detailed investigations. More comparative studies are needed to

include pathologies with direct or indirect involvement of the lymphatic system to develop an accurate interpretation algorithm based on correlation of multiple diagnostic ultrasound and morphological aspects.

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RADIOGRAPHIC DIAGNOSTIC IN TRACHEAL DISORDERS IN SMALL ANIMALS

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Abstract

Between September 2011 and April 2013, 136 pacients with upper respiratory airways conditions were examined in the Roentgendiagnostic Laborabory of the Veterinary Medicine Faculty of Iasi. The radiological examination was performed in latero-lateral and ventro-dorsal recumbency. The primary conditions radiologically diagnosed were: tracheal hypoplasia-1 case, tracheal collapse- 6 cases and traumatic rupture of the trachea-2 cases. There was no diagnosed case with tracheal foreign body or neoplasia. Extrinsec conditions with repercussions on position or caliber of the trachea were represented by stenosis caused by intrathoracic – mostly mediastinal organs pathologies: cardiomegaly- 95 cases, esophageal disorders – vascular ring anomalies 4 cases, megaesophagus 8 cases, transdiaphragmatic hernia- 6 cases, dorsal or ventral cranial mediastinal masses(abcess, neoplasia, the increase in volume of mediastinal and tracheobronhic lymphnodes) and pleural effusions-13 cases. Abnormal (paraphisiological) changes –the mineralization of the trachea- 1 case. Most frequentlysecondary deviation due to mediastinal pathologies have been diagnosed (92,6%). The primary tracheal disorders were diagnosed in 6,6% of the cases.

Key words: *tracheal, radiography, dog*

Introduction

The radiological examination of the trachea is an essential one in the respiratory pathology because it may establish its type and its location. The aim of this paper is to describe the radiological findings in the most often tracheal pathologies.

The radiological examination of the trachea is made in latero-lateral and dorso-ventral or ventro-dorsal incidence and thoracic inlet view. The lateral views bring valuable information regarding the integrity of the tracheal walls (rings), diameter of the tracheal inner space, foreign objects or masses within the trachea [1]. The DV and VD projections have limited value due to the superposition of the mediastinum, spine and sternum, but brings important data concerning lateral deviations. The thoracic inlet view actually shows the shape and diameter of the trachea at the thoracic inlet, being elective for the tracheal collapse.

Material and method

In the Roentgendiagnostic Laborabory of the Veterinary Medicine Faculty Iasi, between September 2011 and April 2013, 135 patients with upper respiratory airways conditions were examined in latero-lateral, ventro-dorsal and cranio-caudal tangential view.

Radiological examination was performed for cervical and thoracic trachea. In order to establish the presence of a primary tracheal disease, traject, diameter, integrity or obstructions of the organ have been searched. For secondary diseases, intra and extra thoracic organs or masses compressions, esophageal diseases with tracheal compression,

transdiaphragmatic herniations, with topographic changes in the thoracic cavity, or pleural liquid have been taken in consideration.

Results and discussions

Between September 2011 and April 2013, 136 cases of tracheal affections have been radiological diagnosed:

- Primary affections: tracheal collapse (6 cases – 4,4%), tracheal hypoplasia (1 case – 0,7%), tracheal rupture (2 cases – 1,5%), intralumenal tracheal masses (0 cases), foreign body (0 cases);
- Secondary affections: deviation and stenosis – mediastinal (cardiac, esophageal, vascular ring abnormality, mediastinal masses such as lymphonodes or tumors), or pleural liquid accumulation (126 cases – 92,6%);
- Tracheal ring mineralization (1 case – 0,7%);

Primary disorders

Tracheal hypoplasia is a congenital disease evolving with at least 50% tracheal lumen narrowing, along it`s length. Tracheal rings are almost complete, and the dorsal muscle is atrophied [2] . Brahicephalic breeds such as English or French bulldog are predisposed, but it can occur in other breeds like Labrador retriever, German Sheppard or Basset hound aswell[3]. It has been rarely diagnosed in cats. Radiological signs are: uniform narrowing of the trachea, thickening of the tracheal wall and sometimes retard growth of the trachea, which can be lately compensated.

Tracheal collapse s a progressive and degenerative disease of the tracheal rings cartilage, which produces a dynamical flattening of the trachea, advancing from mild (25% of the initial diameter) to severe (75% of the initial diameter), or total (100%). Often, toy breeds are affected, and it is diagnosed in young patients (congenital collapse), but it can occur in adults aswell (secondary collapse to obstruction of the high airways, laryngeal paralysis, laryngeal masses or vegetations). Collapse can occur in inspiration when the cervical region is affected, in expiration, when the thoracic trachea is affected, or mixed, when the trachea is affected over it`s all length, and can extend to the main bronchi. Radiological signs in lateral recumbency, inspiratory and expiratory phase show a differential narrowing between the cervical and thoracic trachea (cervical collapse in inspiratory phase and thoracic collapse in expiratory phase) [4]. Usually the ventral wall of the trachea remains straight and the dorsal wall appears irregular.

Tracheal trauma can end with the wall rupture. Biting or piercing wounds, foreign bodies perforations, or damage from medical maneuvers (intubation, tracheotomy) can generate ring fracture, necrosis, or ligament and ring lysis. Rarely, wall tumors can damage and pierce the tracheal wall. In all cases, the air passes from the airways between the muscular fascia and in the thoracic mediastinum and involves subcutaneous emphysema, pneumomediastinum and pneumothorax. Radiological signs are all of an inflammation, high radioopacity around the wound, loss of tracheal wall integrity, subcutaneous emphysema, pneumomediastinum and pneumothorax.

Tracheitis usually do not reveal specific radiological signs, but can be associated with other radiologic diagnosed diseases like tracheal rupture, foreign bodies, or medical maneuvers damage.

Secondary disorders

Tracheal deviation occurs secondary to cervical or thoracic diseases such as: volume modifications of neighboring organs, neoplasia (esophagus, thyroid), pulmonary masses, cardiomegaly, diseases of mediastinal organs (megaoesophagus, vascular ring anomalies-PRAA, the most common vascular ring anomaly in dogs [5], mediastinal masses, lymphomas, mediastinal or pleural liquid collections etc).

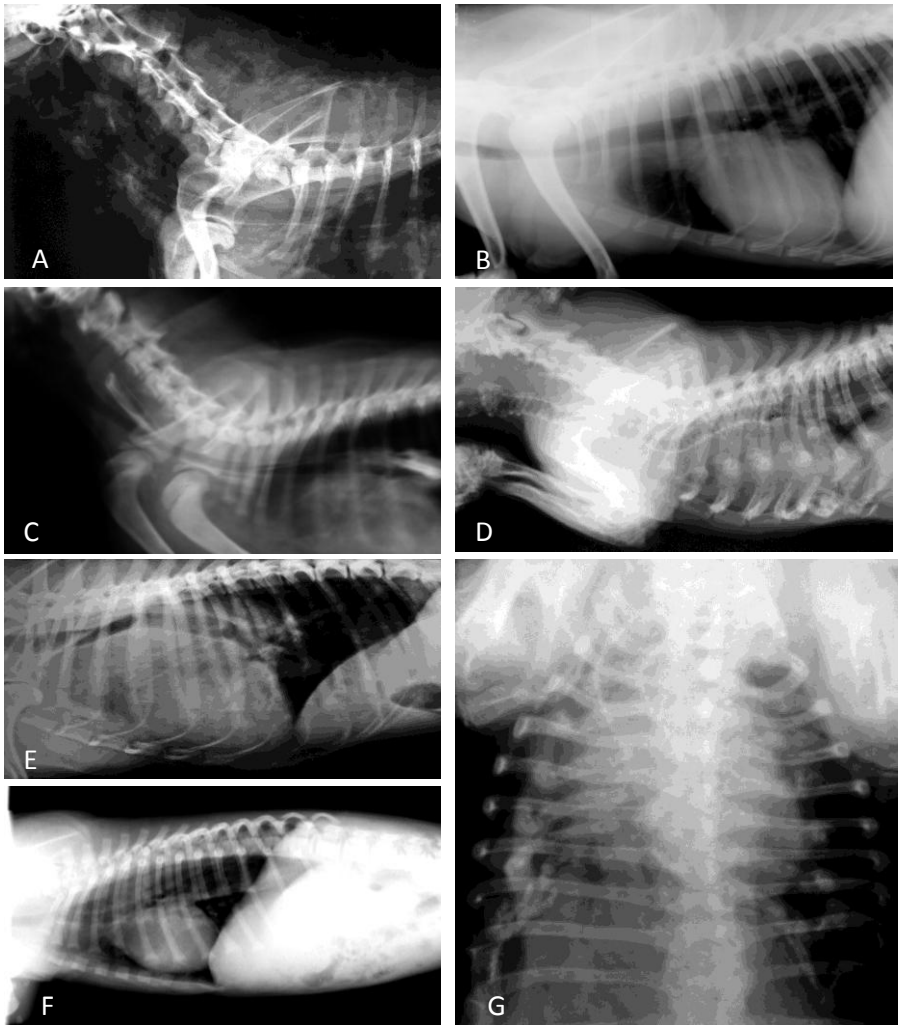

Fig.1 : primary and secondary tracheal diseases : A. cervico-thoracic image, lateral recumbency showing the rupture of the ventral tracheal wall with subcutaneus emphysema; B. thoracic image in lateral recumbency showing a 50% narrowing of the tracheal lumen for a 3 cm distance before the first rib – tracheal collapse; C. cervico-thoracic image, lateral recumbency showing a tracheal lumen narrowing from 4th thoracic vertebrae to the main bronchi bifurcation – tracheal hipoplasia; D. cervico-thoracic image, lateral recumbency showing ring opacity and displacement of the organ – tracheal mineralisation; E. thoracic image, lateral recumbency showing dorsal displacement of the trachea because of a precardiac mediastinal mass ; F. thoraic image, lateral recumbency showing ventral displacement of the trachea because of an oesophageal dilatation ; G. thoracic image, ventro-dorsal recumbency showing right lateral displacement of the precardiac portion of the trachea by a mediastinal mass and ring mineralisation

Mineralization of the tracheal rings usually occurs due to excessive deposit of minerals in tracheal cartilage rings, with age, in larger dogs and chondro-distrophic breeds and most of the times has no pathological significance. Rarely a metastatic calcification can be observed, due to metabolic processes (calcification can also affect the soft tissue - ex. Cushing's syndrome) [1].

Tabel 1. Tracheal diseases radiologically diagnosed according to etiology and pathology

No.	Disease type	Pathology	Number
1.	Primary	Hypoplasia	1
		Tracheal collapse	6
		Traumas	2
2.	Seconday	Tracheal displacement	126
3.	Other	Mineralisation	1
4.	TOTAL		136

Often, the primary diseases of the trachea cause radiological visible damage to other organs. Thus, depending on the severity, the collapse and tracheal hypoplasia lead in general to lung injury from pulmonary edema to collapse and atelectasis. Tracheal trauma determine emphysema, pneumomediastinum, pneumothorax, pneumoperitoneum or pneumoretroperitoneum. Also, stenosis can be seen, due to local inflammation of ligaments and respiratory mucosa.

From all examined cases, the following diseases were radiological diagnosed: hypoplasia, tracheal collapse and tracheal trauma as primary pathologies.

From all secondary diseases, tracheal deviation is by far the most common. Drift direction, the curvature of tracheal lumen and the headquarters of the deviation bring important data regarding the organs responsible for this disease. In other words, the aspect of the trachea narrows the differential diagnostic of mediastinal organs pathology.

Thus, the positional changes of the trachea in cervical level are usually ventral, most often due to thyroid mass, vertebral or paravertebral mass.

In the cranial mediastinum, the dorsal deviation of the trachea is the result of either the increase in volume of the mediastinal lymph node or thymus (lymphoma, thymoma). At the basis of the heart, the trachea can be pushed ventrally by mediastinal dorsal masses or the esophageal distension. Heart base tumors and pulmonary artery dilatation push the trachea dorsally in this portion.

The tracheal bifurcation (carina) and main bronchi origin is the main place of deviations due to trachea-bronchial lymph nodes or volumetric changes of heart. The increase in volume of the lymph nodes deviate the carina ventrally, instead left heart dilatation or global cardiomegaly causes dorsal deviation of the trachea.

As a paraphysiological change, was diagnosed a patient with tracheal rings mineralization.

Due to the anatomical position and structure of the trachea, primary diseases are few in number. Of these, with the highest share were diagnosed the tracheal collapse(4,4%),

followed by trauma(tracheal rupture), with a percentage of 1,5% and tracheal hypoplasia(0,7%).

The pathology with the largest share (92,6%) is represented by the secondary, namely the extrinsic compressions of the trachea, especially cardiomegaly and pulmonary neoplasia, which can compress either by consistency and pressure on the organ, or through the mass of liquid released into pleural cavity.

The deviation of the trachea due to vascular ring anomalies has a small percentage (2,94%) as the underlying cause (persistence of right aortic arch or persistence of subclavian artery) is also rare.

Conclusions

1. The number of secondary pathologies(deviation of the trachea) is higher than the number of primary pathologies, due to the large number of compressions from neighboring organs or intrathoracic / cervical masses.
2. Most tracheal diseases can be radiological diagnosed because of the contrast between the tracheal lumen and wall of the organ. Thus, changes in shape and in volume are most common, but also those related to trajectory and wall integrity can be diagnosed radiological.
3. Depending on the place of the positional changes occurred on X-ray, in conjunction with the patient's signs, narrow, most of the times, the differential diagnosis to a small number of pathologies. Even when radiological examination fails to identify the exact cause of the disease, it reduces the differential diagnosis to a small number of diseases and guides the clinician to further investigation, which make the radiological investigation very important in cardio-respiratory medicine.

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A STUDY ON THE PREVALENCE OF DIROFILARIAIMMITIS INFESTATION ON STRAY DOGS IN GALAȚI COUNTY

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Abstract

The aim of this study is to assess the prevalence of heartworm at stray dogs in Galați County, Romania. Dogs were screened for presence of heartworm antigen using the PetCheck® ELISA on blood samples (N=50) collected from dogs in shelter during May-June 2013. Thirty dogs were heartworm antigen positive, with a prevalence value of 60%. For detection of microfilariae in the canine peripheral blood, it was performed a direct blood smear. The results were analyzed in order to test the presence of heartworm antigen against the following independent variables: percentage of time spent outdoors, pet coat length, age, prevention status and gender. Based on these two criteria: time spent outdoors (100%) and the fact that all pets have not received any sort of heartworm preventative medication we may provide explanation for the higher heartworm prevalence.The length of a dog's coat was significant in heartworm infection: 25 short-haired (N=39, prevalence 64,1%), 5 long-haired (N=11, prevalence 45,4%) dogs were positive for heartworm. The association between gender and heartworm at infected dogs was: 26 females were positive (N=38, prevalence 68,43%) and 4 males were positive (N=12, prevalence 33,3%).Dogs less than 9 months of age were excluded because of extremely low probability of patent infections.

***Keywords**: Heartworm, microfilariae, PetCheck Canine, Heartworm, Antigen Test.*

Introduction

Heartworms (*Dirofilaria immitis*) are mosquito's vectored filarial nematodes of significant importance on the veterinary community and pet owners as parasites of domestic dogs (*Canis lupus familiaris*). At dogs and other canines, adult male and female worms reach maturity inside the pulmonary arteries, and an untreated patent infection is often fatal. (Laura L., Crosbie, 2010).

Different species of culicid mosquitoes act as an intermediate stage in order to complete their life cycle. When taking a blood meal from a microfilaremic host, the mosquitoes become infected and the microfilariae develop to the third-stage larvae (L3) inside the malpighian tubules of the mosquitoes, which are deposed on the host while the mosquito is taking a blood meal, becoming sexually mature within a few months inside the main pulmonary arteries and right ventricle (Cancrini and Kramer, 2001).

Studies carried out in the last 10 years suggest an enhance of cardiopulmonary dirofilariasis at dogs toward Central and Northern Europe. Several factors can exert an influence on the spreading of the disease, such as movement of infected animals, the introduction of new species of mosquitoes able to act as vectors, the climate change caused by the global warming, and development of human activity in new areas. (Morchon R., Mellado I.,E. et al.,2011)

In Romania, the average prevalence was 35% rising to 67% in some areas (Olteanu, 1996). The study, published by Sofia et al. (2007), reported the presence of *Dirofilaria* spp. in

23.07% of the studied dogs, which provides evidence of the current presence of the parasite in the country, which is considered an endemic country. (Morchon R., et al., 2012). In 2007, at the department of Parasitology, Faculty of Veterinary Medicine, Iaşi were diagnosed cases of cardiovascular and subcutaneous heartworm disease caused by *Dirofilaria immitis* and *D.repens* collected from stray dogs in Brăila and Tulcea Counties. Four dogs were serologically diagnosed positive for dirofilariosis, in Tulcea County. Necrosis examination showed in one of the individuals, inside the cardiovascular system a number of 25 adult parasites with a body of 8-33cm. (Acatrinei D. et al.,2008). In the year of 2008, the first case of combined forms of dirofilariosis was reported in Romania.(Pasca S.,et al.,2008).

The first case report of canine heartworm disease in Iasi was diagnosed in March 2009. Since then, 27 new cases were diagnosed owned by Iasi citizens and 41 in stray dogs from shelters and public kennels.(Acatrinei D., et al., 2010).

The presence of *D. immitis* at dogs constitutes a risk for the human population. In a human host the parasite is the causative agent of the pulmonary dirofilariasis and in many cases produces benign pulmonary nodules which can initially be misidentified as malignant tumors (Simón et al., 2005).

The aim of this study was to assess prevalence of heartworm infection on stray dogs in Galați County. The main hypothesis evaluated, was that the heartworm prevalence increases at un-owned dogs without prophylaxis and on dogs that spent their time outdoors. We focused on the southeast of Moldavia on shelter dogs (never sampled before) due to the suitable conditions for heartworm vector development. It has been demonstrated experimentally that infectious L3 development requires 8–10 days at 28–30°C, 11–12 days at 24°C, and 16–20 days at 22°C. Below 14°C, the development arrests, although it can be restarted when the ambient temperature increases above this threshold (Cancrini and Gabrielli, 2007).

In literature it says that dogs with longer or thicker coat may be less likely to acquire heartworm due to the fact that a mosquito might be less likely or able to take a blood meal from a pet whose skin is difficult to access. This study has confirmed the theory, because only 5 long haired dogs were found positive with heartworms, (N=11), the rest of 25 dogs infected were short haired (N=39).

Materials and methods

Overview

The study was carried out from May to June 2013. Blood samples from stray dogs in Galați County, Romania, were randomly selected from a local shelter and tested for presence of heartworm antigen using ELISA (PetCheck®). For each individual animal were recorded data about the potential exposure factors.

2.2 Sample collection

Blood was collected from un-owned dogs at least 9 months of age. Dog less than 9 months of age were excluded because the probability of patent infections for this age group is extremely low. Most dogs were bled via the external saphenous vein; anesthesia was not administrated and the animals that resisted physical restraint were no sampled. At least 2 ml of blood sample from each dog was collected in a plasma separator tube and the plasma was stored afterwards at freezing temperature (-20^0C) until time of testing.

For each studied animal were provided data about gender, coat length, prevention, age, outdoors. (Table 1) It was assumed that all un-owned dogs spent 100% of their time outdoors during day and night. Coat length was registered as short (39) and long (11) during the time of sample collection. None of the dogs in the shelter have been receiving anti-heartworm medication. From the total of 50 canine blood samples, 38 were female and 12 male.

Table 1. Factors evaluated for impact on heartworm prevalence

Analyzed factor	Categories	
Gender	Male	Female
	12	38
Coat length	Short hair	Long hair
	39	11
Prevention	No	
Age	9 months- 12 years	
Outdoors	Yes	

Immunodiagnostic

Plasma samples from this study were tested for the presence or absence of *Dirofilaria immitis* antigen, using an ELISA, the Canine Heartworm Antigen Test Kit PetCheck®(Westbrook, Maine USA). This PetCheck® test has the highest sensivity and specifity of all available tests, particularly on low worm burden infections (Courtney and Zeng, 2001). The samples were tested on a plate frame plus two wells for the positive and negative controls using procedure #2: Laboratory Protocol. This procedure uses and requires washing equipment and a spectrophotometer. The measurements and the records of the optical density (OD) values at 650 nm for samples and controls were accomplished through the Magellan Soft. For the assay to be valid the P-N (OD Positive Control- OD Negative Control) should be greater than 0,150. In addition, the negative control OD value should be less than or equal to 0,150.

Results and discussion

A total of 50 canine blood samples were collected from shelter dogs in Galaţi. Of all the samples tested for the presence of heartworm antigen, 30 (60%) were positive. (Table 2)

Table 2. Heartworm positive dogs (female/male)

	N	Heartworm			
		Positive	% Positive	Negative	% Negative
Female	38	26	68,43	12	31,57
Male	12	4	33,3	8	66,6
Total	50	30	60	20	100

N= total blood sampled

There was a difference in heartworm infection rates between female (N=38, 26 positive dogs, prevalence 68,43%) and male (N=12, 4 positive dogs, prevalence 33,3) recorded in Table 2.

Table 3. Heartworm positive dogs (short/long hair)

No. Negative samples Coat length		No. Positive samples Coat length		Positive %	
Short	Long	Short	Long	Short	Long
39	11	25	5	64,1	45,4

It can be noticed the difference between the length of a dog's coat in heartworm infection: 25 short-haired and 5 long-haired. (Table 3)

Table 4. Heartworm OD (optical density) values

OD Values - Female% 0, 852-2635		OD Values-male % 0, 85-1,588				
Dog number	OD.	Positive				
		Female	Male	Coat length	Prevention	Outdoors
Unknown	1,588			Short	No	Yes
0893	0,952			Short	No	Yes
0899	2,527			Short	No	Yes
0940	0,172			Short	No	Yes
Unknown	1,864			Long	No	Yes
Unknown	2,253			Long	No	Yes
8897	0,217			Short	No	Yes
0916	0,85			Short	No	Yes
Unknown	2,419			Long	No	Yes
Unknown	2,304			Short	No	Yes
0894	2,068			Short	No	Yes
0466	0,175			Short	No	Yes
1975	0,418			Long	No	Yes
0903	0,349			Short	No	Yes
0914	0,183			Short	No	Yes
Unknown	2,35			Long	No	Yes
0901	2,467			Short	No	Yes
0932	2,81			Short	No	Yes
1110	2,287			Short	No	Yes
0939	2,328			Short	No	Yes
4294	0,196			Short	No	Yes
1107	2,328			Short	No	Yes
0920	0,196			Short	No	Yes
0930	2,317			Short	No	Yes
0615	2,273			Short	No	Yes
Unknown	2,328			Short	No	Yes
1123	0,196			Short	No	Yes
Unknown	2,317			Short	No	Yes
Unknown	2,273			Short	No	Yes

The dogs that were positive are recorded in Table 4 with their gender, coat length and optical density values.The selection of dogs for this study was based on two hypothesis: it was expected that dogs which spent at least some time outdoors would be more likely to acquire heartworm than dogs which spent less time outside; it was further hypothesized that dogs with no prevention would be more likely to acquire heartworm than dogs which had an anti-heartworm medication, may provide some explanation for the higher heartworm prevalence that was found in this study.

All positive samples have been recorded with an optical density (OD) values (Table 4). The results were tabulated through Magellan Soft, and the interpretation was read after OD (optical density) values. The heartworm positive female had OD values greater than OD values of the male. This could create thehypothesis that OD values of heartworm positive female is correlated with the high level heartworm infection.

For the detection of the microfilariae in the canine peripheral blood a direct blood smear was performed.

This study presents the first data gathered from a shelter in Galați County. We focused on the southeast of Moldavia, due to the existence of favorable circumstances for the development of the vector in this region. No previous study has been conducted in this city with these goals. The overall prevalence of heartworm in this study (60%) was higher than expected. Regardless of methodology, there are important factors that may be involved in the high prevalence heartworm. Vector distribution and density may have been high, leading to an increase in available mosquito breeding sites. For our region, to this date, no inventory of fauna species culicids exists more recently than13 years.

The hypothesis that female dogs are less likely to acquire heartworm it is wrong due to the fact it is contradictory to our data found in this study which reveals 26 heartworm positive female dogs (N=38), to 4 heartworm positive male dogs (N=12). This data indicates the fact that mosquitoes bite has not revealed discrimination between genders but rather the exposure was achieved equally. In fact, regarding the animal habitat from the shelter (2-3 in cages outdoors) we might have the explanation for the higher prevalence at heartworm positive female.

The statistical analysis related an association between gender and heartworm positive dogs and their correspondent of optical density values. The range of OD values at heartworm positive females were larger and more varied (0, 852-2635) than the range of OD values at heartworm positive males (0,85-1,588). These values may be a potentially important variable related to the presence of heartworm if they indicate a connection between the level of heartworm infestation and high OD values. Through the achievement of this research, we can confirm the link between level heartworm infestation and high OD values.

The most accurate test currently available is the PetCheck® assay (Courtney and Zeng, 2001) used in this study. This way, the very small numbers of adult worms can be detected, even single worm infections. This kind of testing made it possible to detect infections in which no microfilariae are present: the so-called "Hidden infections."(Laura, L., Crosbie, 2010).

Conclusions

1. The overall prevalence of heartworm (60%) in Galati County was higher than expected, therefore we considered Galati County a hyper endemic area.

2. Finally, based on the results of this study, it is possible to confirm that the heartworm infection is increased on stray dogs which spend their time outdoors (N=50, positive=30, outdoors =100%) and on those which have not been receiving any type of heartworm prevention. A particular interest may be the role that un-owned dogs resident in shelters may play within the maintenance of heartworm transmission.

3. By contrast, this study found that heartworm positive dogs were more female (positive=26, prevalence= 68,43%) than male (positive=4, prevalence 33,3%), therefore based on these results we can confirm that dogs were equally exposed to mosquitoes bites.

4. There was no association between the length of a dog's coat and heartworm positive dogs: (25 short haired were positive and 5 long haired were positive), according to specific literature which specifies that heartworm infection is diagnosed more often to short haired dogs.

5. The increasing number of heartworm positive dogs found in this study in Galati County, represents a risk for the human population. Further studies on human heartworm infections are absolutely essential.

6. Finally, based on these results, we may predict the occurrence of heartworm infection on humans, in Galati County, therefore our intent will be to achieve a serological screening on human blood samples in this area.

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GLYCAEMIC CURVE ASSESSMENT, A MONITORING TOOL FOR ADEQUATE INSULIN THERAPY FOR DIABETES MELLITUS IN CATS

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Abstract

Blood glucose curve is one the most important test that needs to be considered when dealing with a diabetic patient. The test holds a few key points that still impose difficulties when interpreted. An accurate interpretation of a blood glucose curve helps clinicians to establish an appropriate treatment protocol. The main aspects considered when interpreting a blood glucose curve, are the initial blood glucose before the insulin administration, the onset of insulin action, the action peak, the nadir, defined as the lowest point of the blood glucose after the administration of insulin and the length of insulin action. In order for a blood glucose curve to be reliable, other primary or secondary pathologies that might interfere with the insulin activity need to be excluded or addressed if present. Serial blood glucose curves are essential when dealing with a poor response to the insulin treatment and for an adequate insulin therapy protocol. Also when the dose or type of insulin are altered, or as routine periodic evaluation test. Clinicians are able to establish an appropriate dose of insulin, with minimal risks, on the base of blood glucose curve data.

Key words: *cat, diabetes, glucose curve, portable blood glucose meters, Somogy*

Introduction

Recognizing and addressing diabetes in cats involves a number of factors that relate primarily to the case history, intensity of clinical signs and physical examination (Van de Maele, Rogier et al. 2005).

Diabetes control and remission are highly dependent on early diagnosis and adequate glycaemic control. Maintaining a close to normal range as long as possible, gives the pancreatic β cells the possibility to regain their secretory capacity(Michiels, Reusch et al. 2008, Roomp and Rand 2009, Zini, Moretti et al. 2009). Contributing factors in diabetes remission were not fully elucidated. However, tight glucose control could be considered the most important factor. Some authors also have considered the age to be an important characteristic, showing that geriatric cats are more likely to enter remission than those younger. This theory was explained by a slower progression of the disease(Zini, Hafner et al. 2010).

A number of tools are being used as indicators for blood glucose (BG) control, like fructosamine and glycated haemoglobin, which reflect glucose concentration over the preceding 1 to 3 weeks and up to 120 days respectively(Van de Maele, Rogier et al. 2005, Zini, Hafner et al. 2010). High concentrations of both parameters indicate a poor BG control but they do not point the flaw in the treatment protocol. In turn, blood glucose curve (BGC) is a highly avalible test and easy to perform with portable blood glucose meters (PBGM), able to provide valuable information about the insulin activity onset, activity peack and lenght, a possible Somogy effect and insulin over or underdosage. Performing a blood glucose curve is considered vital for newly diagnosed diabetic patients, when specific treatment is initiated, when insulin dose is considered too low and clinical signs of PU/PD persists or conversely,

when the dose is too high and clinicians and owners deal with life threatning hypoglycemia episodes. Also BGC should be performed when other clinical signs indicate inadequate glycemic control, or simply as a rutine periodic checkout (Rucinsky, Cook et al. 2010). Blood glucose curve gives the clinicians the possibility to detect the exact issue that lead to treatment failure and the ability to address the appropriate cause, in order to obtain an adequate glycaemic control. However, before performing a BGC the nature of each case, sensitivity to insulin and also stress factors should be evaluated. Also a primary or secondary endocrinopathy which can reduce the body's ability to restore glycaemic homeostasis should be investigated. Associated endocrinopathies act either by antagonistic activity to the insulin, by reducing the availability of insulin receptors, or by competition towards the receptors, automatically reducing the treatment response and life expectancy of each patient (Niessen, Petrie et al. 2007, Niessen 2010).

Materials and methods

A number of 18 client owned diabetic cats admitted in the small animal clinic of the veterinary teaching hospital of the University of Iasi (Romania) were included in the study. The inclusion criteria required owner agreement for hospitalization and continuous monitoring for at least one week. Owners consent was obtained, along with the local ethical committee approval.

Glucose determination was performed with Accu-Chek Active (Roche), PBGM designed for human medicine. Portable glucose meters designed for human medicine are used on a routine base in the veterinary medicine, and were proved to be enough accurate for blood glucose determinations (BGD) (Zini, Moretti et al. 2009). The device used in this study is able determine glucose concentration in the range of 10 to 600 mg/dl or 0.6 and 33.3 mmol/L. All values outside these parameters are indicated as "Lo" for the concentrations below 10 mg / dl and "Hi" for those over 600 mg / dl. Because there was no data available regarding the performance of Accu-Chek Active in cats, the glucose measuring accuracy was evaluated by comparing the results with those obtained on a standard spectrophotometric biochemical analyzer, Auto Focus 200 Chormay. All cases were hospitalized for at least 3 days prior to the BGC performing, in order to accustom the patients with the new environment and reduce the stress hyperglycaemia.

For the PBGM, in each cat, capillary blood samples were obtained from the inner pinna. The sampling site was sanitized, dried and an oil-based ointment was applied to prevent the droplet of blood flow through the hair. A volume of 2-3 μL of blood was expressed on the inner pinna and transferred on the snap test on each determination. For the reference spectrophotometric biochemical analyzer a minimum 1 ml of blood were collected from the jugular vein in to dry tubes coated with cloth activator. All samples were separated by centrifugation for 5 minutes at 3000 rpm. The type of insulin administrated in the study was human *premixed insulin* **Mixtard 30,** with 30% short-acting and 70% intermediate-acting isophane insulin. Samplings for PBGM and the reference method were collected and performed at the same time, before meals and before insulin administration at 0 h, followed by every two hours sampling on the course of 12 hours and the glycaemic curves were performed for each cat included in the study.

The results were statistically correlated Pearson with the Statistical Package for the Social Sciences, version 17 (SPSS). Clarke error grid analysis was constructed in Matlab

Software (Edgar Guevara – Ecole Polytechnique de Montreale). Also, the mean difference and standard deviations were calculated.

The error grid analysis was used to compare the predicted values obtained with the PBGM and the values obtained on the reference method. The measurements were divided in five zones annotated from A to E. The first two zones A and B corresponded to accurate results, which can guide the protocol treatment to correct decisions, while the results that fall in the zone C, D and E correspond to high errors that would lead to inadequate treatment (Zini, Moretti et al. 2009).

Results and discussions

All BGC were conducted inside the clinics in the absence of the owners, thus stress hyperglycemia cannot be ruled out. Stress hyperglycaemia was explained as an adaptation reaction to extreme conditions and was based on a surge of adrenaline, a stimulation of the sympathetic nervous system and an increase of the catabolic hormones(Tappy 2008). Catabolic hormones, especially glucagon and adrenaline, lead to degradation of glycogen, blood sugar increase and reduced use of glucose via insulin-mediated processes. Thus, the stress or "fight or flight" reaction allows the body to provide a greater amount of energy substrate for central nervous system and skeletal muscles, at the expense of parenchymatous organs(Stumvoll, Chintalapudi et al. 1995, Van Cromphaut 2009). In order to reduce the stress hyperglycaemia, all patients were allowed a tree day accommodation period. In cases where the results were dubious, the BGC were repeated. By removing the cat from their environment, especially those who rarely leave the usual habitation, at the same time if the examination is taking place in a noisy room, with more people around it, will increase the amount of stress, contributing directly to falsely elevated glycaemic values and the false impression that high insulin doses are required and therefore increased risk of hypoglycaemic episodes.

The first consideration regarded when constructing a BGC is the initial glycaemic measurement, prior to insulin administration. This value should be considered decisive for the initial insulin dose. A second factor and the most important considered for the therapy protocol establishment is the nadir, defined as the lowest point in blood glucose decrease, expressed as mg/dl or mmol/L. A nadir lower than 100 mg/dl, could be encountered in different type of situations. The first and the most probable is insulin overdose, followed by reduced food intake and dosage overlap. By observing the nadir, clinicians also have the ability to detect the Somogy effect, defined as rapid drop of blood glucose, caused by high doses of insulin. Glucose concentration usually drops below 65 mg/dl, followed by a rapid increase that usually exceeds the initial pre-insulin concentration. This phenomenon is explained by the perception of the situation as a life threatening crisis state and the liver responds by a rapid increase of BG via glycogenolysis and gluconeogenesis. While the BG concentration rises sharply in a short time, beta cell failure does not allow the production of a sufficient amount of insulin that is needed in order to restore euglycemia state (Van Cromphaut 2009). On the other hand if the nadir is over 170 mg/dl, a series of factors should be considered, like a low dose of insulin, high food intake, poor administration skills of the owner, insulin resistance or even stressful environment. Blood glucose nadir is considered to be ideal when it falls in to the range of 100 to 170 mg/dl.

The next consideration is the activity peak and the length of the time of action. Insulin activity length is expressed in hours and is considered from the time of the insulin being injected until the BG starts increasing again and reaches or exceeds the initial BG.

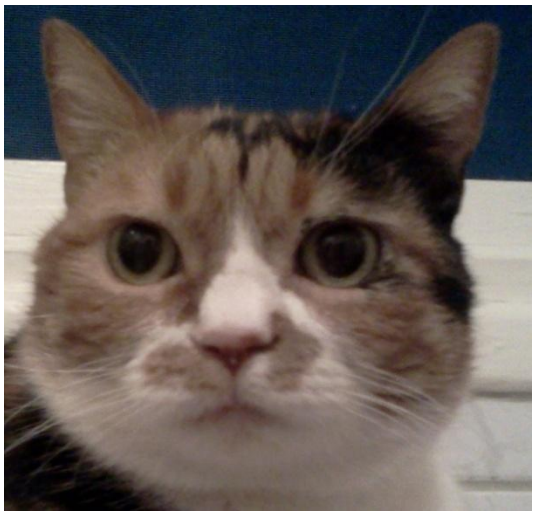
In our protocol, in cases where insulin action was shorter than 8 to 10 hours, clinical signs persisted and the mixed insulin was switched to a lent acting type (Lantus). Also if the activity peak was observed earlier than 6:00 hour post-insulin, the therapy protocol was altered from mixed insulin to a lent acting insulin type, whereas when the action peak installed 6 to 8 hours post-insulin, the insulin type was considered to be ideal and treatment was continued as such. Also if the nadir fit in to the 100 to 170 mg/dl interval the dosage was considered to be appropriate and the protocol was continued as such. All key points and possible situations that can be encountered when performing a BGC are described in table 1.

Tabel 1. Results interpretation for blood glucose curve.

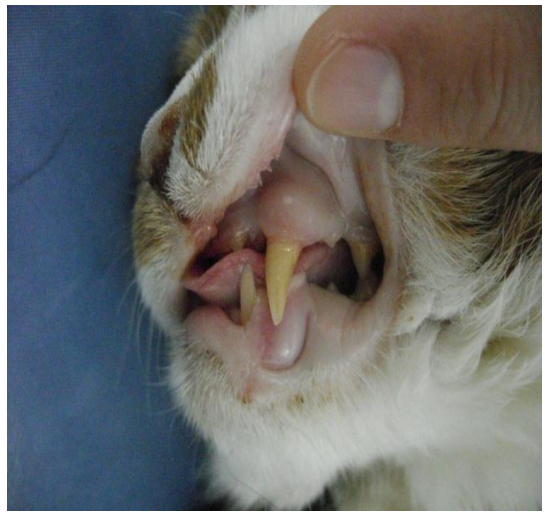
Nadir	Results interpretation	**Insulin Peak**	Results interpretation
<100 mg/dl	Insulin dose should be reduced in order to avoid hypoglycaemic states. Repeat BGC	**<6 hour**	Treatment should be changed to a lent type of insulin. Repeat BGC
100-170 mg/dl	Could be considered ideal range of blood sugar control, the risk of hypoglycaemia is low.	**6-8 hour**	Treatment could be continued with the same type of insulin, administered twice a day if blood glucose nadir is found in the desired range
>170 mg/dl	Shows poor blood glucose control and a graduate increase of the insulin dose is recommended. Repeat BGC	**>8-12 hour**	If the nadir is satisfying, insulin can be administered once daily

For the cats observed in our study, clinical signs improved in a considerably short period of time. Clinical signs improved within 2 weeks from the initiation of the study for 15 cases (83.3%), while 2 cats (11.2%) required up to 3 weeks. One cat revealed marked insulin resistance and the diabetic clinical manifestations persisted without any sign of improvement. Further investigations on this case have shown that insulin resistance and clinical signs persistence were secondary to acromegaly (Photo 1 a, b). The blood glucose curve for this cat is plotted along with the insulin peak and nadir and insulin action (Fig. 1).

The PBGM proved to be highly accurate and adequate to be used for BG monitoring in diabetic cats. All data in this study were positively correlated Pearson and Spearman, with a correlation factor of 0.997 and 0.991 respectively. The amount of blood required for the manual glucose monitor was easily obtained from external pinna vein in all cases, thus the technique can easily be performed in most cases by a single person, including the owners. Hematoma formation on the collection site is highly reduced compared to venous blood sampling. Only the acromegalic cat displayed high aggressiveness and required more than one person for contention. All results obtained with the PBGM registered minimal variations compared to those determined with the standard biochemical analyzer from the jugular vein, with a range of 5 to 15 mg / dl, with a mean difference of 6.11 mg/dL and standard deviation of 5.48mg/dl.



a.



b.

Fig 1: Domestic shorthaired female cat, 14 years of age, diagnosed with acromegaly, with broad facial features (a) and inferior prognatia (b).

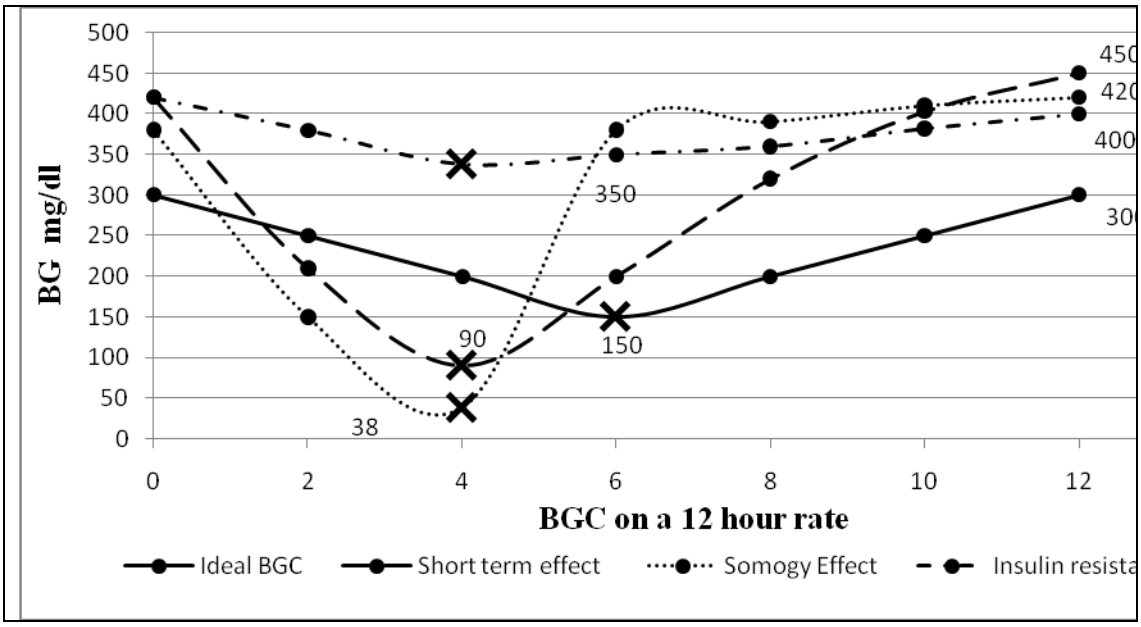


Fig 2: Key points and situations encountered on BGC

The Clarke error grid approach is used to assess the clinical significance of differences between the glucose measurement technique under test and the venous blood glucose reference measurements. The method uses a Cartesian diagram, in which the values

predicted by the technique under test are displayed on the y-axis, whereas the values received from the reference method are displayed on the x-axis. The diagonal represents the perfect agreement between the two, whereas the points below and above the line indicate, respectively, overestimation and underestimation of the actual values. Zone A (acceptable) represents the glucose values that deviate from the reference values by ±20% or are in the hypoglycaemic range (<70 mg/dl), when the reference is also within the hypoglycaemic range. The values within this range are clinically exact and are thus characterized by correct clinical treatment. Zone B (benign errors) is located above and below zone A; this zone represents those values that deviate from the reference values, which are incremented by 20%. The values that fall within zones A and B are clinically acceptable, whereas the values included in areas C-E are potentially dangerous, and there is a possibility of making clinically significant mistakes (Zini, Moretti et al. 2009). The results obtained in this study (Fig.3) are encouraging, as only 5.5% of result fit in the B area. However results that fall in this area are still considered acceptable and treatment protocol guided by this results has a low potential of failure.

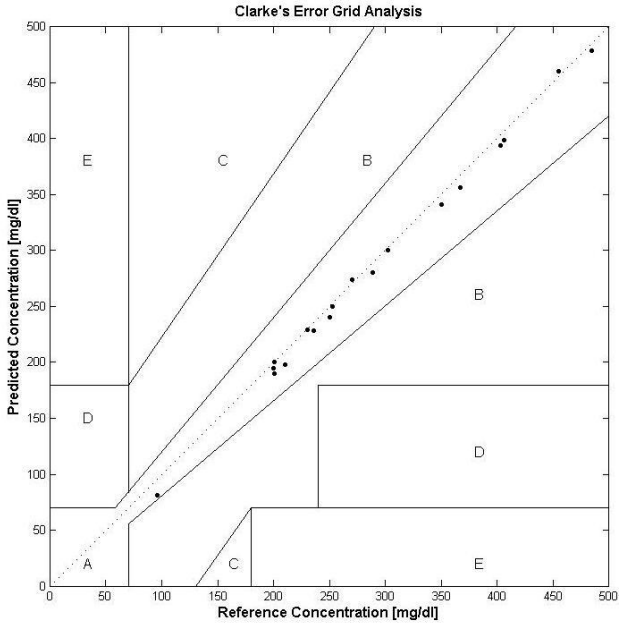


Fig. 3: Clarke`s error grid analysis

Only after taking in to account all the key points of a BGC, a treatment protocol, with an appropriate type and dose of insulin, a suitable administration frequency, can be established for each individual. Also by constructing an accurate image of each case the insulin therapy could imply minimal risks.

Improvements have been observed in the attitude of owners when dealing with their pet after establishing the diagnosis of diabetes mellitus. Most cat owners agree on the acquisition of a PBGM and for home BG monitoring and are committed for continuous

monitoring the amount of water and food intake of the pet, actions considered vital for the establishment of a proper treatment protocol and a positive evolution of the disease.

Conclusions

Serial blood glucose curves give the possibility to exert a tight glycaemic control, by allowing an accurate monitoring and an adequate insulin dosing. Close monitoring and periodic evaluations improve the quality of life and extend life span of the individual.

Even if BG measurements are considered to be insufficient, the technique is a low cost and highly available method, proved to be very handy for pet owners.

All these are important factors in the long-term monitoring of the cat, and real data can be obtained in terms of removing the suspicion of stress hyperglycemia.

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THE EFFECTS OF CURCUMA (*CURCUMA LONGATA*) AS NATURAL TENDERIZER ON POULTRY MEAT

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Abstract

Forty day old chicks Arbor Acres CP-707 were used randomly in this experiment, to study the effects of various levels of Curcuma (Curcuma longata) as natural tenderizer on poultry meat, were studied for six weeks. Research using Completely Randomized Design (CRD). The dietary treatments are: R0 basal diet as control (0% curcuma meal), R1 basal diet + 2% curcuma meal, R2 basal diet + 4% curcuma meal and R3 basal diet + 6% curcuma meal, and each treatment were repeated five times. Results indicated that for the meat fat in broiler which was added curcuma meal in the ration will have less fat to the broiler meat (1.65% - 1.92%) versus 1.99% for basal diet. And for the meat tenderness; the highest meat tenderness was get from the broiler with basal diet + 6% curcuma meal (187,2 mm/g/10sec) and the lowest was get from the broiler with basal diet + 0% curcuma meal (151.6 mm/g/10sec). The results of the study indicated that there is beneficial effect of dietary inclusion of curcuma (Curcuma longa) powder at 0, 2.0, 4.0 and 6.0 per cent on meat fat and meat tenderness of broiler.

***Keywords** : curcuma meal, meat fat, meat tenderness*

Introduction

Texture of foods is mostly determined by the moisture and fat contents, and the types and amounts of structural carbohydrates and proteins. Changes in texture are caused by loss of moisture or fat, and coagulation or hydrolysis of protein (Fellows, 1990). Lean meat contain a very high amount of protein and water and very little fat. In chicken, the protein content are 21 g/100 g; fat 3 g/100g and water 75 g/100g (Simonsen, et al, 1988 in Cross and Overby, 1988). Meat usually cooked before being eaten, and in gastronomic terms, meat is rare if cooked to an internal temperature of 60^{o}C and well done if cooked at 80^{o}C. The endomysial tissue begin to shrink at 50^{o}C and complete at 70^{o}C. When the heating prolonged, the collagen fibres become swollen and gradually denature; and above 70^{o}C will caused further disruption of collagen, and eventually the collagen will solubilized as gelatin. The fat content of meat and meat products has to be judged primarily from the standpoint of calories (Hostetler and Landmann, 1968; Schmidt and Parrish, 1971 in Cross and Overby, 1988).

Tenderness is the process of partial relaxation of the fibres. Resolution of rigor is due to enzymatic activity and physical stretching of the muscles fibres attached to bones. Tenderness is measured by use of specialized laboratory equipment or by a taste-panel (Bell and Weaver, 2002). Contrary to popular belief, what the animal is fed does not directly influence tenderness. Many factor influence meat tenderness. The most important factors are genetics, age of the animal, location of the cut on the carcass, processing, method of cooking and degree of doneness (Epley, 2011).

Curcuma (Zingiberaceae) is a large genus of rhizomatousherbs distributed in tropical and subtropical regions especially in India, Thailand, the Malay Archipelago, Indochina, Northern Australia and Indonesia. Many species have been cultivated, and their powdered rhizomes have been widely used as flavours in native dishes and ingredients in many traditional medicines to treat various ailments (Jantan, et al, 2012). Curcuma longa has significantly greater total polyphenols, flavonoids and anthocyanidins and anti-oxidant activity (Trinidad et al, 2012). According to Jantan et al, (2012) the three curcuminoids

showed strong inhibition on LDL peroxidation, with curcumin and demethoxycurcumin showing comparable antioxidant activity and more potent than bisdemethoxycurcumin. The three curcuminoids showed strong inhibition on LDL peroxidation. The present study was in accordance with previous studies which indicated that the absence of one methoxy group (demethoxycurcumin) on the phenyl ring did not have effect, but the absence of both methoxy groups (bisdemethoxycurcumin) resulted in decreased antioxidant activity in curcuminoids. The phenolic hydroxyl and the methoxyl groups on the phenyl ring and the 1,3-diketonesystem are important structural features for antioxidant activity. The fermentable dietary fiber from Zingiberofficinale and Curcuma longa was shown to produce only the short chain fatty acid, propionate which was significant for both samples indicating protective effect for cholesterol-lowering. Propionate release in the colon after dietary fiber fermentation is readily taken up by the liver. Its action is to inhibit the limiting enzyme HMG Co-enzyme reductase for cholesterol synthesis(Trinidad, et al, 2012). Accordng to Basavaraj (2011), results of meat parameters such as live weight (g), and carcass weight (g), dressing percent, meat to bone ratio are lower and chemical composition of meat, there is no significance between control and curcuma treatments, because the broiler rabbit are on summer stress.

Materials and Methods

Forty broilers, day old chicks Arbor Acres CP-707 were assigned randomly and studied for six weeks. Research using Completely Randomized Design (CRD). They were randomly allotted to four dietary treatment groups of ten chicken broilers in each group namely R-0, R-1, R-2 and R-3. The dietary treatments are: R-0 basal diet as control (0% curcuma meal), R-1 basal diet + 2% curcuma meal, R-2 basal diet + 4% curcuma meal and R-3 basal diet + 6% curcuma meal, and each treatment were repeated five times. Carcass composition of meat were analyzed for the fat content. The broiler carcass tenderness was established by meat tenderness instruments.

Results and Discussions

The effect of Curcuma meal on broiler meat fat.

In Table 1, there are the results of the effect of curcuma meal in ration on broiler meat fat.

Table 1. The effect of curcuma meal in ration on broiler meat fat (%)

Replication	R-0	R-1	R-2	R-3
I	**2.01**	**1.92**	**1.69**	**1.64**
II	**1.99**	**1.93**	**1.73**	**1.64**
III	**1.97**	**1.91**	**1.70**	**1.66**
IV	**1.98**	**1.91**	**1.71**	**1.65**
V	**2.00**	**1.93**	**1.72**	**1.66**
Average	**1.99**	**1.92**	**1.71**	**1.65**

Notes :
R-0 basal diet as control – 0% curcuma meal,
R-1 basal diet + 2% curcuma meal,
R-2 basal diet + 4% curcuma meal and
R-3 basal diet + 6% curcuma meal

From Table 1, the average of meat fat are between 1.65% to 1.99%. The highest meat fat is from R-0 the basal diet without curcuma meal (1.99%) and the lowest is from R-3 that using basal diet plus 6% curcuma meal (1.65%). It means that adding curcuma meal in the ration will give less meat fat to the broiler. According to Jantan et al, 2012 the three curcuminoids showed strong inhibition on LDL peroxidation, with curcumin and demethoxycurcumin showing comparable antioxidant activity and more potent than bisdemethoxycurcumin. The fermentable dietary fiber from Curcuma longa was shown to produce only the short chain fatty acid, propionate which was significant for the samples indicating protective effect for cholesterol-lowering. Propionate release in the colon after dietary fiber fermentation is readily taken up by the liver. Its action is to inhibit the limiting enzyme HMG Co-enzyme reductase for cholesterol synthesis (Trinidad, et al, 2012).

The effect of Curcuma meal on broiler carcass tenderness.

In Table 2, there are the results from using of curcuma meal in ration, to the broiler carcass tenderness. The highest carcass tenderness was get from the broiler that fed R-3 basal diet with 6% curcuma meal (187.2 mm/g/10sec) and the lowest was get from the broiler that fed basal diet R-0 (151.6 mm/g/10sec).

Table 2. The effect of curcuma meal in ration on broiler carcass tenderness (mm/g/10 sec)

Replication	R-0	R-1	R-2	R-3
I	**146.0**	**141.0**	**182.0**	**192.0**
II	**162.0**	**171.0**	**178.0**	**195.0**
III	**142.0**	**154.0**	**175.0**	**185.0**
IV	**149.0**	**177.0**	**185.0**	**182.0**
V	**159.0**	**162.0**	**172.0**	**184.0**
Average	**151.6**	**163.0**	**178.4**	**187.2**

Notes :
R-0 basal diet + 0% curcuma meal, as control,
R-1 basal diet + 2% curcuma meal,
R-2 basal diet + 4% curcuma meal and
R-3 basal diet + 6% curcuma meal.

From Table 2, adding curcuma meal has effect to the broiler carcass tenderness. And the tenderness will increase when the curcuma meal level percentage more higher. In R-0 (basal diet + 0% curcuma meal), the tenderness are 151.6 mm/g/10sec, will increase when the level of curcuma meal are 2% (R-1 = 163.0 mm/g/10sec); and in R-2 (basal diet + 4% curcuma meal) the tenderness is 178.4 mm/g/10sec; compared to the R-3 basal diet + 6% curcuma meal in ration (187.2 mm/g/10sec). Tenderness of the carcass was increase, because the curcuma has enzymatic activities. Because of the activities of the enzyme, even the fat content in the meat decreased, but the meat tenderness will higher than the meat with no curcuma in the ration. Tenderness is the process of partial relaxation of the fibres. Resolution of rigor is due to enzymatic activity and physical stretching of the muscles fibres attached to bones (Bell and Weaver, 2002). According to Jantan et al, 2012 the three curcuminoids showed strong inhibition on LDL peroxidation, with curcumin and demethoxycurcumin showing comparable antioxidant activity and more potent than bisdemethoxycurcumin.

Conclusions

The effect of curcuma in the ration on poultry meat, will decreased the meat fat, but it also will increased the tenderness of poultry meat. So, the curcuma can be used as natural tenderizer on poultry meat.

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THE USE OF ACTINOMICETES PRODUCTS IN THE FIGHT AGAINST AMERICAN FOULBROOD

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Abstract
It is known that actinomycetes actively synthesize products with antibacterial, antifungal, antiviral and antitumoral proprietes. Summary of actinomycetes antibiotics are characterized by a broad antimicrobial spectrum. Antibiotics are widely used on microbial diseases in human and veterinary medicie. Their advantage of actinomycetes is in the relatively low toxicity to humans and animals in comparison with the chemical compounds and the specific action of ability to penetrate the tissues and high activity against pathogens. Scientists from several countries dealing with the isolation of new strains of streptomycetes, which synthesizes substances with antimicrobial properties. Thus, the selection of antibiotic-producing microorganisms isolated from various regions, has been isolated a new strain of streptomicetes summarizing the antibiotic. Object of the research were actinomycetes strains of the genus Streptomyces isolated from soil and kept in CNMN Moldova ASM. Streptomycetes strains are maintained in the laboratory on solid Czapek medium with glucose at temperature +4^{0}C. As test strains were used pathogens Paenibacillus larvae, Aspergillus flavus and Aspergillus niger causing American foulbrood and aspergillosis in Apis mellifera.

Keywords: *actinomycetes, strains, antimicrobial activity, bee, sensibility.*

Introduction

In recent years aimed at alarming decrease in the number of bee families not only in our country but also in the United States and in some European countries, which means that global health is in danger of bees. Welfare bee families is adversely affected by the intensive use of pesticides and fungicides in agriculture and chronic exposure to acaricide. Honeybees are attacked by parasites (*Varroa destructor*, *Tropilaelaps spp*., *Nosema spp*.), fungi (*Ascosphaera apis*), bacteria (*Paenibacillus larvae*, *Melissococcus plutonius*), numerous viruses in any period of life. All this in any way reduce the productive capacity of bee families.

Increasing incidence of American foulbrood, loss of natural resistance of bees and increasing resistance of bacteria to common antibiotics, has increased lately interest in natural tolerance of bee families to American foulbrood pathogen [2, 3, 4, 8].

Antibiotics ranks among the most important group of physiologically active substances. Are described about 8,000 antibiotics isolated from natural sources, particularly from soil microorganisms. Contemporary objectives in antibiotics are primarily related to the need to combat resistance to pathogens existing preparations [2, 11]. To solve this problem resorting to screening of new groups of broad spectrum antibiotics, with a mechanism of action, pharmacokinetics and favorable pharmacological properties, which means that the search for new strains of actinomycetes is current assets and current.

It is known that actinomycetes actively synthesize products with antibacterial, antifungal, antiviral and antitumor. Summary of actinomycetes antibiotics are characterized by a broad spectrum antimicrobial. For example, the substance (I) has been isolated from the culture fluid and mycelium of *Streptomyces sp*. 702, *S. aureus* retain development, *B.cereus*,

B. subtilis, *B. thuringiensis*, *S. cerevisiae*, *F. viride*, *A. niger*, *Mucor sp*., *E. coli* and *F. vasinfectum* [5].

Until now scientists from several countries dealing with the isolation of new strains of streptomycetes, which synthesizes substances with antimicrobial properties. Thus, the selection of antibiotic-producing microorganisms isolated from various regions of Bangladesh, has been isolated a new strain – *S. bangladeshensis sp*. wich sinthesize the new antibiotic bis-(2-etilhexil)phthalate [1].

In the search for new compounds that inhibit sterol biosynthesis enzymes was isolated strain *Streptomyces sp*., wich sinthesize unsaturated fatty acids containing β-lactam ring. Preparation have antifungal activity and can be used in the treatment of fungal infections in humans.

In order to eliminate infectious diseases bee Various remedies are medicines - antibiotics, sulfanilamide, fago-vaccine, inactivated vaccine [6, 7]. Often, the selection of more efficient preparations, it is necessary to investigate new antibiotics against pathogens that are sensitive, specific isolated from outbreaks of infection. In this respect of particular interest actinomycetes as potential producers of new substances with diverse chemical antibiotic properties.

Material and methods

Object of the research were actinomycetes of the genus *Streptomyces* strains isolated from soil and kept in National Collection of Nonpathogenic Microorganisms ASM. Streptomycetes strains are maintained in the laboratory on solid Czapek medium with glucose at temperature $+4^0$ C (refrigerator).

As test strains were used pathogens *Paenibacillus larvae*, *Aspergillus flavus* and *Aspergillus niger* causing American foulbrood and aspergillosis in *Apis mellifera*.

Antimicrobial characteristics were studied by disc diffusion method using agar blocks [9, 10]. Streptomycetes strains were seeded in Petri dishes in lawn on Czapek agar. Over 5-7 days strain could be used to prepare blocks that are inserted into the Petri dishes with test strains.

As control were used discs impregnated with tetracicline and neomicine. Assessment of the antimicrobial activity was performed over 24 hours for bacteria and 72 hours for fungi, by measuring the increase in diameter of the valve.

Rezults and discusions

The results showed that most strains of streptomycetes tested possess antibacterial activity against the pathogen *P. larvae*. Strains of *Streptomyces sp*. 49 and *Streptomyces sp*. 63 deserve attention, the diameter of the zone of growth of *P. larvae* retention was 26.7 and 29.9 mm respectively.

Antimycotic activity clearly showed only two strains of actinomycetes. Retention areas increase *Streptomuces sp*.10(12) made up to 19.5 mm and 17.0 mm from *A.flavus* and *A.niger*. Strain *Streptomuces sp*.6 (66) growth of fungi retained 25.0 and 29.0 mm respectively.

Table 1. Sensitivity of the to bee's pathogen strains to streptomycetes

Strains of streptomycetes	The diameter of the growth restraint, mm		
	Paenibacillus larvae	*Aspergillus flavus*	*Aspergillus niger*
Streptomuces sp. 6 (9)	0	0	0
*Streptomuces sp.*10(12)	0	19,5	17,0
Streptomuces sp. 11	12,9	0	0
Streptomuces sp. 14	14,7	0	11,0
Streptomuces sp. 16 (66)	0	25,0	29,0
*Streptomuces sp.*19	11,0	0	0
Streptomuces sp.33	11,6	0	0
Streptomuces sp.42	0	0	0
Streptomuces sp.44	16,5	0	0
Streptomuces sp. 49	26,7	0	0
Streptomuces sp.63	29,9	0	0
Streptomuces sp. 73	11,0	0	10,0
Streptomuces sp. 120	0	0	0
Streptomuces sp. 145	0	0	0
Streptomuces sp. 178	13,6	0	0
Streptomuces sp. 198	13,0	0	11,0
Streptomuces sp. 208	0	0	growth sporulation restraint
Streptomuces sp. 210	21,7	0	0
Streptomuces sp. 212	0	0	0
Streptomuces sp.214	0	0	0
Streptomuces sp.227	0	0	0
Streptomuces sp.228	0	0	0
Antibiotics			
Tetracycline	41,0	-	-
Neomycin	33,0	-	-

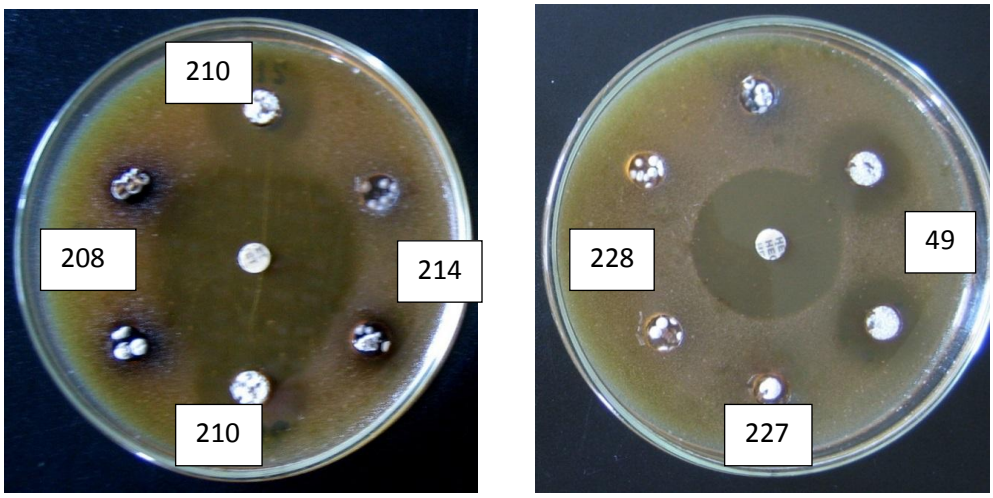

Fig. 1. Retention growth zones of *P. larvae* at some strains of streptomicetes

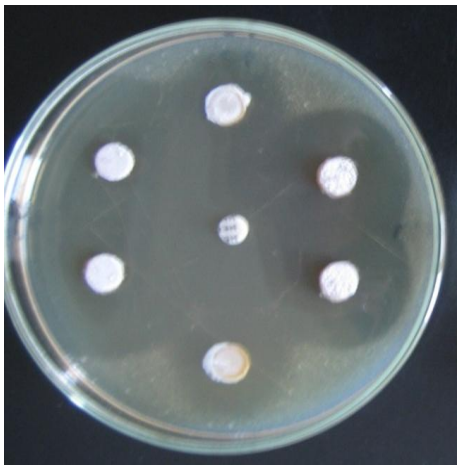

Fig.2. Retention growth zones of P.larvae by *Streptomices sp*. 63

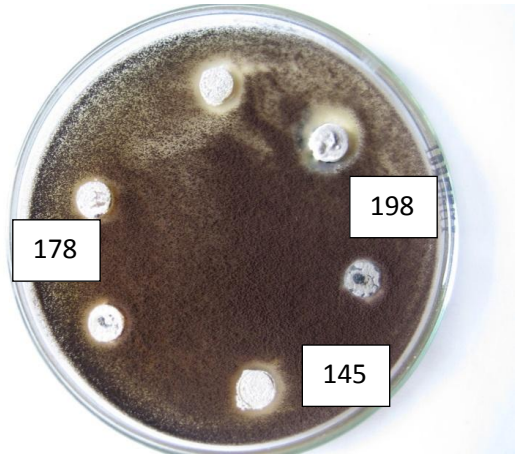

Fig.3. Retention growth zones of *A. niger*, by strain *Streptomices sp*. 198

Conclusions

1. The streptomycetes strains isolated from Moldovan soil,s produs a biologically active substance which also possesses antagonistic action against some pathogenic microorganisms of bees.
2. Following the scriningului of 22 strains of streptomycetes, 3 strains were revealed which actively increase of growth retention of American and European foulbrood (P. larvae).

3. Based on the results we assumed that biomass of this streptomzcetes strains can be used for elaboration of biological products for prophylaxy and treatment of bees bacterial diseases.

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INVESTIGATION OF ANTIMICROBIAL ACTIVITY OF SOME *LACTOBACILLUS SALIVARIUS* STRAINS ISOLATED FROM DENTAL ROOT CANAL AND OF TWO PROBIOTIC *LACTOBACILLUS* STRAINS BY INTESTINAL ORIGIN

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Abstract
In this study, the antimicrobial activity of some Lactobacillus salivarius strains isolated from dental root canal and of two probiotic Lactobacillus strains by intestinal origin has been examined. Lactobacillus salivarius G1 strain isolated from dental root canal showed antimocrobial activity as probiotic Lactobacillus salivarius strain against Lactobacillus sakei LMG 2313 indicator strain.

Keywords*: Lactobacillus salivarius, dental canal, probiotic*

Introduction

A strong antagonistic activity is developed by Lactic acid bacteria (LAB) against many microorganisms including food spoilage organisms and pathogens. These bacteria can produce different antimicrobial compounds including organic acids, hydrogen peroxide, diacetyl and bacteriocins which play a major role in the safety and extending the shelf life of the products which contain them (2, 3, 7).

Bacteriocins are extracellular peptides or proteinaceous antimicrobial compounds, which exert a bactericidal effect against closely related bacteria [4, 5]. The bactericidal activity of bacteriocins is produced by destabilization of the functions of the cytoplasmic membrane of the target cells, altering the permeability properties of the membrane. The C-terminal region is an important zone of target cell specificity.

Lactobacillus species are primarily used as probiotics, but can also be used as starter cultures in various fermented foods and the produced bacteriocins have an important role in bioconservation of foods (6).

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 (*Lactobacillus salivarius* probiotic and *Lactobacillus rhamnosus* GG). All strains were grown in MRS medium and were incubated at 37ºC for 24h, in 5% CO_2 atmosphere.

For determining the antimocrobial activity of *Lactobacillus* strains agar well diffusion assay was used. Petri dishes with CASO agar that were previously inoculated with 100µl from each indicator strain of 24 hours old nutrient broth culture of individual test bacteria were poured. Once solidified, Petri dishes were stored for 2 hours at 4°C. Four wells of 5mm diameter were made and filled with 10µl of *Lactobacillus* culture supernatant. The inoculated plates were kept at 4°C for 2 hours and then incubated at 37°C for 24 hours. Inhibition zones around the wells were measured [Aslim B., 2004].

Table 1. Bacterial indicator strains used in this experiment

Specie	Strain symbol	Incubation temperature °C
Mycrococcus luteus	NCIMB 927B	30
Pseudomonas fluorescens	NCIMB 9046	30
Bacillus cereus	NCIMB 9373	30
Enterococcus aerogenes	NCIMB 10102	30
Lactobacillus sakei	LMG 2313	30
Enterococcus faecium	NCIMB 11508	37
Staphylococcus epidermidis	NCIMB 12721	37
Salmonella enterica subsp. enterica serovar Thyphimurium LT 2	NCIMB 11943	37
Escherichia coli	NCIMB 11843	37
Staphylococcus aureus	ATCC 1448	37
Staphylococcus aureus	NCDO 949	37
Klebsiella pneumoniae	NCIMB 13218	37

Results and discussions

The obtained results were showed in table 2.

Table 2. The antimicrobial activity of *Lactobacillus salivarius* strains isolated from dental root canal (G-1 and G-2) and the probiotic strains *Lactobacillus salivarius* probiotic and *Lactobacillus rhamnosus* GG

Indicator bacterial strain	L. salivarius probiotic				G-1				G-2				LGG			
	10 µl	50 µl	100 µl	C	10 µl	50 µl	100 µl	C	10 µl	50 µl	100	C	10 µl	50 µl	100	C
Mycrococcus luteus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas fluorescens	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bacillus cereus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Enterococcus aerogenes	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactobacillus sakei	+	++	+++	-	+	++	+++	-	-	-	-	-	-	-	-	-
Enterococcus faecium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus epidermidis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Salmonella typhimurium LT 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Escherichia coli	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus aureus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus aureus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Klebsiella pneumoniae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

C= control. – =no inhibition zone; += small inhibition zone; ++= moderate inhibition zone; +++= strong inhibition zone.

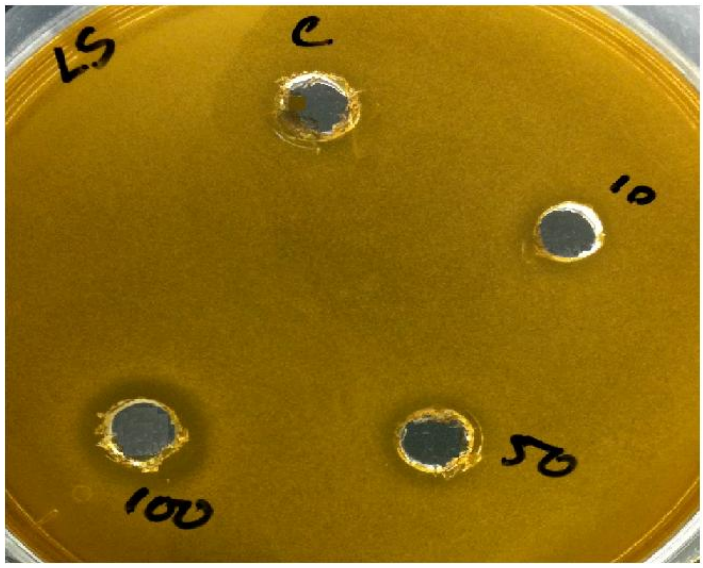

Fig. 1. Antimicrobial activity manifested by G-1 *Lactobacillus salivarius* strain against LMG 2313 *Lactobacillus sakei* indicator strain

The review of the results from the table 2 shows that the probiotic *Lactobacillus salivarius* strain had an antimicrobial activity against L*actobacillus sakei* LMG 2313 indicator strain at all quantities of culture supernatant (10 µl, 50 µl and 100 µl).

Only G-1 strain from the *Lactobacillus salivarius* strains isolated from dental root canal, manifested antimicrobial activity at all quantities of culture supernatant, against same *Lactobacillus sakei* indicator strain LMG 2313 (Fig. 1).

Lactobacillus salivarius G-1 strain isolated from dental root canal and *Lactobacillus rhamnosus* GG probiotic strain as control wells (containg MRS liquid medium) showed no antimicrobial activity.

Conclusions

1. 1.*Lactobacillus salivarius* G1 strain isoleted from dental root canal as well as *Lactobacillus salivarius* probiotic strain showed antimicrobial activity at all quantities of supernatant against *Lactobacillus sakei* LMG 2313 indicator strain.
2. *Lactobacillus salivarius* G2 strain isoleted from dental root canal and *Lactobacillus rhamnosus* GG had no antimicrobial activity for any indicator strain.

Acknowledgments

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REPORT CONCERNING RESULTS OF PROFICIENCY TESTING LABORATORY ON ASSAY OF TOBRAMYCINE AND NYSTATIN BY MICROBIOLOGICAL METHOD

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Abstract

The present study describes the results obtained by the Microbiological Control Laboratory from Institute for Control of Biological Products and Veterinary Medicines after participating in the proficiency testing scheme study on microbiological assay of nystatin and tobramycin. The proficiency testing scheme was organized by European Directorate for the Quality of Medicines and Health Care. The microbiological method consisted of a cylinder-plate agar diffusion assay using Bacillus subtilis ATCC 6633 for tobramicine and Saccharomyces cerevisiae for nystatin as the test microorganism. The means of results were 108, 70 % of label claim for nystatin and 104,70 % of label claim for tobramicin. The Z- scores were 0,14 for tobramycin and 1,40 for nystatin, the assigned value used for booth samples was 105,4 % for tobramycin and 101,7 % for nystatin. The performance of Microbiological Control Laboratory was very good for both samples.

Keywords: *Proficiency testing schemes (PTS), Inter-Laboratory Comparisons, tobramycin, nystatin*

Introduction

Participation to proficiency tests (laboratory evaluating interlaboratory tests) is considered mandatory for laboratories accredited according to ISO/IEC 17025. Checking analyses results with those of other laboratories is one of the most important quality control elements. Confidence that a laboratory consistently produces reliable results is of major importance to the laboratory itself and the organization it belongs to.

The Microbiological control laboratory from ICBMV is regularly taking part in Proficiency testing schemes organized by EDQM. For PTS 130 22 laboratories participated that needed to determine the percentage content of tobramycin injection and nystatin suspension from samples labeled A and B, according to European Pharmacopoeia, 2.7.2, The diffusion method.

Materials and methods

The principle of the method has, as a basis, a dose-response model in which the antibiotic concentration is proportional to the inhibition zone of microorganism growth.

Sample A: tobramicine injection (25 mg/2,5 ml), reference substance, water R -Solvent used in preparing the stock, buffer solution pH 8.0, microorganism test - Bacillus subtilis ATCC 6633, nutrient agar.

Sample B: nystatin oral suspension (100000 IU/ml), reference substance, dimetihylformamide, potassium dihydrogen orthophosphate - Solvents used in preparing the stock, microorganism test – Saccharomices cerevisiae, nutrient agar.

The plates were prepared with media and microorganisms test needed for each samples.

After medium solidification, 4 metal cylinders were placed on the plates surface using sterile pens.

The stock and working samples as well as the reference substance dilutions were prepared according to EDQM protocol. To assess the validity of the assay 3 different doses (tobramycin: 12 IU/ml, 6 IU/ml, 3 IU/ml and nystatin: 100 IU/ml, 50 IU/ml, 25 IU/ml) of the reference material were used together with an equal number of doses of the test substance having the same presumed activity as the solutions of the reference material. After preparing the working dilution, 0.4 ml of the standard and test sample solution were poured in their corresponding cylinders. The plates were left for 1 – 4 hrs at room temperature as a period of pre-incubation diffusion. The plates were incubated for about 18 hrs at 35 to 37˚C. Care was taken while transferring the plates from laminar bench to incubator. After incubation, the diameter of the zone of inhibition was measured using a micrometer.

The potency of the sample was calculated and the results were reported as percentage of the label claim in Excel data sheet .The precision of the assay was such that the fiducial limits of error were not less than 95% and not more than 105% of the estimated potency.

Results and discussion

In the present studies, Microbiological assay estimated the quantity of tobramycin and nystatin present in the sample A and B. Table 1 shows the content of Tobramycin in sample A, for 3 independent determinations .

Determination	SAMPLE A (Tobramycin injection) % of label claim
1	105,10
2	103,80
3	105,20
Mean	104,70
SD	0,7810
RSD	0,7517

The content was calculated taking into account that each 1000 IU is found to be equivalent to 1 mg of tobramycine.

The mean of Potency was 104,70 %, standard deviation was 0,7810 and relative standard deviation was 0,7517.

Table 1 shows the content of Nistatin in sample B, for 3 independent determinations.

Determination	SAMPLE B (Nystatin oral suspension) % of label claim
1	109,91
2	107,25
3	108,96
Mean	108,70
SD	1,3479
RSD	1,0439

The content was calculated taking into account that each 5825 IU is found to be equivalent to 1 mg of nystatin.

The mean of Potency was 108, 70 %, standard deviation was 1, 3479 and relative standard deviation was 1,0439.

The results of the studies were sent to EDQM for examination. After the examination of the data received from laboratories involved in this study, EDQM has calculated, for each sample, the mean value, the standard and relative deviation and the Z- score. For consistency, data are commonly reported using a cut-off value, often <-2 and >+2 Z-scores. The rationale for this is the statistical definition of the central 95% of a distribution as the "normal" range, which is not necessarily based on the optimal point for predicting functional outcomes.

For sample A, the mean of all the results (from 22 laboratories) was 105,7 per cent with a standard deviation of 5,28 per cent and for sample B the mean of all the results (from 22 laboratories) was 96,5 per cent with a standard deviation of 23,80 per cent, according to EDQM.

For the Microbiological Control Laboratory the Z- score was – 0,14 for tobramycin and + 1,40 for nystatin. The assigned value used for samples was 105,4 % for tobramycin and 101,7 % for nystatin.

Conclusion

1. The results obtained by the Microbiological Control Laboratory has found that the Z scores are in the established interval (– 0,14 for tobramycin and + 1,40 for nystatin).
2. The performance of the Microbiological Control Laboratory was very good for both samples and EDQM sent the ”Attestation of participation in proficiency testing scheme’ in 13/09/2012.

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PERSPECTIVE EUROPENE IN DOMENIUL PRODUSELOR MEDICINALE VETERINARE

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Rezumat

Legislatia europeana care guverneaza produsele medicinale veterinare este in curs de revizuire de catre Comisia Europeana. Noua legislatie va reglementa domeniul pentru cercetarea, dezvoltarea si autorizarea de noi produse medicinale veterinare si va stabili modul in care se va efectua distributia produselor medicinale veterinare atat pentru animalele de ferma cat si pentru animalele de companie. Se asteapta ca proiectul legislativ revizuit sa fie postat spre consultare publica in acest an, iar forma finala sa fie adoptata in anul 2014.

Cuvinte cheie*: legislatie, revizuire, produs medicinal veterinar*

Decizia de modificare a legislatiei europene care guverneaza produsele medicinale veterinare, respectiv Codul produselor medicinale veterinare (Directiva 82/2001/CE, modificata cu Directiva 28/2004/CE) a avut la baza o serie de sesizari si propuneri venite din partea reprezentantilor industriei farmaceutice.

Propunerea de revizuire a legislatiei veterinare a fost pusa in discutie prima data in anul 2008, iar principalele motivatii care au stat la baza initierii procesului de revizuire legislativa au fost imbunatatirea functionarii piete europene unice, reducerea volumului de lucru administrativ, simplificarea procedurilor de autorizare si imbunatatirea disponibilitatii produselor medicinale.

La cele mentionate anterior, a contribuit si faptul ca la nivelul Statelor Membre, Directiva este implementata neuniform, iar cerintele suplimentare nationale au dus la cresterea volumului de lucru administrativ si la reducerea eficientei procedurilor de autorizare europene.

Acest lucru a fost confirmat de comunicatul Comisiei catre Consiliu si Parlamentul European (2009) in care se preciza *„It is estimated that 32 % of administrative burdens of EU origin are the result of the decision of some Member States to go beyond what is required by EU legislation (goldplating) and of the inefficiency in their administrative procedures."* (Action Programme for Reducing Administrative Burdens in the EU).

Procesul de revizuire vizeaza intreaga Directiva insa principalele subiecte supuse dezbaterii se refera la:

1. Protectia datelor

Cerintele actuale privind protectia datelor sunt insuficiente. Acest lucru a condus la lipsa de investitii în dezvoltarea de produse noi. Companiile care investesc bugete substantiale în medicamente inovatoare trebuie să se asigure ca investitia lor este viabilă iar scopul dezvoltarii unui astfel de produs medicinal este justificat.

Dupa punerea in aplicare a Codului produselor medicinale veterinare (Directiva 82/2001/CE, modificata cu Directiva 28/2004/CE) s-a constatat o scădere drastică a productiei de produse medicinale inovatoare si din acest motiv este necesara reglementarea protectiei adecvate a datelor pentru companiile care investesc si dezvolta produse inovatoare.

Imbunatatirea reglementarilor privind protectia datelor este necesara, de asemenea pentru produsele destinate utilizarii la pesti, albine, specii minore, etc.

2. Procedura de autorizare

Aceasta propunere presupune simplificarea sistemului actual de autorizare a produselor medicinale veterinare astfel incat toate eforturile repetate actuale sa fie eliminate la nivelul statelor membre UE, prin aplicarea unei singure proceduri europene de obtinere a autorizatiei de comercializare: conceptul 1-1-1.

Conceptul 1-1-1 presupune o singura solicitare avand la baza o singura documentatie tehnica a produsului, o singura evaluare stiintifica si o singura decizie pentru acordarea autorizatiei de comercializare, care sa permita comercializarea produsului in toate cele 27 de state membre, astfel încât medicii veterinari din Europa sa poata avea acces la toate medicamente disponibile în Uniunea Europeană.

3. Ambalarea si etichetarea

Aceasta problema trebuie abordata cu maxima responsabilitate deoarece raportarile de la industria farmaceutica arata ca 34% din totalul activitatilor administrative sunt reprezentate de aceasta activitate.

Propunerea include simplificarea cerintelor legale privind ambalarea si etichetarea si abordarea unui sistem flexibil pentru aceasta activitate, prin utilizarea pictogramelor informative si abrevierilor standard.

4. Farmacovigilentă, monitorizarea si raportarea reactiilor adverse

Revizuirea legislatiei are in vedere si simplificarea cerintelor referitoare la farmacovigilenta in scopul corelarii acestora cu riscul asociat fiecarui produs, simplificarea sistemului de raportare a reactiilor adverse, in paralel cu imbunatatirea masurilor de supraveghere si sanctionare de catre autoritatile competente.

Noile reglementari de farmacovigilență veterinară trebuie sa fie adecvate specificului, nevoilor şi resurselor din rețea.

5. Distributie

Industria farmaceutica veterinara consideră că sistemul de distributie din fiecare tara este suficient dezvoltat pentru a satisface cel mai bine nevoile tării respective. În Danemarca, de exemplu, medicii veterinari nu pot furniza medicamente, acestea fiind disponibile numai prin intermediul farmaciilor, în timp ce în Germania medicamentele sunt disponibile doar prin intermediul medicilor veterinari. Toate medicamentele destinate animalele de fermă sunt vandute "doar pe baza de prescriptie medicala". În Marea Britanie, vanzarea acestei categorii de produse medicinale veterinare este permisa si altor persoane calificate adecvat care prescriu si furnizeaza anumite medicamente pentru prevenirea si tratarea bolilor la animale de fermă.

Revizuirea legislativa va lua în considerare propunerea de armonizare a procesului de distributie a produselor medicinale veterinare la animale.

6. Utilizarea in cascadă

Prevederile legislative actuale ofera posibilitatea diferita de utilizare a produselor pentru specii la care nu a fost autorizat produsul medicinal veterinar sau in conditiile in care se aplica perioada minima de asteptare stabilita. Noua legislatie va asigura o abordare armonizată a procedurii "în cascadă" în întreaga Comunitate si va stabili o cale mult mai practica pentru deteriminarea unei perioade de asteptare corespunzatoare care sa asigure siguranta consumatorului .

7. Rezistenta antimicrobiana

Este cunoscuta importanta reducerii incidentei cazurilor de rezistenta antimicrobiana prin implementarea unor strategii europene pe termen lung care sa implice constientizarea medicilor veterinari si a utilizatorilor.

Rezistenta antimicrobiana este o chestiune foarte complexă, care poate avea impact asupra sănătătii si bunăstarii animalelor. Ca urmare, revizuirea legislatiei va avea in vedere si acest aspect care va include reglementari privind dezvoltarea si autorizarea produselor antimicrobiene, informatii privind vanzarile de antimicrobiene, restrictiile privind utilizarea antimicrobienelor, cerinte privind avertizarile referitoare la antimicrobiene, revizuirea legislatiei privind hrana medicamentata, etc.

8. Armonizarea produselor existente

Revizuirea legislatiei trebie sa includa un proces simplu si eficient pentru armonizarea voluntara si obligatorie a sumarului caracteristicilor produselor care sunt autorizate in tarile UE. In prezent unele produse sunt autorizate în unele tări, dar nu si în altele, precum si conditiile de autorizare pentru produse identice pot fi diferite.

Punctele prezentate anterior constituie doar o parte dintre subiectele puse in discutie la revizuirea Codului comunitar privind produsele medicinale veterinare.

Se asteapta ca proiectul legislativ revizuit sa fie postat spre consultare publica in acest an, iar forma finala sa fie adoptata in anul 2014.

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DATA REQUIREMENTS FOR REMOVING THE TARGET ANIMAL BATCH SAFETY TEST (TABST) FOR IMMUNOLOGICAL VETERINARY MEDICINAL PRODUCTS IN THE EU

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Abstract

This data requirements to be submitted by the marketing authorisation holder (MAH) in order to waive the target animal batch safety test for the release of a batch of this product onto the market. The European Pharmacopeia (Ph. Eur.) General monograph, Vaccines for Veterinary Use (0062), was revised in January 2004 (Supplement 4.6, Ph. Eur., 4th Edition) to state that for an established vaccine the routine application of the safety test may be waived by the competent authority in the interests of animal welfare when a sufficient number of consecutive batches have been produced and found to comply with the test, thus demonstrating consistency of the manufacturing process.

Keywords: *Target Animal Batch Safety Test (TABST), Immunological Veterinary Medicinal Products (IVMP), Batch Safety Test, Good Manufacturing Practice (GMP), Periodic Safety Update Report (PSUR)*

The European Pharmacopeia (Ph. Eur.) General monograph, Vaccines for Veterinary Use (0062), was revised in January 2004 (Supplement 4.6, Ph. Eur., 4th Edition) to state that for an established vaccine the routine application of the safety test may be waived by the competent authority in the interests of animal welfare when a sufficient number of consecutive batches have been produced and found to comply with the test, thus demonstrating consistency of the manufacturing process. Significant changes to the manufacturing process may require resumption of routine testing to re-establish consistency if the competent authority considers that the changes introduced could adversely affect product safety. The requirements laid out in this guideline apply equally to both well established products and those that have been recently authorised. In the light of the changes to the Ph. Eur. monograph, The Committee for Medicinal Products for Veterinary Use (CVMP) has developed this guideline for harmonising the data requirements for removing the target species safety test. The ability to waive this requirement relies, at least in part, on the assurance of product safety that is given by the current EU requirement for pre-authorisation safety tests (single dose, overdose, repeat dose).

Points to consider for removing the batch test for target animal safety

In general it is sufficient to evaluate existing information which is available from routine batch quality control and pharmacovigilance data, without the need for any additional supplementary studies. The data which should be presented to support such an application are presented below. However, this should not be taken as an exhaustive list, and should in all cases be accompanied by a summary report which brings together all of the data presented in terms of an overall assessment of the risk that waiving the requirement will represent.

1. The characteristics of the product and its manufacture

Directive 91/412/EEC covers the principles and guidelines of Good Manufacturing Practice (GMP) and provides assurance that products placed on the market place have been manufactured in a consistent and suitable manner. However, because of the inherent variability of biological products, there is a requirement for final product testing to provide further assurance that the product has been manufactured to the appropriate specifications. The target animal batch safety test on final product provides some assurance that the product will be safe in the target animal species even when administered at an overdose using the route of administration most likely to demonstrate a safety concern.

If the batch safety test is to be deleted as a final product test, a summary report should be provided which would encompass the inherent variability of manufacture of the product, the intrinsic safety margin and the validation which was undertaken to provide the necessary assurance that the product would always be manufactured to an acceptable level of quality and safety.

This would include the characteristics of the product, taking account of whether it has live and/or inactivated viral and/or bacterial components and for live components any residual virulence has been shown to be safe. The type of excipients, adjuvants and preservatives incorporated in the final formulation should be considered.

The applicant should also take account of the range of specifications for the manufacture of the product and how the extremes of the ranges and variability of the final formulation may influence the safety profile of the final product. In exceptional circumstances, where the safety threshold of the product is narrow, the applicant should justify that any issues related to batches at the limits of these parameters could be detected in the absence of the batch safety test. An example of this would be to examine the target ranges of endotoxin, which may be permitted in the final formulation, and how slight alterations in production conditions may influence the subsequent levels of endotoxin and the impact this may have on the safety profile of the resulting formulation.

For those circumstances when *in vivo* batch tests are conducted in the target species for reasons other than the target animal safety test, it is recommended that manufacturers use these tests to gain data of the safety of the vaccine in the target species.

2. Information available on the current batch safety test

Batch data should be submitted on at least 10 consecutive batches from separate final bulks, unless justified. These data may be submitted in the form of a table that lists the results for all finished product tests to demonstrate that the batches consistently met the agreed specifications and also details the antigen bulks used. The applicant should examine the variability of the local and systemic reactions observed in the batch safety test results and the nature of these reactions in relation to those observed in any developmental studies submitted in support of the marketing authorisation. The conduct of the trial shall be in accordance with the Ph. Eur. requirements in operation at the time when the tests were performed. There should be a thorough examination of any batches that have failed the batch safety test and this information, along with an explanation as to the reasons for failure, should be submitted to the regulatory authorities. Information on the number of years the product has been marketed in the EU should be provided.

3. Pharmacovigilance data

A satisfactory pharmacovigilance system should have been in place over the period during which the batches for which data are submitted were on the market.

Pharmacovigilance data to support the removal of the batch safety test should be provided. Marketing authorisation holders should follow the Note for Guidance for veterinary pharmacovigilance for marketing authorisation holders. The most recent Periodic Safety Update Report (PSUR) for the product should be submitted in the variation application to support the removal of the batch safety test. The summary report should include an overall assessment of the product, which would include taking account of the number of batches manufactured, the number of years the product has been on the market, the number of doses sold and the frequency and seriousness of any reactions relating to the safety in the target species and any investigations into the likely causes of these events.

4. Other data

Applicants should summarise the variation applications that have been submitted during the life of the marketing authorisation and any effects these changes may have had on the quality and safety profile of the product in the different categories of animals to which the product is given.

Reference

1. EMA/CVMP/IWP/810769/2011 Guideline on data requirements for removing the target animal batch safety test for immunological veterinary medicinal products in the EU

PHYLOGENETIC ANALYSIS OF THE FELINE CORONAVIRUSES FOUND IN HEALTHY CATS AND IN CATS WITH FELINE INFECTIOUS PERITONITIS BASED ON THE NUCLEOCAPSID PROTEIN

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Abstract

The coronaviruses are higly spread but not always fatal. In cats are two biotypes of coronaviruses, one is the enteric type and the other is the feline infectious peritonitis type, which is said to occur by mutations from the enteric one. The current means of molecular biology can not discriminate the two. Feline infectoius peritonitis is a fatal disease described in cats which is considered to be an immune vasculitis and can evolve in different forms. The diagnose is always diffucult to establih. To investigate the phylogenetic similarities and differences in the nucleocapsid proteins between the two biotypes we collected samples of feces, blood, ascite and pleural fluids from both healthy and feline infectious peritonitis diseased cats both sexes and with ages between 4 months and 6 years. We succeeded to obtain the amplification of the nucleocapsid coressponding gene in 10 from 23 samples by using the rt-pcr methode. Using the transeq program from the European Bioinformatic Institute site (www.ebi.ac.uk) the virtually proteins were obtained and then the alignment and the tree construction based on maximum parsimony was made by using the MEGA5.1 program. For tree bulding we used six feline proteins, a porcine and a canine one taken from GenBank. All sequences obtained presented deletions and mutations which led to the synthesis of aminoacides which changed the structure of the corresponding protein. Three of them presented insertions also and they formed a separate branches.

Key words: *coronavirus, nucleocapsid, phylogenity*

Introduction

The coronaviruses have a large genom made of up to 29 kb forming structural and non-structural genes. For cats are two biotypes of coronaviruses that can be highly pathogenic: the enteric type and the feline infectious peritonitis virus (Pedersen, 2009). The feline infectious peritonitis is a fatal disease for cats and can evolve in two forms-wet or dry. The diagnosis is difficult to establish. The coronaviruses are also known to exceed the barrier of species suffering mutations and increasing their virulence (Vincent et al., 2009). The exact places in the genom where this mutations occur are being study because they are important for undrestanding the increase or the decrease of the pathogenicity. For cats there is no safe measure for preventing the coronaviral infection because no vaccin has proved so far its efficiencyThe coronavirus has a spherical to pleomorphic shape being an enveloped virus. On the surface of the small envelope are presented the spike proteins which has an important role in the attachement and entry to the host cells. The coronaviral genom which is a linear, single-stranded, non-segmented, positive sense molecule is content in a nucleocapsid protein which has a disputed role in the packaging of the RNA (LePoder, 2011). The most abundent protein is the matrix protein which is placed between the envelope and the nucleocapsid (Narayanan,2000).

The nucleocapsid protein has a 50kDa weight and is the only protein to be phosphorylated being the most abundend viral component present in two forms-a large one

and a small one in the infected cell (Cologna R., 2000). Only the large one is able to incorporate the RNA genom in the new virions. The exact role of the small species of these proteins is not known but it is believed to be formed by proteolysis of the large species (Huang Q.,2004).

Materials and methods

The samples were collected in the periode of march 2010-february 2013 from cats clinically healthy or from cats suspected of feline infectious peritonitis. For the study were collected 23 samples of feces, blood, abdominal and pleural fluid from 8 cats PIF suspected and 9 cats clinical healthy or with mild digestive disorders. From the 17 animals investigated 13 were males and 4 were females with ages between 4 months and 6 years. Feces were collected on rectal swabs and stored in Eppendorf tubes with PBS at -80˚C. The blood samples were collected on EDTA tubes and the abdominal and pleural fluids on simple sterile tubes.

The amplification by classic RT-PCR

The samples were analized using the rt-PCR tehnique. First the viral RNA was extracted from samples using the QIAamp Viral RNA Mini kit, from Qiagen according to the manufacturer's protocole. For extraction a minimum of 140µl from each sample was required. The RNA samples were stored at -80˚C. For detecting the presence of coronavirus in these samples the viral amplification was necessary. The rt-pcr was performed using the One-Step RT-PCR kit (Qiagen) and several pairs of primers (sens and anti-sens). The 205-211 pair (p205: GGCAACCCGATGTTTAAAACTGG, p211: CACTAGATCCAGACGTTAGCTC) amplified a well conserved region in the group alpha-coronavirus (Herrewegh si col, 1995). The expected amplicon had a 223 pb lengh.

For the detection of the nucleocapsid gene was used the Ncons primers sens and anti-sens. These pair amplifies a segment of 400 pb from the nucleocapsid gene of the coronaviruses (Herrewegh si col, 1995).

Table 1.

Primer	Nucleotide sequence (5'-3')	GC percentage	Direction	Number of nucleotides
205	GGCAACCCGATGTTTAAAACTGG	47,82%	sens	23
211	CACTAGATCCAGACGTTAGCTC	50%	antisens	22
Ncons5'	AACAAACACACCTGGAAGA	40%	sens	19
Ncons3'	GTGTCATCAAACACATCTGT	42,1%	antisens	20

The amplification protocole included the revers-transcription step for which a temperature of 50˚C was required, the Taq DNA polymerase activation which was made at 95˚C for 15 minutes, 46 cycles of amplification and a final extension at 72˚C. A cycle consisted in 3 steps: denaturation, annealing and extension. The annealing temperature was 52˚C for both pairs of primers. For each sample 24µl of mix and 4µl RNA was prepared. The negative control was represented by free water which was used instead of sample and for the positive control 2µl of porcine coronavirus- transmissible gastroenteritis virus RNA. The amplified samples were mixed with 3µl of a loading dye buffer containing bromphenol blue and then put in a 2% agarose gel. The electrophoresis was performed using a 85V electric

current for an hour. The gel containing the samples was observed using an UV light (BIORAD DOC) after 20 minutes of ethidium bromide immersion.

Obtaining the amplified nucleotide sequences

The positive samples were cut out from the gel and sent to UMR1161, ENVA where the amplicons were extracted using a Qiagen commercial kit MinElute Gel Extraction. The purified DNA was recovered in 20µl RNase- DNase free water. The DNA samples were sent for the sequencing at Eurofins Company, Germany and the results were available online.

The phylogenetic tree construction

The sequences obtained were compared with the ones stored in the GenBank database for detecting if the amplified products coresponded to the coronavirus family. Using an informatic program (transeq) and based on the nucleotidic sequences the coresponding proteins were virtually obtained. All proteins were aligned using the ClustaW program. Both Transeq and ClustalW were used by accesing the European Bioinformatic Institute site (www.ebi.ac.uk). The phylogenetic trees were obtained using the Mega5.1Beta4 program.

For building the phylogenetic tree making an alignment was necessary. For that were used the obtained proteins and other coronaviral proteins obtained from cats, dogs and cows and stored in GenBank. The WSU 79-1683 was obtained from a cat with a fatal enteritis, the FIPV 79-1146 was collected from a cat which suffered a neonatal death being highly pathogenic strains of the enteric and sistemic biotypes. The DQ848678, FJ943764 and FJ917622 were obtained from cats with feline infectious peritonitis and the FJ943761 was obtained from the feces of a healthy cat (the names used correspond to their accesion number in GenBank). The AY436636 was obtained from the liver of a giant panda and was stored as a canine coronavirus giant panda strain and we used a transmissible gastroenteritis virus also.

Results and discusions

From 10 samples tested with the 205/211 primer pair only 4 were positive (two feces samples and two ascite fluids). All 23 samples were tested for the presence of the nucleocapsid gene using the Ncons primer pair by performing 2 rt-PCRs (fig. nr.1). From these 10 samples were positive, from which four samples were of ascitic fluid, 3 pleural fluids and 3 fecal samples.These were collected from both healthy cats and feline infectious peritonitis suspected cats.

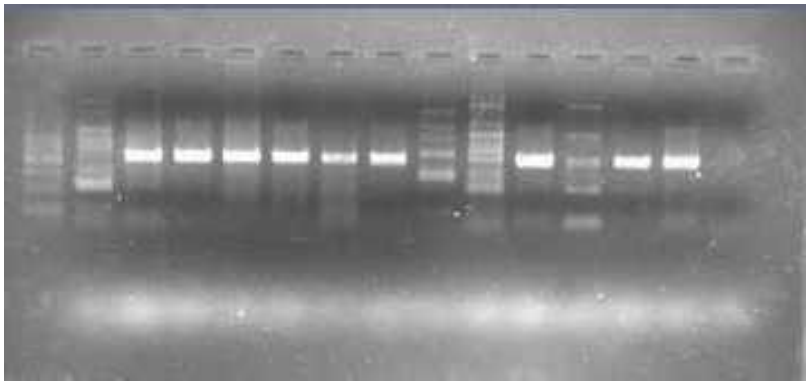

Fig. 1. RT-PCR NCONS

The corresponding sequences of the amplicons were analysed. All 10 amplicons obtained presented high similarities with the feline nucleocapsid genes stored in GenBank. The proteins for each samples were obtained using the Transeq program from European Bioinformatic Institute site and then aligned using the Mega5.1 program (fig. nr.2). The proteins obtained presented similarities when compared with the proteins of the other feline and non-feline coronaviruses stored in GenBank. All the proteins we obtained presented deletions and mutations which leaded to different aminoacides in the translation fase. These were highlighted in various colours by the program Align Mega5.1 we used for alignment. Samples 5RO, 6RO and 7RO also presented insertions in different positions translated in aminoacides not present for the other coronaviral proteins we used for building the tree (fig.nr.3). The strains collected from the healthy cats are coloured in yellow and the others in red. The samples 2RO, 3RO,4RO,10RO collected from sick cats and 16RO from a healthy cat showed similarities. The 13RO and 12RO presented similarities with the strains of both low and highly pathogenic feline coronaviruses. The strains that presented insertions obtained from cats with feline infectious peritonites formed separate branches.

Fig.2. The alignment of the proteins from our study and from GenBank

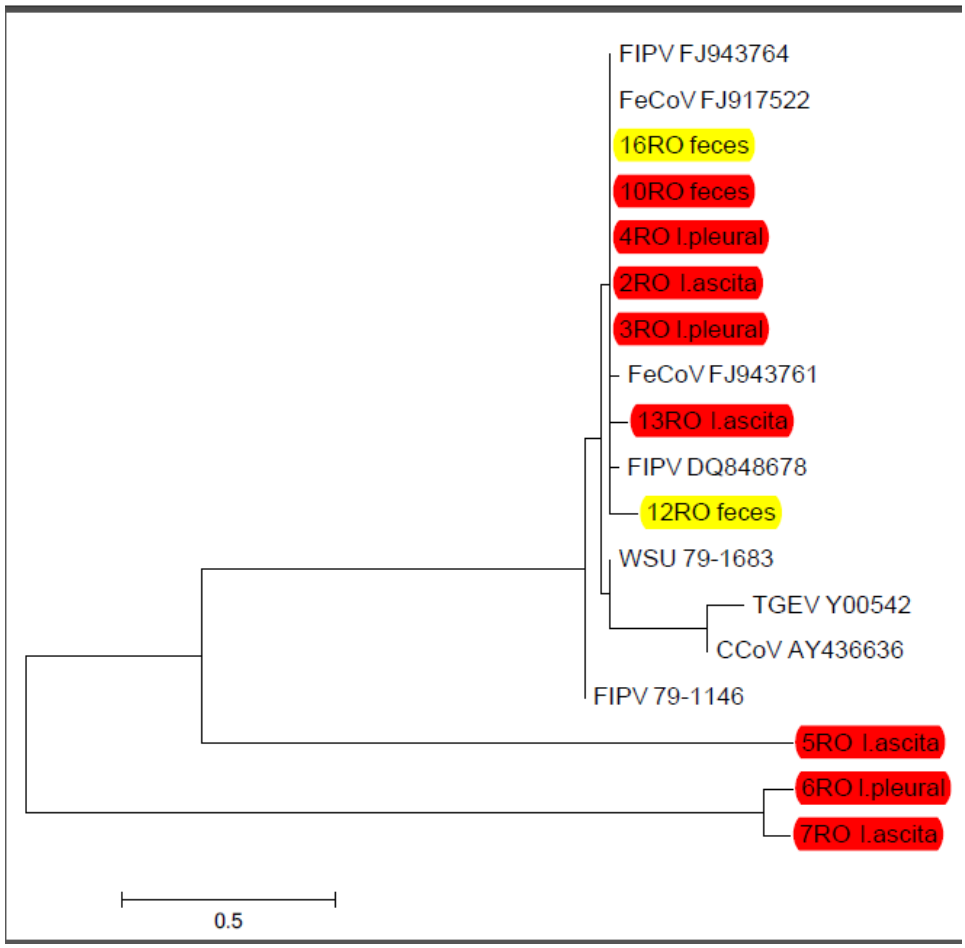

Fig. 3. The phylogenetic tree constructed on the maximum parsimony based on the N proteins

Conclusions

From 23 samples tested for the presence of the coronavirus using a pair of primers for N gene 10 were positive. From these 8 were collected from cats with feline infectious peritonitis and 2 from healthy eliminator cats.

The virtual proteins were obtained for the positive samples based on the nucleotide sequence. Using these proteins a phylogenetic tree was constructed based on maximum parsimony. This showed similarities between samples collected from both healthy and sick cats.

All nucleotide sequences presented deletions and mutations which leaded to the synthesis of different amino acids in the translated proteins which changed their structure.

Three samples also presented insertions and they formed separate branches in the phylogenetic tree.

Acknowledgments

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DETECTION OF THE FELINE CORONAVIRUS IN THE ORGANS OF CATS WITH FELINE INFECTIOUS PERITONITIS USING RT-PCR METHODS

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Abstract

There are two biotypes of feline infectious coronavirus. One is located in the intestinal tract and it is called the enteric type (FECoV) and the other one is a systemic type called feline infectious peritonitis virus (FIPV). The capacity of systemic dissemination is due to the ability to infect macrophages which is determined by mutations that occurs in the spike, ORF 3 and ORF 7 regions in the coronaviral genom. For determine the presence of the coronavirus in different organs of 4 cats we used the real-time pcr and for amplify the ORF 3c segment we used the rt-pcr method and several pairs of primers. The positive samples were extracted from the elecrophoresis gel and sent for sequencing to the Eurofins Company. Using the real-time pcr we find the presence of coronavirus in 16 from 33 samples. From 13 samples tested with several pairs of primers for ORF 3c, 11 were positive and we succed to obtain the nucleotide sequence for 3 intestine, 2 lymphnode, one lung and one pancreas samples using the sequencing method.

Keywords: *cat, coronavirus, ORF 3c, sequencing.*

Introduction

There are two biotypes of feline infectious coronavirus. One is located in the intestinal tract and it is called the enteric type and the other one is a systmic type called inefctious peritonitis virus. The two viruses have large similarities and can not be distinguished easily (Pedersen, 2009a). The most accepted hypotheses is that the sistemic type derived from the enteric type (Vennema, 1998). That is due to the mutations that can occur in the replication stage.

The feline coronaviruses have a large RNA genom and four types of proteins that structure the nucleocapsid, the envelope, the matrix, the spikes. The cats can be contaminated by the oronasal way, from where the viruses are spread in the intestinal tract. They have the ability to infect enterocytes where they multiply causing disturbances (Kipar, 2010). The infected cats can show signs of enteritis. In some cases these enteritis can be unnoticed, in others can be serious, sometimes deadly. The evolution of the infection varies depending on the virulence of the strain and the animal status. The enteric biotype always remains at this level of the enteric tract. In some cases mutations take place and the new formed viruses present the ability of infecting macrophages (Pedersen, 2009b). In these way the coronaviruses are disseminated in the entire organism determing the so called feline infectious peritonitis. Others speaks about the existence of several quasispecies of coronaviruses that circulate in the nature and which present different forms of virulence (Battilani M., 2003; Chang H-W, 2011).

The mutations, the deletions that occur influence the spike structure which is the viral component that has an important role in the attachemnt to cell. But the mutations that occurs here are not the only ones responsible of changing the tropism (Rottier, 2005). There are other parts of the coronaviral genom incriminated in the increasing the virulence like the

ORF 3C, ORF 7a and ORF 7b (Chang, 2010). These genes are responsible of encoding nonstructural proteins which are belived to be important in spreading the virus through the body (Balint A, 2012). FECoV replicates in the intestinal tract and needs an inatct ORF 3C gene. When mutations occur at this level the 3C protein function changes or even became unfunctional determing the ability of the coronaviruses to infect macrophages and in this manner to spread sistematically (Chang. 2010).

Materials and methods

We collected organs from four cats with clinical signs of feline infectious peritonitis and with positive results for the rt-pcr performed on the biological fluids such as acitic and pleural fluids. The serological tests showed high anticoronaviral-antibody titers also. The necropsy was performed and samples from intestine, heart, lung, liver, lymph nodes, kidney, pancreas and spleen were taken.

The Rneasy Mini kit from Qiagen and 30 mg from each organ were needed for the viral extraction. For each sample were added 8µl of β-mercaptoethanol (not contained in kit) and 800µl buffer RLT from the kit. For tissue disruption we used the FastPrep24 MPBIOMEDICALS machine and tubes with beads. After homogenization the samples were centrifugated and the supernatant was collected. The viral RNA was dissolved in 30µl of pure water and stored at -80˚C.

For testing the presence of the coronavirus in the samples the real-time rt-pcr was performed. For this were used an amplification kit- QuantiTect SYBR Green RT-PCR from Qiagen and a Lyght Cycler from Roche. The 205-211 pair (Herrewegh A, 1995) was used for amplification of a well conserved region in the coronavirus family. For each sample were used 17µl mix containing 1µl from each primer-sense and antisens and 3µl of viral RNA to be tested. The revers-transcription nedeed a 52˚C temperature for 40 minutes and 45 cycles of 40 seconds at 94˚C, 40 seconds at 50˚C and another 40 seconds at 72˚C. No final extension was required. The positive controll was RNA of a porcine coronavirus used pure and in two dilutions 1/10 and 1/100. As negative controll free water was used.

The amplification of the 3C gene was obtained using 3 pairs of sense and antisense primers. The pair 3CFW-3CRV amplified a fragment of 850pb. When a negative result was obtained another pair of primers 3CInt5'-3CRV or 3CFW-3CInt3' was used for the amplification of a shorter fragment of 520pb. The nucleotide structure of the primers showed a composition of 32-50% for the GC. The nucleotide sequences are shown in table no1 as described by Chang (Chang,2010).

Table 1. Primers composition

Primer	Nucleotide sequence	Sense
3CFW	CAAGTACTATAAA ACGTAGAAGMAG	Sense
3CRV	CAGGAGCCAGAAG AAGACACTAA	Antisense
3Cint5'	ATGGCATTGTGACA GCAACTG	Sense
3Cint3'	GAGCCGTGAGAAC TTCTCAT	Antisense

For the classical rt-pcr was used the amplification One-Step RT-PCR kit from Qiagen and different thermocyclers. The steps used for amplification were the ones described in the manufactacturers protocole. The first step consisted in the revers-transcription of the viral RNA in DNA strains at 50˚C for 30 minutes. The temperature used for the annealing step was 50˚C for all primers. A final extension of 10 minutes at 72˚C was required. For the positive control RNA from a feline coronavirus was used and free water for the negative control.

Electrophoresis was performed in a 2% agarose gel and TBE 1X buffer using a 90V, 200mA current for 50 minutes.

The results were observed using the DocGel instrument from BIORAD. The positive samples were cut off from the electrophoresis gel and the DNA was extracted using a comercial kit-MiniElute Gel Extraction from Qiagen. The obtained DNA was solubilized in 20 µl of free water. The samples were sent for sequencing to Eurofins Company (Germany). The results were available online.

Results and disscusions

Using the real-time rt-pcr method the coronavirus was detected in the intestine, lymphnode, lung, kidney and liver collected from cat no1. The spleen was negative for coronavirus in this case.The intestine and heart were positive for the cat no 2, but lung, liver and spleen samples were negative.We found coronavirus in cat no 3 small intestine, lung, spleen, large intestin, pancreas, lymphnode and liver. The kidney, lymphnode and heart samples were negative. For cat no 4 only the intestine and lymphnode samples were positive.

Amplifing the samples using the 3CFW-3CRV primers we obtained positive results for cat no3 lung, cat no 4 lung and intestine.

For the 3Cint5'-3CRV we obtained positive results for cat no1 lymphnode, cat no2 intestine, cat no3 pancreas, intestine, lung, lymphnode and cat no 4 intestine. Using the 3CFW-3Cint3' no positive results were obtained.

The sequencing for the cat no 3 lung, cat no 4 lung and intestine amplified with 3CFW-3CRV failed.

For the 7 samples amplified using the pair 3Cint5'-3CRV corresponding results were obtained for the cat no1 lymphnode, cat no2 intestine and cat no3 pancreas and lymphnode. The nucleotides sequences were obtained for the both sense and antisens strands. For cat no 3 lung and intestine and cat no 4 intestine samples the nucleotides sequences were too short.

Table 2. The pb lenght and the presence of mutations and deletions in the amplified samples

Nr.crt.	Sample	Lenght (pb)	Mutations	Deletions
1	Cat no1 lymphnode	494	Yes	43
2	Cat no2 intestine	186	Yes	36
3	Cat no3 lymphnode	442	Yes	43
4	Cat no3 pancreas	405	Yes	43

The nucleotide sequences lenght range between 186 and 494 pairs of bases. In all four cases mutations and deletions were present in these fragments of the feline ORF 3C. These deletions and mutations can determinate changes in the protein translation, function

and virulence. Chang conclude that an intact 3C protein is very important for the viral replication in the gut (Chang, 2010). In this case the intestine 3C gene fragment presented a smaller number of deletions compared with the other strains obtained from lymphnode and pancreas samples but the obtained nucleotide sequence was also shorter.

The number of mutations and deletions observed in the ORF 3C fragment sustain the distribution of the coronavirus in the entire organism. Although further investigations are nedeed to determine if these changes in the viral genom affects the aminoacid translation and more the protein function.

There is no usual method that can discriminate between the FECoV and the FIPV strains which shows high genetic similarities. The fact that FECoV replicates and stays in the intestinal tract and that the presence of the coronavirus was determined in several organs of cats with clinical signs of feline infectious peritonitis claim that the identified viruses are FIPV strains.

Conclussions

The feline coronavirus was found in small intestine, large intestine, lymphnode, lung, kidney, liver, heart, spleen, pancreas samples collected from four cats using the real-time rt-pcr method.

Three samples amplified using the 3CFW-3CRV were positive but the nucleotide sequence could not be obtained because the sequencing results failled.

Using rt-pcr and sequencing methods the presence of deletions and mutations was found in the fragments from the feline ORF 3C lymphnode, intestine and pancreas samples which could explain the systemic distribution of the virus.

The coronavirus detection in the organs of cats suggest the presence of the FIPV strains in this cases.

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METHODOLOGY OF THE ENVIRONMENTAL RISK ASSESSMENT RELATED WITH THE USE OF VETERINARY MEDICINAL PRODUCTS

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Abstract

An environmental risk assessment (ERA) aims the environmental protection and is mandatory for all new applications for a central or national marketing authorisation irrespective of the underlying legal basis. Risk assessment is a two phase evaluation of the possible fate, exposure and effects of the product. Phase I of ERA is based on a completion of a decision tree with 19 questions. If the answers to those question don't stop the assessment in this stage, then it advances to the phase II. It is used a two-tiered approach Tier A and Tier B. The first tier, Tier A, makes use of simpler, less expensive studies. If the ERA cannot be completed then it progresses to Tier B to refine the ERA. If there is still an indication of risk on completion of the Tier B assessment, then it is recommended the discussion of the dossier and proposals for further data or risk mitigation.

Key words: *Environmenal Risk Assessment, Veterinary Medicinal Product*

1. Structure of ERA for Veterinary Medicinal Products

Risk assessment is an evaluation of the possible fate, exposure and effects of the product. As a whole, the risk assessment is structured around the risk quotient approach as described in VICH guidelines GL6 (Phase I) and GL38 (Phase II). The risk quotient (RQ) is defined as the ratio between the predicted environmental concentration (PEC) and the predicted no-effect concentration (PNEC). If reliable monitoring data are available, these may replace the predicted values. The risk quotients indicate the likelihood of adverse effects occurring. In Phase I, the investigator shall assess the potential extent of exposure of the environment to the product, its active substances and other ingredients, taking into account:

- The target species, and the proposed pattern of use
- Characteristics of the constituents of the VMP
- The method of administration

In Phase I several exemptions from further testing are incorporated. When these exemptions do not apply, and trigger values are exceeded, one enters Phase II.

As appropriate, further investigation may be required of:

- Fate and behaviour in soil, water and dung
- Effects on aquatic organisms
- Effects on other non-target organisms

The Phase II assessment starts at Tier A with a base data set on fate and effects that allows for risk characterisation. If a risk cannot be excluded the assessment proceeds to Tier B.

2. Exposure of VMPs to the environment

The route and quantity of a VMP entering the environment determines the risk assessment scenarios that are applicable and the extent of the risk assessment. Emission can occur at various stages in the life cycle of the product. However, with the exception of certain topicals or those added directly to water, most VMPs first pass through the animal to which it

is administered. Generally the most significant environmental exposure results from excretion of the active substance being the parent and/or its metabolites. Following excretion, residues are generally assumed to be uniformly distributed in the environment.

The route and quantity by which a VMP enters the environment determines the type of assessment (Phase I or Phase II) and the scenarios to be used. Dosage, route of application, type of target animals, excretion, route of entry into the environment and agricultural practice all influence the point at which environmental exposure occurs. The main scenarios are:

- Removal of material containing the product (manure, dirty water, fish farm effluent)
- Excretion via faeces and urine (grazing animals)
- Spillage at external application and/or direct exposure outdoors

3. Environmental distribution

The route of distribution and the fate in the environment are important for the final exposure concentration. For veterinary medicinal products, the predominant routes of exposure for the terrestrial and aquatic environment are through the application of manure, dung and urine. Distribution of the product occurs within the directly exposed compartment(s) and between different compartments. The terrestrial environment is exposed via:

- Direct excretion of dung and urine;
- Loss from animals treated topically;
- Spreading of contaminated slurry and/or sludge.

The aquatic environment is exposed via:

- Leaching, run-off and drainage from manured land;
- Direct spillage and/or feed spillage;
- Direct excretion into water (pasture animals);
- Direct application in water (aquaculture);
- Direct discharge of waste water into surface water (indoor aquaculture);
- Release from Sewage Treatment Plants (indoor aquaculture).

In Phase I, the potential for environmental exposure is assessed based on the intended use of the VMP. It is assumed that VMPs with limited use and limited environmental exposure will have limited environmental effects and thus stop in Phase I. Phase I also identifies VMPs that require a more extensive ERA under Phase II.

The Phase I ERA for a VMP makes use of the decision tree. To use the Phase I decision tree, the applicant works through the questions until they arrive at a question which allows them to conclude that their product qualifies for a Phase I report. If there is no information on a particular question, the question is ignored and the applicant continues to the next question.

4. Predicted environmental concentration - Question 17 from the decision tree - „Is the predicted environmental concentration of the VMP in soil (PECsoil) less than 100 µg/kg?"

In Phase I the total residue approach is applied. This means that the total amount of the dose applied is excreted from the animal and data on metabolism/excretion should not be taken into account. Food producing species can be raised indoors for all or a major part of

their lives or they can be kept outdoors for all or a major part of their lives. The calculation of the initial PEC in soil is performed when more than a "small number of animals" are treated.

Table 2. Percentage herd treatment for various groups of VMPs

Product group	% herd treatment
Anthelmintics	100
Products for treatment of diarrhoea in calves, lambs and pigs (excluding products administered in feed and water)	30
Coccidiostatics	100
Ectoparasiticides	100
Intramammary preparations:	
for drying off	100
in lactating animals	25
Antibiotics (feed and water medication)	100
Antibiotics (injectable)	
all pig treatments	50
respiratory infections in cattle	50
foot rot in sheep	100
Teat dip and sprays	100
All products for poultry	100
All products for fish	100

a) PECsoil initial for intensively reared animals

Intensively reared animals are those which are housed indoors throughout the production cycle so treatment with the VMP is carried out in housing and the active residue is excreted in the stable and is incorporated in the manure. This active residue reaches the environment when the manure from the stable is spread onto land.

Calculation of the PECsoil initial for intensively reared animals is dependent on the quantity of manure containing active residue, which can be spread onto land. Based on the EUROSTAT database a nitrogen load of 170 kg N /ha is on average the maximum load in most EU countries.

The PECsoil initial should be calculated using the following equation:

$$PEC_{sol\ initial} = \frac{D \times Ad \times BW \times P \times 170 \times Fh}{1500 \times 10000 \times 0.05 \times Ny \times H} \times 1000 \qquad \text{Eq 1}$$

Where:

PECsoil initial = Predicted Environmental Concentration in soil [µg.kg^{-1}]

D = Daily dose of the active ingredient [mg.kgbw^{-1}.d^{-1}]

Ad = Number of days of treatment [d]

BW = Animal body weight [kgbw] (see Table 3.)

P = Animal turnover rate per place per year [place^{-1}.y^{-1}] (Table 3.)

170 = EU nitrogen spreading limit [kg N.ha^{-1}]

Fh = Fraction of herd treated [value between 0 and 1] (see Table 2.)
Ny = Nitrogen produced in one year per place [kg.N. place^{-1}.y^{-1}] (see Table 3.)
H = Housing factor either 1 for animals housed throughout the year or 0.5 for animals housed for only 6 months (see Table 3.)

In this equation the only inputs required from the user are the dose rate and the number of administrations of the veterinary medicine in a course of treatment. These parameters will be available from the product's SPC.

Table 3. Default values for use in calculating the PECsoil for intensively reared animals

Animal type	Number of animals raised per place per year	Bodyweight (kg)	Nitrogen produced in 1 year per place (kg.N.y^{-1})	Housing factor[1]
Calf	1.8	140	10	1
Dairy cow	1	425	60	0.5
Cattle (0-1 year)	1	200	18	0.5
Cattle (>2 years)	1	450	35	0.5
Weaner pig (to 25 kg)	6.9	12.5	2.25	1
Fattening pig (25-125 kg)	3	65	7.5	1
Sow (with litter)	1	240	262	1
Broiler	9	1	0.23	1
Laying hen	1	1.6	0.35	1
Replacement layer	2.6	0.8	0.24	1
Broiler breeder	1	1.7	0.69	1
Turkey	2.7	6.5	0.9	1
Duck	7	1.6	0.41	1
Horse	1	400	35	0.5
Rabbit	8	1.4	0.352	1

b) PECsoil initial for pasture animals

Pasture animals are those, which are on pasture throughout the production cycle so treatment with the veterinary medicine is carried out in the field and the residue of the veterinary medicine, is excreted directly onto the soil.

Calculation of the PECsoil initial for pasture animals is dependent on the number of animals kept on any area of land. This parameter is known as the stocking density and is expressed in animals per hectare.

$PEC_{sol\ initial} = \frac{D \times Ad \times BW \times SD \times Fh}{} \times 1000$ Eq 2 1500 x 10000 x 0.05

where:
SD = Stocking density [animal.ha^{-1}] (see Table 4.)

Table 4. Default values for use in calculating the PECsoil for pasture animals

Animal type	**Stocking density (animals.ha^{-1})[1]**	**Bodyweight (kgbw)[2,3]**
Dairy cow	3.5	600
Beef cattle	9.5	330
Sheep (adult ewe)	15	80
Lambs	25	36
Horse	3	600
Pony	5	250
Goat	15	60

5. Phase II

The aim of the Phase II (and in Phase I) is to assess the potential for VMPs to affect non-target species in the environment. It is not possible to evaluate the effects of VMPs on every species in the environment that may be exposed to the VMP following its administration to the target species. The taxonomic levels tested are intended to serve as surrogates or indicators for the range of species present in the environment.

It is used a two-tiered approach to the environmental risk assessment. The first tier, Tier A, makes use of simpler, less expensive studies to produce a conservative assessment of risk based on exposure and effects in the environmental compartment of concern. If the ERA cannot be completed with such data, due to a prediction of unacceptable risk, then the applicant progresses to Tier B to refine the ERA. In some cases, it may be possible to implement a risk management option instead of moving to Tier B.

At the beginning of Phase II a Tier A base data set on the fate and effects of the VMP is produced by the applicant. This data set is a key element of the assessment procedure allowing for the rapid identification of hazards and/or risks associated with the use of the product.

At this point, it is important to make use of all available documentation relevant to the environmental risk assessment of the product. This includes physico-chemical data, relevant pharmacological toxicological and toxicokinetic studies and information on degradability or persistence of the active ingredient under relevant conditions.

These properties will vary between the parent compound and the individual excreted metabolites, for example, the latter may be more water-soluble than the parent compound and may be more mobile and/or more persistent in the environment.

Consideration of the excretion data is not initially recommended at Tier A, where a total residue approach should be taken and a PECinitial should be estimated. It should be assumed that the VMP is excreted 100% as parent.

The specific test guidelines/protocols recommended in Phase II are those finalized by OECD/ISO. This has the advantage of ensuring that environmental studies are current and broadly acceptable to regulatory authorities on a worldwide basis.

a) Tier A - PEC refinement

In Phase II Tier A the PECs are initially calculated based on the total residue approach and compared with the PNEC derived from the base set of toxicity tests. If the RQ is above one, the adjustments presented below can be used to refine the PECs.

Depending upon the scenario and the characteristics of the active ingredient being studied, a number of options may be available to refine the exposure assessment. Broadly speaking, these refinements fall into one or more of the following categories:

- Refinement based on metabolism
- Refinement based on the excretion pattern
- Refinement based on degradation in manure/slurry
- Refinement based on degradation in soil

b) Tier B Testing - Environmental Fate Studies

If the log Kow is ≥4, evidence from absorption, distribution, metabolism and excretion (ADME) and biodegradation studies and molecular mass should be considered to see whether there is the potential for bioaccumulation to occur. If so, then a bioconcentration factor (BCF) study is recommended to be carried out at Tier B. Evidence of bioaccumulation from ADME studies would be the presence of high concentrations of the active in fat compared to other tissues and/or the slow depletion of the residue from fat tissue. In view of the fact that in general the activity of enzymes involved in the transformation of xenobiotics decrease at lower trophic levels, the lack of accumulation in mammals does not automatically exclude the potential for accumulation in fish.

If there is still an indication of risk on completion of the Tier B assessment, e.g. for VMPs which
still have an RQ >1 or the BCF ≥ 1000, then the applicant is recommended to discuss their dossier
and proposals for further data or risk mitigation with the regulatory authority.

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APPLICATION OF A REAL-TIME PCR FOR QUANTITATIVE DETECTION OF *CAMPYLOBACTER JEJUNI* IN FRESH MEAT

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Abstract

The aim of this study was to detect and quantify Campylobacter jejuni in fresh meat samples by a TaqMan PCR assay and to establish the specificity and detection limit of this assay in naturally and artificially contaminated meat samples. The detection of Campylobacter jejuni in meat has performed by ISO 10272/2007standard method and confirmed by real-time PCR assay (Applied Biosystems), targeting the VS1gene. The assay use specific primers and a probe for and the detection performed by fluorescence curves analysis. From 52 sample of poultry meat, 9 (17,3%) were positive for Campylobacter jejuni, but no pork meat sample was positive with this pathogen. We also confirmed 10 strains of C. jejuni, isolated from children diarrheic feces. The specificity of the TaqMan PCR assay was 100%, and detection limit was 10 ufc/ml in naturally enriched samples and 100 ufc/ml in artificially contaminated samples. These results shows that Campylobacter jejuni could serve as a potential risk for consumers if proper hygienic and cooking conditions are not maintained, also, rapid and efficient methods, such as real-time PCR are required for food pathogens detection.

Kay words: *detection, Campylobacter jejuni, real-time PCR, meat*

Introduction

Campylobacter jejuni is recognized worldwide as a leading cause of diarrhea and food-borne gastroenteritis. This organism is carried in the intestinal tract of a wide variety of wild and domestic animals. In most cases, the host is a carrier that does not exhibit symptoms, but it may have acquired immunity through an earlier *C. jejuni* infection (Yang et al., 2003). The microorganism has been isolated from animals and birds, milk, beef, pork and poultry meat. Food of animal origin contaminated with *C. jejuni* is a primary source of *Campylobacter* infection in man (Adak et al., 2005; Sulonen et al., 2007)

As food safety has become an increasing concern for consumers, there is a growing need for fast and sensitive methods for specific detection and identification of zoonotic microorganisms. The PCR technique has several advantages over classical bacteriology with respect to detection limit, speed, and the potential for automation and has succesfully been applied to the detection of *Campylobacter spp.* (Lund et al., 2004) .

Materials and methods

Classical microbiological method

The 69 (52 poultry and 17 pork meat samples) were first processed by classical microbiological methods to obtain the presumptive strains of *C. jejuni* and quantified by TaqMan real-time PCR.

Food samples were performed in Food Microbiology Laboratory (Iasi Veterinary Medicine Faculty), according to the ISO. 10272/2007. The samples were first serially diluted and 1 ml was added into 9 ml of Preston broth and incubated at 42ºC, 18 hours. An aliquot of 0,1 ml was used for selective isolation on Karmali and mCCD agar and incubated at 42°C, 48 hours to observe the typical colonies of *Campylobacter jejuni*.

Detection limit of the assays

The detection limit was tested with five serial dilutions of DNA (10-10^5 CFU/ml) of *Campylobacter jejuni* from naturally (enriched) and artificially contaminated samples. A standard curve was obtained plotting the log of the number of CFU/ml against the Ct value. The DNA samples were analized in triplicate. For artificially contamination, 1 g of minced meat was inoculated with 9 ml of each dilution of a reference strain of *C. jejuni*.

DNA extraction

The extraction of bacterial DNA was performed with PrepMan Ultra (Applied Biosystems) from enrichment media. One ml was transferred in the 2-ml microcentrifuge tubes and centrifuged for 3 minutes at 14000 rpm, to pellet bacteria and residual food or other debris, and then the supernatant was removed. The cell pellets were resuspended in 100 µl reagent and placed in a 100ºC heating block for 10 minutes, and cooled at room temperature for 2 minutes. 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

Oligonucleotide primers, TaqMan probe (table 1) and real-time PCR conditions were adopted from a previous study (Yang et al., 2004), targeting a 358-bp amplicon from the VS1 gene of *C. jejuni*.

Reactions and data analysis were performed in the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems). 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 45 cycles: 95ºC 1 s., 56°C 10 s and 72ºC 20 s.

Table 1. Primers and TaqMan probe for real-time PCR
(Yang et al., 2004)

Microorganism	Target gene	Primers and TaqMan probe structure
Campylobacter jejuni	*VS1gene*	5'-GA AT GA AA TT TT AG AA TG GG G-3' 5'-GA TA TG TA TG AT TT TA TC CT GC-3'
	TaqMan probe	FAM-TT TA AC TT GG CT AA AG GC TA AG GC T-TAMRA

Results and discussions

From 52 samples of poultry meat, 9 (17,3%) were positive for *Campylobacter jejuni* (table 2). None of the pork meat sample was contaminated with this pathogen. The prevalence of *C. jejuni* in poultry meat is low related with other study: Stoyanchev et al., (2007) found a prevalence of 35,2% in froozen carcasses and 74,8 % in chilled carcasses in Bulgary; Han et al., (2007) in Korea found a prevalence of 68,3% *Campylobacter spp*. in poultry meat, and 37,7% of *C. jejuni*; Lake and al., (2007) in New Zeeland found a prevalence of 89% *C. jejuni* and *C. coli* in poultry meat. In a study made by Vishin et al., (2011) on pork meat, the results indicated that after low temperatures exposure, the major part of *C. jejuni* cells died or stayed alive but with damaged cell functionality. It was established that the microorganisms did not grow in chilled or frozen meat, but were able to survive during the storage period.

Table 2. The prevalence of *C. jejuni* in poultry and swine meat

Samples types	Samples no.	Positive samples		Negative samples	
		No.	%	No.	%
Poultry meat	52	9	17,3%	43	82,6%
Swine meat	17	0	0%	17	100%

To confirm the 9 presumptive *C. jejuni* strains, we used TaqMan real-time PCR, targeting the VS1 gene. The specificity of the primers and TaqMan probe was 100%. The inclusivity and exclusivity were 100%. No false negatives, false positives or cross-amplification were observed during specificity testing. All the 9 presumptive strains of *C. jejuni* were confirmed. We also tested one reference strain and 10 strains isolated hospital from children with diarrhea. For the exclusivity test we used 4 strains of non-*C. jejuni*. The results are presented in table 3.

Table 3. Specificity of TaqMan real-time PCR for VS1 gene (C. jejuni)

C. jejuni strains	**Tested No.**	**CT Mean**	***non – C. jejuni* strains**	**Tested No.**	**Results**
***C. jejuni* poultry meat**	9	**+ 20,9- 34,9**	***C. coli***	1	**-**
***C. jejuni* 200**	1	**+ 26,8**	***C. lari***	1	**-**
***C. jejuni* 243**	1	**+ 20,8**	***E. coli*** calves feces	2	**-**
***C. jejuni* 246**	1	**+ 22,4**			
***C. jejuni* 157**	1	**+ 27,6**			
***C. jejuni* 159**	1	**+ 25,1**			
***C. jejuni* 136**	1	**+ 23,9**			
***C. jejuni* 189**	1	**+ 21,8**			
***C. jejuni* 86**	1	**+ 35,5**			
***C. jejuni* 132**	1	**+ 28,5**			
***C. jejuni* 134**	1	**+ 25,1**			
C. jejuni ATCC 33560	1	**+ 29,1**			

To establish the PCR detection limits, triplicate reaction for each serial dilution were prepared. The standard curve of *Campylobacter jejuni* ATCC 33560 showed a linear correlation between the values of C_t (threshold cycle) and cell numbers (cfu/ml). The Mean C_t values ranged between 18,2 to 37,7 in naturally contaminated sample and 22,05 şi 35 in artificially contaminated sample. The detection limit of the real-time PCR assay was 10 ufc/ml in naturally contaminated meat and 100 ufc/ml in artificially contaminated meat. The results are showed in table 4. Yang et al., (2004) quantified *C. jejuni* in carcasses, with a detection limit of 1CFU/ml.

The use of sensitive, quantitative methods for the detection of *C. jejuni* during food processing could be used to determine points in the food production process where contamination occurs and where controls could be introduced to reduce or eliminate *C. jejuni* from retail food products, thereby reducing the risk to the consumer (Sails et.al., 2003).

Table 4. Detection limit of *C. jejuni* in naturally and artificially contaminated samples

C. jejuni UFC/ml	DNA from enriched naturally contaminated samples			DNA from artifcially inoculated samples		
	CT Mean	SD	Q ng/µl	CT Mean	SD	Q ng/µl
10^5	18,2± 0,2		1,1	22,05±0,1		1,3
10^4	22,8±0,5		0,11	26,2 ± 0,2		0,13
10^3	25,1 ±0,3		0,01	29,7 ±0,2		0,01
10^2	39,3 ± 0,3		0,001	32,5 ±0,3		0,001
10^1	37,7 ±0,2		0, 0001	35±1,5		0

Conclusions

1. The prevalence of *C. jejuni* in poultry meat was 17,3%. None positive sample was find in pork meat. Foods of animal origin, in particular poultry, have been identified as a risk factor for human infection.
2. Real- time PCR is an alternative method that can verify the presence or absence of *C. jejuni* in enriched samples. It is a fast, specific and sensitive method which can be performed in 24 h.

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EPIDEMIOLOGICAL INVESTIGATIONS ON CLASSICAL SWINE FEVER EVOLUTION IN PIGS AND WILD BOARS IN IAŞI COUNTY

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Abstract
Data compilation on the possible presence of classical swine fever in pigs and wild boar is an important prerequisite for epidemiological surveillance of this emerging disease.Epidemiological investigations were carried out between 2007 and 2010. Serological methods and direct immunofluorescence on bone marrow smear were used as laboratory tests. All samples tested were collected from swine farms and back-yard pigs as well as from wild boars in Iași County.The results of classical swine fever surveillance and diagnosis using serological and virological methods on swine samples collected in Iasi County during 2007-2010, highlights the absence of positives cases of classical swine fever.

Key words: *classical swine fever, ELISA, direct immunofluorescence*

Introduction

Classical swine fever (CSF) is caused by an enveloped RNA virus belonging to the genus *Pestivirus* of the family *Flaviviridae*. This virus is related to other two ruminant *Pestiviruses* bovine viral diarrhea virus (BVDV) and border disease virus (BDV). This relationship has serious implications for diagnosis due to cross-reactions that can lead to false positive results. Outbreaks can cause heavy losses in pig production and severely hamper international trade with animals and animal products. The socio-economic consequences of CSF outbreaks are severe and therefore CSF is a notifiable disease to the Office International des Epizooties (*) and to the European Union (EU) (**). Due to its economic impact, classical swine fever (CSF) ranks among the most important diseases of domestic pigs.

This disease is characterized by a per-acute, acute, sub-acute, chronic, atypical or unapparent course (Robertson, 1994) depending on the virulence and dose of the virus and the age and breed of the pigs, besides other host and environmental factors (Moennig et al., 2003)

CSF is conventionally diagnosed on the basis of clinical signs and necropsy lesions. However, the high degree of variability in the clinical picture and the resemblance of the clinical syndrome to many other diseases precludes reliable clinical diagnosis. Confirmation of the presumptive diagnosis needs to be done by standard laboratory tests (Pearson, 1992).

Serology is routinely used for diagnosis and surveillance and whenever it is suspected that CSFV may be present in a pig population. Antibodies are first detectable 2–3 weeks after infection and persist in surviving animals lifelong. Antibodies are a good indicator that a CSFV infection may have been present in the pig herd. The most commonly used tests for antibody detection are virus neutralization tests (VNT) and enzyme-linked immunosorbent assays.

ELISAs for the detection of antibodies against the viral E2 glycoprotein are either designed as blocking (Wensvoort G., 1988) or as indirect ELISAs (Moser C., 1996). They are widely used to screen for antibodies during and after outbreaks, for monitoring of CSFV

infections in wild boar (Vengust G., 2006) and to test coverage of immunization of wild boars after vaccination (Kaden V., 2005). Envelope glycoprotein E2 is the main immunogen, essential for replication) van Gennip HG, 2002). Moreover, it was shown that it plays a role in viral adsorption to host cells together with other surface proteins, namely E RNS and E1 (Wang Z, 2004).

The fluorescent antibody test (FAT) is used to detect CSFV antigen in cryostat sections of tonsils, spleen, kidney, lymph nodes or distal portions of the ileum. Tissues should be collected from animals and transported without preservatives under cool conditions, but not frozen. Cryostat sections are stained directly with anti-CSF immunoglobulin conjugated to fluorescein isothiocyanate (FITC) or indirectly using a secondary FITC conjugate and examined by fluorescence microscopy.

Materials and Methods

Epidemiological investigations were made during 2007-2010. Serological tests (ELISA) and fluorescent antibody test (FAT) were used as laboratory methods. The samples were collected from domestic swine farms and back-yard pigs as well as from wild boars in Iaş i County. As serologic test were used: CHEK - CSF-MARKER is an immunoassay kit (EIA) rapid, simple, specific and sensitive for detection of antibodies to glycoprotein E^{rns} of *Pestiviruses* (classical swine fever virus, bovine diarrhea virus and border disease virus) and CEDITEST CSFV E2 ELISA.

Results and discussions

Our serological and virological investigation on classical swine fever in 2007 revealed the presence of pigs vaccinated against CSFV infection. From Iaş i County were collected 3654 serum samples, of which 2814 were found positives for CSFV antibodies. From pig farms were tested 301 serums, of which 10 (3,32%) were found positives for antibodies anti-CSFV. Positive samples were from sows vaccinated against classical swine fever. Using FAT were tested 384 bone marrow samples collected from swine and wild board. All samples tested were found negative for CSFV.

Table 1. Classical swine fever surveillance by ELISA and FAT in Iasi County in 2007

TEST	Sample type	Farms	Back-yard pigs	Wild boars		
				<1	1-2	>2
FAT	Samples tested	38	158	54	78	56
	Positive	-	-	-	-	-
	Negative	38	158	54	78	56
ELISA E2	Samples tested	-	3353	8	14	14
	Positive	-	2804	-	-	-
	Negative	-	549	8	14	14
ELISA E^{rns}	Samples tested	301	-	-	-	-
	Positive	10	-	-	-	-
	Negative	291	-	-	-	-

The data presented in Table. 2 reveal the results of classical swine fever surveillance in 2008 in Iasi County. Using immunoassay methods were tested 2389 samples collected

from back-yard pigs, 91,50% (2186 samples) of them being identified positives for CSFV antibodies. This seropositivity is due to successive vaccination campaigns against classical swine fever. 503 serum samples were collected from farm pigs in Iasi County. The serum samples were tested using two ELISA kits for detection of CSF-antibodies. None of the tested samples were found positives for antibodies against CSFV. Using FAT were analyzed 3010 tissue samples collected from pigs (85 samples) and wild boars (225 samples). All samples tested were identified as negatives for the presence of CSFV.

Table 2. Classical swine fever surveillance by ELISA and FAT in Iasi County in 2008

TEST	Sample type	Farms	Back-yard pigs	Wild boars		
				<1	1-2	>2
FAT	Samples tested	9	76	41	64	120
	Positive	-	-	-	-	-
	Negative	9	76	41	64	120
ELISA E2	Samples tested	47	2389	38	53	97
	Positive	-	2186	-	-	-
	Negative	47	198	38	53	97
ELISA E^{rns}	Samples tested	456	-	-	-	-
	Positive	-	-	-	-	-
	Negative	456	-	-	-	-

The results of classical swine fever surveillance in Iasi County during year 2009 are presented in table no. 3. Serological investigation in farm pigs consisted in testing 982 serum samples. Using CEDI CSFV E2 ELISA were identified 1,27% positive samples (12 out of 940) and with CHEKIT – CSF- MARKER were identified 30,95% (13 out of 42) positive samples. All positive samples were collected from young pigs originating from sows vaccinated with inactivated and marker vaccine. Serological investigation revealed the presence of vaccinated back-yard pigs in Iasi County. Using CEDI CSFV E2 ELISA were found positives for CSFV antibodies 95,49% of the serum tested (7158 out of 7496). Serological investigation on wild boars revealed 9,52% seropositive serums (16 out of 168), representing vaccinated animals.

Using FAT reaction were tested 56 samples from pigs and 251 wild boars. All samples tested were identified negative for the presence of classical swine fever virus.

In 2010 were performed virological and serological exams for classical swine fever surveillance in Iasi County. Detection of CSFV antibodies was made using CEDI CSFV E2 ELISA. The results of serologic surveillance revealed the absence of positive farm swine. Using the same method were tested 20 serums from back-yard pigs, all being identified as positive for antibodies against gE2 of CSFV. Moreover were tested 39 serum samples collected from wild boars, 5,12% (2 out of 39) were found positive for CSFV antibodies. This seropositivity is due to successive vaccination campaigns against classical swine fever. Using FAT method were tested 1064 samples collected from farm pigs, 21 samples from back-yard pigs and 129 from wild boars in Iasi County. All samples tested were identified negative for the presence of classical swine fever virus.

Table 3. Classical swine fever surveillance by ELISA and FAT in Iasi County in 2009

TEST	Sample type	Farms	Back-yard pigs	Wild boars		
				<1	1-2	>2
FAT	Samples tested	52	4	53	92	106
	Positive	-	-	-	-	-
	Negative	52	4	53	92	106
ELISA E2	Samples tested	940	7496	49	52	67
	Positive	12	7158	6	-	10
	Negative	928	338	43	52	57
ELISA E^{rns}	Samples tested	42	-	-	-	-
	Positive	13	-	-	-	-
	Negative	29	-	-	-	-

Table 4. Classical swine fever surveillance by ELISA and FAT in Iasi County in 2010

TEST	Sample type	Farms	Back-yard pigs	Wild boars		
				<1	1-2	>2
FAT	Samples tested	1064	21	28	30	71
	Positive	-	-	-	-	-
	Negative	1064	21	28	30	71
ELISA E2	Samples tested	440	20	9	21	9
	Positive	-	20	-	2	-
	Negative	440	-	9	19	9

Romania's integration in the political and administrative structures of the European Union consists primarily in legislation implementation, legislative transposition and, secondly, the establishment of new institutional framework or in adapting and improving the implementation of existing EU legislation requirements at nationally. Since 2001, when he resumed formal international notification of classical swine fever to date have been addressed and implemented various strategies that focused on reducing the number of outbreaks of classical swine fever and even eradicate it.

Conclusions

The results of the surveillance and diagnosis of classical swine fever using serological and fluorescent antibody test (FAT) in domestic pigs and wild boars in Iasi during 2007-2010 highlights the absence of cases of classical swine fever.

Recognition of the free status of Romanian territory against some animal diseases that can directly or indirectly influence trade in live animals and products is the primary purpose of surveillance activities, prevention and control of animal diseases.

A compilation of data on the possible presence of classical swine fever in domestic pigs and wild boar is a prerequisite epidemiological essential. It is necessary to implement work protocols and readily available diagnostic methods to facilitate a low cost, using secure technology and laboratory equipment available.

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CLASSICAL SWINE FEVER VIROLOGICAL SURVEILLANCE ON WILD BOARS IN SUCEAVA COUNTY

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Abstract
Since 2007, Romania has developed monitoring, control and eradication of classical swine fever approved annually by European Commission, with the following main objectives: changing epidemiological status of Romania, in order to include it in Part I of Decision 2008/855/EC. The trade with pigs, fresh meat and pork products on EU markets it is possible only by acquiring free status of classical swine fever.The study was conducted between 2007and 2012. Virological tests were performed on samples collected from wild boars in Suceava County. As laboratory methods were used: direct immunofluorescence (IF) on bone marrow smears and RT-PCR for the detection of viral RNA performed on set of organs. All wild boars samples tested by RT-PCR and immunofluorescence during the six years surveillance were found negatives for classical swine fever virus.

Key words: *classical swine fever virus, RT-PCR, immunofluorescence*

Introduction

Classical swine fever (CSF) is a highly contagious and often fatal disease of swine, affecting domestic and wild pig populations. Classical swine fever virus (CSFV), the causative agent of CSF, is a member of the genus *Pestivirus*, which belongs to the *Flaviviridae* family (Wengler, 1995). CSFV is an enveloped virus with a 12.5-kb single-stranded RNA genome of positive polarity (Horzinek, 1991).

The disease occurs in many regions of Asia, Central and South America and parts of Europe and Africa. Some countries have eradicated the disease (Australia, USA, Canada, within the EU), yet it keeps recurring sporadically (South Africa, Germany, Netherlands, England) (Moennig, 2003).

Due to its economic impact, classical swine fever (CSF) ranks among the most important diseases of domestic pigs. Clinical and pathological signs are highly variable, and diagnosis must be confirmed by laboratory tests (Floegel-Niesmann, 2003). Tonsils or blood are generally taken as samples and targeted for diagnosis and surveillance programs (Uttenthal, 2003). Routine testing of meat has also been suggested due to the long-lasting virus infectivity in this material under cold storage conditions (Edwards, 2000). Since the early 1990s the development of new molecular tests has led to an improved sensitivity in CSFV detection (Handel, 2004). Reverse-transcription PCR (RT-PCR) and, more recently, real-time RT-PCR (rRT-PCR) assays have permitted the detection of infection regardless of the clinical stage, starting from early infection, during the incubation phase and all along the clinical phase (Ophuis, 2006).

Since 2007, Romania has developed monitoring, control and eradication of CSF approved annually by the European Commission. The main objectives are changing epidemiological status of Romania, in order to include them in Part I of Decision 2008/855/EC, access to the European Community and partner countries markets for trade with live pigs, fresh meat and pork products, acquiring free status of classical swine fever.

Materials and Methods

The study was made between 2007 and 2012. For surveillance of wild boar for classical swine fever were collected: samples of sternum and organs. Samples were collected from: all hunted wild boars and from wild boar found dead.

Samples were examined in the laboratory of veterinary and food safety in Iaş i County as follows: RT-PCR assay for the detection of specific viral genome; fluorescent antibody test (FAT); virus isolation test cell cultures in all positive cases.

Virological tests were performed on samples taken from wild boars found dead and hunted in Suceava County. The tests used were according to the Methodology methods in the diagnosis of classical swine fever.

Diagnostic test kits used to identify viral antigen by fluorescent antibody test (FAT) on sternal marrow smears were: *Gamarom* or *Ceditest ®CSFV kits*.

The reagents of the detection kit are: antiserum conjugated swine fever with a fluorochrome (most used FITC); smears containing infected cells (positive control) and smears prepared from samples from animals free of infection (negative control); Evans Blue solution, neutral glycerin; thinner (dye Evans-Blue + PBS - set Gamarom); phosphate buffered saline/

Expression of results: positive samples: evidence of large cells with round nucleus represents about 80% of cell surface, blast cells in unusually large numbers, showing fluorescence in the cytoplasm specific color fluorochrome (FITC green when raw or yellowish green), in varying degrees of color, depending on the amount of viral antigen present in each infected cell; negative samples: absence of fluorescence in blastic cell previously described, being identical to negative control;

The methodology for identifying specific classical swine fever virus genome is using One Step RT-PCR technique. PCR protocols for identifying this RNA virus have international recognition and are used in reference laboratories for the detection of CSFV and for differentiation from other pestiviruses of ruminants. Nucleic acid extraction kit was made using "RNeasy Mini Kit" (Qiagen). The One Step RT - PCR kit (Qiagen) was used for nucleic acid amplification. For detection of CSFV, 5′ NTR region of the virus was amplified using the next set of primers: sens primer „a" (50μM) 5' - CAG CTT CAR YGT TGA TTG T - 3' and antisens primer „b" (50μM) 5' – CTA GCC ATG CCC WYA GTA GG - 3'. The results of the analysis are established by agarose gel electrophoresis. Positive samples must have the specific size of 420 pb.

Results and discussions

Classical swine fever (CSF) is of increasing concern in Europe where wild boar appears to play an important epidemiological role. In most parts of the continent, demographic trends are on the increase, due to improvement in game management.

The epidemiological study conducted during 2007-2012 on classical swine fever in wild boars from Suceava County consisted of testing 2290 samples. Using FAT were tested 1784 samples and using RT-PCR were analyzed 506 samples.

In 2007, 236 samples were examined by FAT and 2 samples by RT-PCR. All samples tested were identified as negatives for CSFV.

In the year 2008 were examined tissue samples (bone marrow) from 279 wild boars. Virological testing fluorescent antibody test showed negative response for all samples examined.

In the year 2009 were examined tissue samples (stern marrow and organs) collected from 344 wild boars. 334 samples were tested using FAT technique and 10 using RT-PCR. All samples tested were found negatives for CSFV.

In 2010 were tested 317 samples by FAT and 33 by RT-PCR. There were no positive samples for classical swine fever virus.

Using virological methods (FAT and RT-PCR) were tested 501 samples collected from wild boars in 2011 and 580 samples in 2012. All samples tested were identified as negatives for classical swine fever virus.

Table 1. Surveillance of classical swine fever by RT-PCR and FAT in wild boars from Suceava County

TEST	Sample type	2007	2008	2009	2010	2011	2012
FAT	Samples tested	236	279	334	317	339	279
	Positive	-	-	-	-	-	-
	Negative	236	279	334	317	339	279
RT-PCR	Samples tested	2	-	10	33	162	301
	Positive	-	-	-	-	-	-
	Negative	2	-	10	33	162	301

Classical swine fever virus has no known zoonotic potential; however, its presence in the region has serious economic implications for domestic pig farming and hunting tourism. The disease in the wild boar population was diagnosed and/or serologically confirmed in several Central and Eastern European countries (Rossi, 2005). Wild boar do not appear to be a classic reservoir in most cases, but nevertheless may perpetuate foci of infection over the long term, constituting a real threat for the pig farming industry.

Many researchers have studied the effectiveness of oral CSF vaccination in reducing the risk of epidemics among wild boars. It is important to define the scattering area of the baits to ensure effective delivery of oral vaccines for the control of CSF in wild boars (Ballesteros et al., 2009).

Conclusions

Results on the monitoring and diagnosis of classical swine fever by virological examination in the period 2007-2012 showed no cases of CSF infection in wild boars in Suceava County.

Till 2005, wild boars were vaccinated orally, using vaccine baits (chicken egg is inoculated with live attenuated virus strain C). For each wild boar, vaccination was performed only when the ground was covered with snow.

In November and December of 2008 was conducted emergency vaccination in 22 counties. In 2009, vaccination was carried out in two campaigns in 33 counties. In 2010 and

2011, vaccination was carried out in eight counties of Romania in a buffer zone on the north – east border.

In 2013 will continue the programs for CSF surveillance in pigs and wild boars by clinical examination and laboratory tests to whole Romanian territory.

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IMPACT OF FEED CONTAMINATION WITH MYCOTOXINS (OTA AND ZEA)ON PIG AND HUMAN HEALTH

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Abstract

The mycotoxins are known to affect the animal and human health. Ochratoxin A has been shown to be a mycotoxin is nephrotoxic, hepatotoxic, carcinogenic, immunosuppressive and teretogena. Zearalenoneis anon-steroidalestrogenicmycotoxin thatproduceshormonal disordersin animals thatingestcontaminatedforage, withthe worst effectsin pigs. Consumption of food contaminated with ochratoxin and zearalenone affects the health of farm animals and their productivity leading to its presence in animal products. Early identification and removal of feed and food chain products contaminated with ochratoxin and zearalenone can be achieved by control strategies. This paperwork aims to present the impact on human and animal health, the probable risk of ochratoxin and zearalenone, residues in animal products and control strategies that apply in the feed industry.

Key words: *feed swine Ochratoxin A ,Zearalenone, , human toxicity, animals toxicity*

Introduction

Micotoxins "are fungical metabolites that ingestion, inhalation or absorption through the skin , it can reduce performance and alter metabolism of animals, cause illness or even death in animals (Pit JI, 1996).

At the animals the effects of micotoxicoses are diverse,depending on type of micotoxin consumed, level and duration of intake, animal species, sex, age , breed, immune status, nutritional standing, hygiene, temperature, farm management(Heidler, 2003).

As metabolites of certain types of molds, mycotoxins hav adverse effects on human health and animals that consume feed contaminated with them. Temperature and moisture are key factors .

Livestock in the industrial system is growth on a balanced feed ration, a sufficient amount of essential nutrients and optimal environmental conditions.

Yet, nutritional stress and environmental stress are difficult to avoid this resulting in immunosuppression and decreased resistance to disease consequently lower reproductive and productive performance.

It was established that most mycotoxins are immunosuppressive factor in feed. Researches on mycotoxin-induced immunosuppression have shown that this occurs on both innate immunity and acquired immunity on (Surai, 2004).

The main mechanisms of toxicity of mycotoxins are:

- stimulate lipid peroxidation,
- apoptosis,
- inhibition of protein synthesis of DNA and RNA (figure.1)

So immunotoxicity is the most important consequence of serious mycotoxicoses (Bondy si Pestka, 2000).

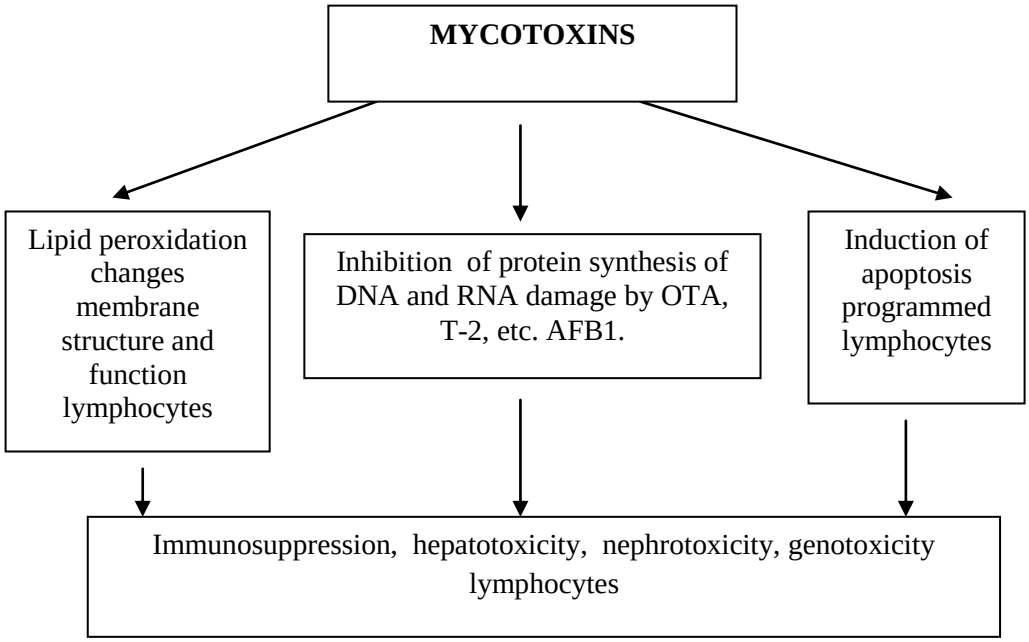

Fig.1. Major mechanisms of mycotoxicity (Surai,2004)

Immunosuppression induced by mycotoxins cause sensitivity of the immune system, the vulnerable cells continue to proliferate and to differ. These cells are those that participate in the activities of complex imunomediate and regulate communication between cellular and humoral components (Corrieri, 1991). High levels of polyunsaturated fatty acids in immune cells and the presence of the sensitive receptors on their surface are an important target for free radical attack (Surai, 2002).

It is assumed that inhibition of DNA, RNA and protein is due to the immunosuppressive action of several mycotoxins (Corrieri, 1991).

Immunosuppression induced by mycotoxins determines:

- reducing T and B lymphocyte activity,
- depresen NK cells activity,
- suppressed immunoglobulins and antibody production,
- reduced complement or interferon activity reduction,
- impaired macrophages.

The consequence of oxidative stress is shown in figure 2.

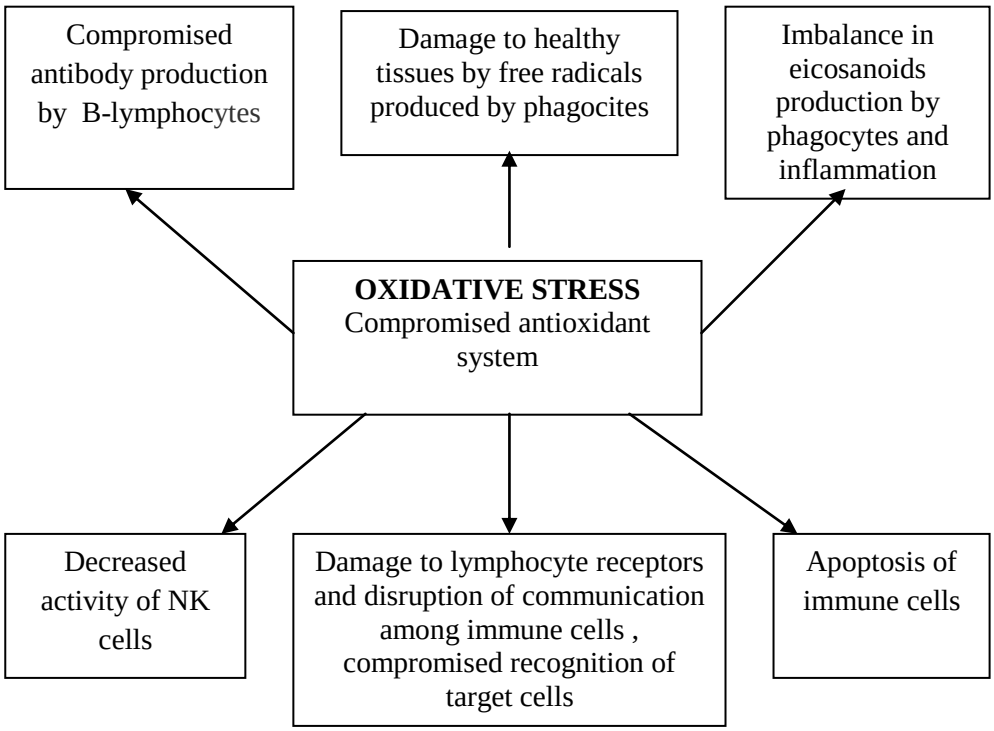

Fig. 2. Oxidative stress and immune system (Surai si col.,2002)

Ochratoxin in swine

Ochratoxin is produced by number of species of both *Aspergillus* and *Penicillium*. In cooler regions, OTA is produced by Penicillium and in warmer regions by Aspergillus (Pohland et al.,1992; Varga et al., 1996). Ochratoxin formes on acidic foods (Cuero et al., 1987).

The factor influence ochratoxicosis are: temperature, humidity, water activity, degree of aeration, substrate biocoenosis. (Pit J I ,1987). Tabel 1

Tabel 1. Growth conditions for ochratoxin production.

Growth conditions	Aspergillus ochraceus	Penicillium verrucosum
Optimum temperature for growth	24 - 37° C	20° C
Optimum temperature for ochratoxin production	31° C	20° C
Optimum growth	3 -10	6.0 – 7.0
Minimum wather activity for ochratoxin production	0,8	0,86

Large amounts of ochratoxin occur during storage of agricultural products due to the humidity (18-24%) favoring mycotoxigen fungal growth (Shotwell et al.,1969 ; Zimmerli and Dick,1996; Campbell et al., 2003) .

The highest amounts were reported in Northern Europe and North America (World Health Organisation 2002).

Rapid drying of agricultural products after harvesting can reduce the production of OTA. (Levi et al., 1974; Urbano et al.,2001; Samson et al., ,2004; Frisvad et al., 2004).

OTA effects on animal health and production

After consumption of OTA contaminated food, major economic impact can be observed on monogastric animals, birds and pigs. The consumption of OTA contaminated food reduces the growth rate and thus lowers animal productivity.

Nephrotoxic action

The effects of consumption of food contaminated with OTA depend on animal health on dose, animal species and the amount ingested.

It is believed that pigs are the most sensible to OTA.(European Food Safety Authority - EFSA, 2006).

Nephrotoxicity of ochratoxin is different from animal to animal.

Consumption of food containing OTA in concentration of 1 to 3 mg/kg resulted in the appearance of nephropathy and kidney cancer in pigs and humans after the installation of the tubular degeneration. The immunosuppressive activity in "natural killer" cells could explain tumour growth (Pfohl-Lenzkowicz et al.,1993; CIRC, 1993; Pfohl- Lenzkowicz et al., 2002) .

The installed kidney necrosis can be explained as a result of increased lipid peroxidation demonstrated for OTA in vivo and in vitro. (Meki AR et al.,2001) .

OTA inhibits the activity of many enzymes from the Krebs cycle, resulting in the decrease of the ATP production and the inhibition of the mitochondrial respiration. (Wei et al.,1985).

The ingestion of OTA produces polyuria and polydipsia. The increased blood urea and creatinine levels draw attention upon the renal impairment.

Consumption of food containing OTA in concentrations higher than 1 mg / kg leads to leukocytosis, increased neutrophils / lymphocytes ratio and decreased hemoglobin and erythrocytes levels. It was observed an impairment in the immune function associated with lymphocyte development and production of interleukins IL-2 (Harvey et et al.,1992).

Administration of OTA in feed over a period of 35 days growing gilts resulted in decreased phagocytic activity of macrophages (Harvey et al., 1992).

On necropsy there are found kidney discoloration and hypertrophy, atrophy and degeneration of proximal convoluted tubules, interstitial fibrosis and sometimes hyalinisation glomeruli. The impairment of the function renal was observed after consumption of OTA contaminated food in concentration of 200-4000g/kg. (Stoev et al., 2002) .

On histology examination there were found proximal tubular lesions and interstitial fibrosis.

After consumption of OTA contaminated food in concentrations higher than those that cause nephrotoxicity,there are found embryotoxicity, immunotoxicity and

teratogenicity(Benforg et al., 2001). On pigs, OTA ingestion lowers resistance to infection (Stoev et al., 2000).

Many European countries have experienced episodes of porcine nephropathy. The pigs intoxicated with OTA showed biochemical lesions: glucosuria, proteinuria, enzimurie, reduced urine concentration, renal insufucienta. (Petkova et al.,1991;Pfohl et al.,2002) .

Ochratoxicosis acute is characterized by nephropathy, enteridis and immunosuppression (Terao and Ohtsubo, 1991)

Carcinogenic action

The administration of OTA contaminated food for 2 years in a group of female pigs has led to kidney cancer. This is due to metabolism and excretion ochratoxins relatively quickly with an RL50 (disposal) in pigs for several days. (Krogh and Role,1992).

Zearalenone in swine

Zearalenone is a non-steroidal estrogenic mycotoxin produced by Fusarium spp. It was reported in many mycotoxicoses to farm animals, especially pigs.

Zearaleona is resistant to high temperatures and can be found in the entire world infecting many cereal crops devices such as corn, oats, barley, wheat, rice and sorghum (Kuiper-Goodman et al., 1987, Tanaka et al., 1988).

Fungal contamination occurs in the field, the corn examination shows a red mold. Mainly toxin production is achieved during storage.

Zearalenone no shows effect chronic toxicity. Between the domestic species the pig is the most sensitive at the zearalenone effect.

Zearalenone effects on animal health and production

Zearalenone is a known phytoestrogen that produces hormonal disorders in animals that ingest contaminated forage, with the worst effects in pigs. Pigs are very sensitive to zearalenone, which produces a syndrome manifested by changes in estrogen and breast tissue of the vulva (congestion, enlarged), abnormal lactation, infertility, pseudopregnancy, abortion, birth of dead or non-viable products,vagianla and / or rectal prolapse.

The most well known toxic effect produced by zearalenone is at the reproductions system level.

Zearalelnone toxicity depends on its interaction or of its metabolities with estrogen receptors.

Absorption in pigs after a single oral dose of 10 mg / kg body mass was estimated to be 80-85%. (Biehl et al., 1993)

Zearalenone and its metabolites were found in plasma of pigs less than 30 min after feeding began. (Kuiper-Goodman et al., 1987, Olsen et al., 1991, Biehl et al. 1993

Following administration of single doses up to 3.5 mg / kg body weight of zearalenone is found in young gilts occurrence of inflammation and vulvar swelling.The ingestion of a dose zearalenone more than 25 mg / kg body weight causes of symptoms of oestrogenism

After consumption of feed contaminated with high concentrations of zearalenone (ZEA) an occurrence in found at the sows, of oestrus prolonged, a syndrome pseudogestation and infertility (Fink-Gremmeles, 1999).

Zearalenone intoxication is characterized by phenomena oestrogenism, swelling vaginal, ovarian atrophy, hypertrophy of the mammary gland (Rainey, 1991).

Teratogenic effect of zearalenone in pigs occurs after consumption of feed contaminated with ZEA higher doses of 20mg/kg and consists of changes in limb development. (Diekman and Green, 1992)

Clinical signs are vulval reddening and swelling, vaginal and rectal prolapse(Etienne and Dourmad, 1994).

The zearalenone was found in the blood of children with precocious sexual development who consumed contaminated food, in Puerto Rico (Saenz de Rodriquez CA, 1984).

The mycotoxins contamination of animal products

The contamination of animal products may occur after consumption of mycotoxins contaminated food or by direct contamination with fungi.

After the consumption of mycotoxins contaminated food there is a rapid absorption of toxins into the bloodstream followed by relatively slow elimination through urine and feces. (Galtier,1991;Mantle,2008).

A team of researchers found that after oral administration of a single dose of 500g/kg of OTA peak plasma concentration at 2 hours is about 30% of the OTA intake. (Vettorazzi et al., 2009) .

The persistence of OTA in plasma is due to enterohepatic circulation and resorption in renal tubules. (Roth et al., 1988; Marquardt and Frohlich, 1992).

On pigs that were fed during the growth with OTA contaminated diet in concentration of 25 g/kg, residue in pork was up to 1 g/kg ((Malgutii et al., 2005) .

After some studies, many researchers have tried to establish a relation between the average concentration of mycotoxins in serum and concentration of mycotoxins in pig's feed.

OTA is considered as a possible causative agent for two chronic diseases: Balkan endemic nephropathy (NEB) and chronic interstitial nephropathy (North Africa).

In Balkan region ochratoxicosis were confirmed in humans. The appearance of Balkan nephropathy was associated with nephrotoxic effect of OTA in humans.

In 1956, this disease was described, for the first time, in a study conducted on a group of 664 hospitalized patients for kidney diseases, in Bulgaria. The Balkan endemic nephropathy was diagnosed in Romania with a spreading area of five outbreaks in Oltenia and one in Banat. (Gluhovschi et al., 1994) .

Following epidemiological studies which demonstrate that OTA can cause in humans a higher incidence of renal tumors and nephropathy, the European Scientific Committee indicates for human alimentation a tolerable food consumption lower than 5 mg/kg/day.

After oral administration, OTA is present in the blood for 35 days (Petzinger, and Weidenbach, 2002) .

After some studies it was observed that renal tumors often appear on food consumption of 70 g/kg/day of OTA (Phofl et al,1993, Phofl et al,2007; Phofl ,2009).

Legislation the mycotoxins in food

Attempts to eliminate mycotoxins in animal nutrition and the food is impossible(Bennett et al., 2003).

There were established regulations and guidelines establish maximum limits for mycotoxins that the: US Food and Drug Administration (FDA), Food and Agricultural Organization of the United Nations (FAO), European Union (EU), the Institute of Public Health of Japan.

The Regulation (EC) No 1881/2006 and in the Commission Recommendation 2006/ 576/ EC has set maximum limits for mycotoxins to basic products: cereals, cereal based products, dried fruits and wine, baby food, coffee.

There were used several strategies to reduce the risk of ochratoxins appearance in food industry, as a result of the transfer or the food chain.

Public health issues are justified on the basis of demonstrated toxic effects caused by contamination with mycotoxins.

Mycotoxins can contaminate feed materials (cereals) before arriving in feed mills due to weather conditions.

It is necessary to develop the capacity determination of mycotoxins level in whole food chaine: plant-animal- animal originated food products. (Savu et al., 2004).

As a precaution, the quality control on each lot is in order.

Feed business operators have to be licensed in accordance with European Commission Regulation no. 183/2005 and to conduct a risk analysis and critical control points in HACCP implementation.

The application of HACCP system in all units that are parts of the the food chain is required. Hazard analysis and critical control points (HACCP) is a scientific and systematic apparatus used to identify:

• Risks associated to a food product regarding food safety;

• Risk monitoring to ensure food innocuousness.

The legislation in food industry aims to reduce, eliminate and prevent a risk to human and animal health. The three components of risk analysis: risk assessment, risk management and risk communication lead to the establishment of efficient and accurate measures of health protection.

European Commission Regulation no. 178/2002 establishes safety requirements regarding feed and feed business operators' responsibilities. Food and feed traceability shall be established at all stages of production, processing and distribution

The official controls carried out in order to ensure the verification of compliance with feed and food under EC Regulation no. 882/2004 had a major role in ensuring food safety.

There were developed laws that establish sampling analyzing methods for the official control of feed and food:

• EC Regulation no. 401/2006 established sampling and analyzing methods for the official control of mycotoxins in food;

• EC Regulation no. 152/2009 established sampling and analyzing methods for the official control of feed.

The control strategies of mycotoxins in food consist in: early identification and elimination of the contaminated products from the food chain.

Material and methods

To minimize the impact of the presence of mycotoxins in feed breeding pigs with direct influence on their determinations were carried out to establish the quality of feed used. This mycotoxins (OTA and ZEA) was evidenced by using ELISA method of working, a rapid

quantitative method of screening. The determination is made based on working kit protocol used is based on the reaction of antigen - antibody. ELISA kit (Enzyme-linked immunosorbent assay-enzyme immunoassay, or EIA) contains:

Microtiter plate consisting of 12 strips with 8 wells each, coated with antigen;

Standards of different concentrations of mycotoxins (5 or 6) with standard Cuba Trace. All reagents and buffers required (Anti-body - specifically of mycotoxin, Conjugate (with enzyme), Substrate Solution, Stop Solution , Washing buffer)

To avoid contamination of samples was taken into account the observance of rules, namely:

-when entering the laboratory, samples were pureed ;

-it was a laboratory sample is stored in the freezer representative until determination;

To obtain valid results has been considered subject to the following precautions:

-all reagents were brought to temperature 20-25 ° C and were mixed before use ;

-these steps were imposed by the kit work in compliance with time forced ;

-to work in the solvent extract preparation - 70% methanol (OTA, ZEA) ;

-were observed using working volumes: 50, 100, 500 and 1000 µl-micropipets;

Upon completion of the determination and use of equipment contributed: centrifuge, shaker, stirrer ELISA, ELISA plate reader at 450nm

Results and discussion

This paper has proposed to address the presence of mycotoxins (OTA and ZEA) in feed intended for pigs in 2010-2012. Samples were representative sample for each lot and have to comply with harvesting.

If consumption of moldy feed containing secondary metabolites such as: Ochratoxin A (OTA), zearalenone (ZEA) swine, especially youth and females may be affected, the impact on reproductive performance.

Toxicity of mycotoxins depends on the source and their dose, duration of exposure and composition.

Samples analyzed samples were represented by the following matrix: combined fodder for pigs, corn beans, bran, ground grain. The results of determinations made are shown in the table 2.

Table 2. Determinative mycotoxins in swine feeds: 2010-2012

Matrices	Nr. Samples			OTA, (µg/ Kg)			ZEA, (µg/ Kg)		
	2010	2011	2012	2010	2011	2012	2010	2011	2012
Mixed fodder for pigs	3	3	13	Ned …0,478	Ned.	Ned …0,47	Ned…16,82	Ned….28,5	Ned.…5
corn beans	6	4	16	Ned.	Ned.	Ned.	5,12…51,64	3,44…30,09	Ned.
bran	3	4	11	Ned.	Ned.	Ned.	Ned. ...35,10	1,12…28,14	Ned.
ground grain	6	5	17	0,36..0,74	0,12..0,24	0,12..0,23	5,34...35,18	Ned. .. 27,41	0,1..0,22

Ned.- nedetectabil

The performed determinations were interpreted according to the European legislation and are thus presented in the table no. 3.

Because the values obtained from analyzes did not exceed the maximum admissible, evidence is not harmful to animal health and human health.

Values obtained from determinations were performed according to the legislation. Because toxic effects of mycotoxins, their highest level in feed for pigs is subject to European legislation Regulation (EC) No 576/2006 Table no.3

Tabel 3. Maximum levels for ZEA and OTA in cereal for pigs feeding

Mycotoxin	**Products intended for animal feed value**	**Value in mg/kg (ppm)**
Zearalenone	Feed materials (*) — Cereals and cereal products with the exception of maize by-products — Maize by-products — Complementary and complete feegingstuffs for pigs	 2 3 0,250
OTA	Feed materials (*) — Cereals and cereal products; Complementary and complete feegingstuffs for pigs	 0,250 0,050

Conclusions

This paperwork approaches about the problem of mycotoxins in relation to toxicity and the mechanisms by which it exerts its toxicity to human and animal health and control strategies used in the feed industry.

The strategies used to reduce, eliminate or avoid the risk of mycotoxins are justified by the demonstrated toxic effects caused by contamination with mycotoxins.

Mycotoxins can have a negative effect on the consumption of nutrients, they inhibit absorption of nutrients by animals. Also potavea a direct negative impact on animal performance

Counteracting mycotoxin problems in a farm animal will increase performance and improve pig producer's profit.

Livestock producers are key factors in the production chain, their responsibility is to counter the problem of mycotoxins

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MYCOBACTERIUM AVIUM SSP. AVIUM STUDIED IN NATURALLY INFECTED HENS BY CULTURE AND PCR IDENTIFICATION

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Abstract
Avian tuberculosis is an important infectious disease which affects all species of birds and the most common etiologic agent is Mycobacterium avium than Mycobacterium gavanese. Besides the economic losses caused by the etiologic agent of this disease the major risk factor is the transmission to humans. Diagnosis of avian tuberculosis is based on the detection of an immune response or the presence of the mycobacteria in the dead or the live bird. The objective of this study is to identify the etiologic agent of tuberculosis in hens by culture and PCR.

Keywords*: avian tuberculosis, PCR, IS901, IS1245*

Introduction

Tuberculosis in bird is caused by several mycobacteria, the most commonly ethological agent are *Mycobacterium avium* classified in the Mycobacterium avium-intracellulare complex [2] and *M. genavense* [21]. *M. avium* is subdivided into four subspecies (subsp.): subsp. *avium*, subsp. *paratuberculosis*, subsp. *silvaticum* and recently subsp. *hominissuis* [2, 13]

Mycobacterium avium subsp. *avium* (*MAA*) cause tuberculosis in a wide variety of domestic and wild bird species [10, 20, 21, and 24] as well as other animals [15, 17]. For human, especially for the immunocompromised ones, the contact with infected birds with *Mycobacterium avium* subsp. *avium* may cause a zoonotic infection [12]. The characteristic repetitive sequence for the most MAA isolates from birds is IS901 and it has been detected only in *M. avium* strains with serotypes 1, 2 and 3 [16, 18], that are apparently more pathogenic to birds than other serotypes [21]. MAA genome is characterized by the presence of 2 to 17 copies of IS901 insertion sequence [5, 8] and one copy of IS1245 [9]. Genomic studies relieved for the repetitive sequence IS1245 described in MAA a characteristic three-band pattern in RFLP- IS1245 analysis [18].

The aim of this study is to identify MAA in cultures isolated from hens and validate the diagnosis of avian tuberculosis by IS901 and IS1245 PCR.

Matherial and method

A total of 12 birds from the same species of domestic hens were examined. All of them were originated from 3 different household from Iaşi and Vrancea County. The majority of them were from a household from locality Vişani, Iaşi County (n=7). Is needed to mention that considering the fact that the birds are originated from households, only 3 of them could

be examined alive and for this it was done the intradermal test with avian tuberculin (Avitubal,25 000U.I/ml, Bioveta) and for the hystopathological exam samples from 4 hens were processed for the Ziehl-Neelsen (ZN) and Masson's trichrome stain. In consequence the following laboratory examinations were performed on the liver samples from all examined 12 hens: mycobacterial culture, DNA isolation and identification of ethological agent by IS901-PCR and IS1245-PCR.

Hystopahology

A total of 9 tissue samples, liver, spleen and intestine, were formalin fixed, embedded, in paraffin blocks and strained by ZN technique for the presence of acid-fast rods (AFR) and Masson's trichrome technique for observing the granulomatose aspect. Read preparations were made with a microscope Leica ICC50 HD and capturing images with the system Acquire Leica Leica Acquire Software.

Culture

For culture examination, approximately 1 g of liver tissue was used [25, 26]. The decontamination was done with oxalic acid and NaOH and the medium for growth was Löwenstein–Jensen (LJ) without mycobactin. To process specimens for culture, the tissue is first homogenized using a mortar and pestle followed by decontamination with 2–4% sodium hydroxide. The alkali mixture is shaken for 10–15 minutes at room temperature and then neutralized. The suspension is decanted at room temperature for 30 minutes and the sediment is used for culture [26]. We also tried the decontamination method with hexadecylpyridiniumchloride 0.75% (HPC) and the cultivation on Herrolds's egg yolk media (HEYM) with mycobactin. Neutralization is not required when using HPC [27]. The samples cultivated on the two culture medias were incubated at 37^0C for 3 months [6].

DNA isolation and PCR conditions

Previous DNA extraction the liver samples (n=10) were homogenized using a stomacher and then mechanically lysed using lysing matrix B tubes. The extraction of DNA was made with the Macherey Nagel Tissue kit. Cultures preparation for MAA identification was done by heat shook.

All samples with macroscopic lesion of avian tuberculosis (n=10) were examined by PCR method for detection IS901 [11, 16] and IS1245 [1, 7], specific sequences for *Mycobacterium avium* subsp. *avium* and *silvaticum* (Table 1).

Table 1. Primers used to identify IS901 and IS1245 sequences

Sequence	Primers			Amplicon size
IS901	Forward	P102	5'-CTGATTGAGATCTGACGC-3'	252 pb
	Reverse	P103	5'-CACCACGTGGTTAGCAATCC-3'	
IS1245	Forward	P1	5'- GCCGCCGAAACGATCTAC-3'	427 pb
	Reverse	P2	5'-AGGTGGCGTCGAGGAAGAC-3'	

For IS901 and IS1245 PCR we used 2 µl of DNA sample and 23 µl PCR mix: 9 µl water, 5 µl tampon 5x, 2.5 µl dNTPs 2mM; 5 µl $MgCl_2$ 25 mM; 2.5 µl sense and antisense primer 10 pmol.μl^{-1} each and 0.1 µl Taq polymerase (Promega). The amplification conditions for both sequences were as follows: initial denaturation 15 min at 94 °C; 35 cycles with 30 s at 94 °C; 30 s at 58^0C, elongation 30 s at 72 °C and final hold 7 min at 72°C.

Results and discutions:

Alergic test

For the tree live hens examined was noted poor body condition. At the read of intradermical reaction, after 24 and 48 hours we observed: for one of them a positive response with the barbital inflammation, for one of them the inflammation of barbital and the intemandibular space and for the last one a false-negative response with the local necrosis.

Necropsy

From all 12 hens 8 of them presented lesions of avian tuberculosis especially in the liver and spleen and 3 of them also in the intestine. Tuberculosis lesions presented various sizes: submiliari to training milestones and larger than an inch. The liver is hypertrophied, yellowish brown color and increased friability; exceeding 3-4 times the normal size. The spleen presented enlarged volume with the presence of granulomatous nodules of considerable size, sometimes more than 1 cm and increased friability. Granulomatous inflammation was diagnosed in all tuberculous lesion tissue samples with the detection of numerous AFR.

Histopathology

Smears realized from organs with lesions and strained ZN showed on direct bacterioscopy the presence of acid fast bacilli. The histopathology findings were those typical of avian tuberculosis: granulomas containing a caseous central necrosis, surrounded by macrophages, heterophils, lymphocytes and moderate amounts of multinucleated giant cells (Langhans type), were present within liver (Figure 1), spleen, and intestine. With the Masson's trichrome stain it can be very well observed the granuloma structure and avian tuberculosis granulomas in different stage of formation (Figure 1).

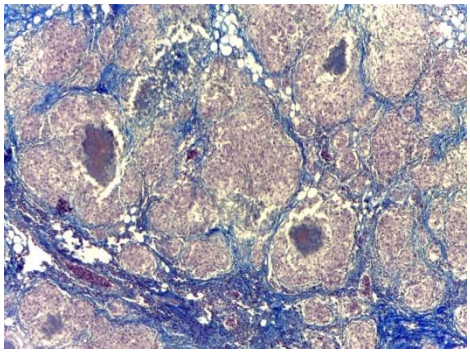

Fig. 1. Chicken liver, tuberculous granulomas in various stages of evolution; Masson's trichrome stain; x100

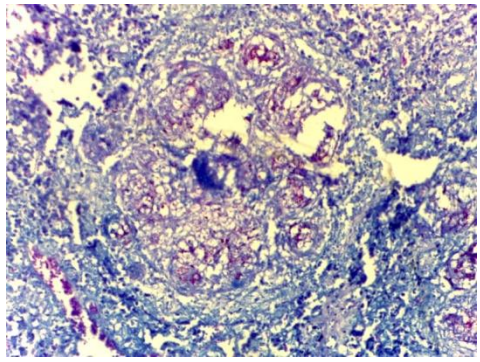

Fig. 2. Chicken liver with tuberculous granulomas and the presence of bacilli AFR in central areas of necrosis; ZN stain; x200

Acid fast bacilli were present within necrotic tissues and cytoplasm of macrophages, these they could be shown with the ZN strain (Figure 2). Due to an oral route transmission, *M. avium* infection often results on gastrointestinal tract, hepatic and spleen lesions, without involvement of lungs, skeletal or other tissues [4, 23].

Culture

For all 12 birds was processed the liver for culture, even if 4 of them didn't presented lesions. In culture, as in the necropsy, 8 of the subjects were positives. The LJ media was verified every day after inoculation and for 2 subjects were observed start growing colonies at the day four. A week after inoculation for the 8 subjects with the lesions described before start growing colonies are observed and in two weeks the culture is observed as a smooth canvas on culture medium slope. The same cultural character was observed on HEYM media with mycobactin J. The first start growing colonies on HEYM media with mycobactin J were observed after one week after inoculations on 7 subjects from 12 and the examination after 2 weeks of the inoculation it was observed smooth mycobacterial colonies for 8 subjects. The culture examination confirmed the others exams done before.

PCR identification

The PCR test for these cases was definitive for diagnosis of the etiological agent as *Mycobcaterium avium* subsp. *avium*.

For the first time it was realized a DNA extraction from the liver from 10 birds and 8 isolates were positive for IS901 and IS1245 PCR, with the visualization of a 252 pb fragment and 427 pb fragment (Figure 3).

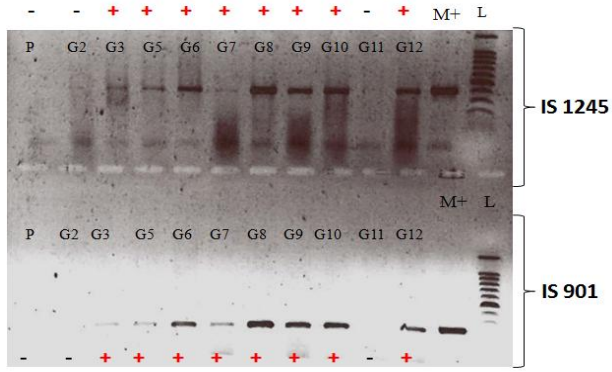

Fig. 3. Figure Analysis of the agarose gel using QuantityOne ® software (BioRad). IS901 and IS1245 PCR performed on isolates from poultry;
G2, G3,G5 - G12 = samples; M + = positive control; L = ladder 100pb

After the 2 weeks of culture incubation and the visualization of colonies it was preceded a ZN stain and a DNA extraction of culture and an IS901 PCR for confirmation of *Mycobacterium avium* spp. *avium* presence.

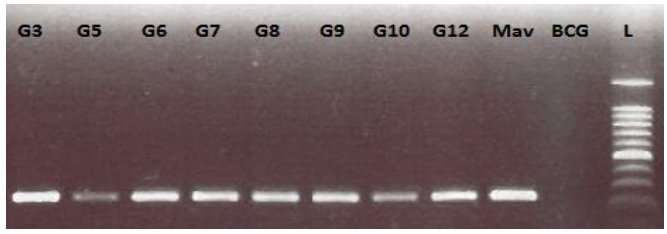

Fig. 4. Analysis of the agarose gel using QuantityOne ® software (BioRad). IS901 PCR performed on extracts isolated from culture; G3, G5 - G12 = samples
Mav = positive control, BCG = negative control, L = 100 bp ladder

As it was expected, after the IS901 PCR amplification, the migration and the agarose gel visualization the amplicons size was about 252 pb (Figure 4).

Conclusions

1. All cases with avian tuberculosis lesions were confirmed as positive for infection with *Mycobacterium* avium *subsp. avium* in culture examination.
2. Using IS901 PCR, we were able to confirm MAA infection in all of the positive culture samples isolates from hens and it can be concluded that bimolecular exams are a faster and specific alternative method to conventional culture.

Acknowledgments

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COMPARATIVE EVALUATION OF THE ANTIFUNGAL ACTIVITY OF VORICONAZOLE AND A NEW PROPICONAZOLE DERIVATIVE (MXP-4509) AGAINST 278 *CANDIDA ALBICANS* CLINICAL ISOLATES

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Abstract

278 C. albicans clinical yeast isolates, collected within the 2010-2011 time-frame from four Romanian tertiary hospitals located in different parts of Romania (Iaşi, Cluj, Tg. Mureş and Timişoara), were investigated. The EUCAST E. Def. 7.1 broth dilution method was used to determine susceptibility. Assigning isolates to a susceptibility category was done using the specific breakpoints. 160 isolates (57.56%) came from superficial infections (SUP), 61 (21.94%) from deep seated infections (DEEP) and 57 (20.50%) from blood stream infections (BSI). Two isolates showed voriconazole resistance. The activity of the two compounds did not vary with infection type (P>0.05). Although slightly inferior, the activity of MXP-4509 was very much comparable to that of voriconazole.

Keywords*: Candida albicans, fluconazole, voriconazole, MXP-4509*

Introduction

Although in the last decades we have witnessed a constant rise in the number and diversity of *Candida* non-*albicans* and non-*Candida* isolates involved in fungal infections [1], *C. albicans* remains the most frequently isolated yeast [2]. Despite the fact that it is one of the more responsive species to classic treatment, resistant isolates or isolates with decreased susceptibility are not uncommon [3].

Although the range of antifungal agents available for the treatment of mycoses has expanded and diversified in the last decades, the azoles remain the most studied and widely used class of antifungals [4]. Reported azole resistance, however, especially fluconazole resistance, is increasing in frequency [5]. This situation shows the need for the development of other more efficient and non-toxic classes of antifungal agents, in the long term, and for the improvement and diversification of the existing ones, in the medium term.

Materials and methods

Antifungal agents: Pure powders of fluconazole and MXP-4509 were used. Voriconazole was used as VFEND. Fluconazole was purchased from Sigma (St. Louis, USA) while VFEND was purchased from Pfizer Ltd. (Sandwich, UK). MXP-4509 was synthesized at the "Petru Poni" Institute of Macromolecular Chemistry (Iaşi, Romania).

The new compound is a bionanoconjugate based on a propiconazole derivative and a ciclodextrin. Its synthesis and characterisation have been previously published [6]. Prior to use the antifungal agents were dissolved in distilled water.

Isolates: A number of 278 *C. albicans* clinical isolates were tested. The isolates were collected in the 2010-2011 time frame, from 4 tertiary hospitals located in various regions of Romania (Cluj, Iaş i, Tg. Mureş and Timiş oara).

The *C. albicans* strain used for quality control belongs to the Romanian Type Culture Collection (RTCC) from the Laboratory of Antimicrobial Chemotherapy of Iaş i, Romania.

Yeast identification: the microorganisms were presumptively isolated using various techniques – germtube test, manual and automatic Vitek 2®, Api Candida®. The isolates were centralized by our laboratory where they were rechecked for purity and they were kept in a 20% glycerol aqueous solution, at -80°C, until analysis.

The identification was redone using ID 32 C® (BioMerieux). The isolates identified as *C. albicans* or *C. dubliniensis* were further analysed by PCR duplex [7].

Prior to susceptibility testing, each *Candida* isolate was cultivated on YPD agar and incubated at 36±1°C for 24-48 h.

Antifungal susceptibility testing: MICs for the three compounds were assessed using the EUCAST (European Committe for Antimicrobial Susceptibility Testing, ESCMID) broth microdilution method described in the EUCAST Definitive Document EDef 7.1 [8]. Briefly, 100 µL of two-fold dilution series of the antifungal agents, at double the final strength, in a modified RPMI-1640 medium (containing 2% glucose), also at double the final strength and at a 7.0 pH, were 1:1 mixed with 100 µL of inocula at double the final density, in 96 well microplates. This way, the final concentrations for each dilution and for the medium as well as the final density for each inoculum were obtained.

The ranges of final antifungal concentrations tested were 0.125 µg/mL - 64 µg/mL, for fluconazole, and 0.0156 µg/mL - 8 µg/mL, for voriconazole and MXP-4509. For the preparation of the inoculum, a yeast suspension in sterile 0.9% saline solution, adjusted to a turbidity equivalent to the 0.5 McFarland standard, was made for each isolate. These initial suspensions were 1:10 diluted with distilled water and used for the inoculation of the microplates that contained the antifungal agents dissolved in medium. The final density of the inocula was 0.5-2.5 × 10^5 CFU/mL [8].

MICs for *Candida* spp. isolates were determined after a 24 h incubation period, at 36±1°C. MICs were established, according to EUCAST E. Def. 7.1, as the smallest concentrations that induced an obvious reduction of turbidity (spectrophotometrically measured 50% growth reduction) compared to the positive control.

Microbiological resistance: The isolates were classified as "resistant" according to the EUCAST Breakpoints, version 6.1, valid from 11/03/2013 [9].

Data analysis: D'Agostino-Pearson omnibus normality test was used to check the normality of the log(MIC) values of each compound or of the differences between the paired log(MIC) values of the two compounds. Since none of the data passed the normality test and sample size was not small, non-parametric tests were used [10].

To analyse whether the MICs of each compound were different between infection types, the Kruskal-Wallis test was used followed by Dunn's multiple comparison test. To compare the MICs of the two compounds the Wilcoxon matched-pairs signed-rank test was run. In case of ties, the method of Pratt for ties was used [10]. The tests were run using a fully functional trial version of GraphPad Prism version 6.02 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Two-tailed *P* values were calculated. *P* values smaller than 0.05 were considered as indicative of statistical significance.

The geometric mean of the MICs was calculated as the antilogarithm of the arithmetic mean of the natural logarithms of the MICs using as a base the following array formula in Microsoft® Excell® 2010: EXP(AVERAGE(LN(x))), where x is a MIC value or a range of values. This formula was extended with various logical arguments using the IF function. Right-censored values (greater than the maximum tested concentration) were considered as the next theoretical value (double of the maximum tested concentration).

Results and discussions

The 278 yeasts tested were isolated from various sources: blood, bronchial aspirate, cerebrospinal fluid, draining tube, peritoneal fluid, sputum, urine, balanitis, bile, bronchial catheter, faeces, onychomycosis, oral infection and vaginal infection. These sources were grouped into three infection types: blood stream infections - BSI (blood), deep seated infections - DEEP (bronchial aspirate, cerebrospinal fluid, draining tube, peritoneal fluid, sputum and urine) and superficial infections - SUP (balanitis, bile, bronchial catheter, faeces, onychomycosis, oral infection and vaginal infection). The largest number of isolates came from superficial infections (160 - 57.56%) seconded by deep seated infections with 61 isolates (21.94%) while blood stream infections came last with 57 isolates (20.50%) (Fig. 1).

Two of the *C. albicans* isolates (one BSI and one SUP) showed voriconazole resistance.

The distribution of MICs for both VOR ($P \approx 0.6051$) and MXP-4509 ($P \approx 0.2536$) was not different between the three infection types.

On *C. albicans* isolates VOR had a statistically significant superior activity to that of MXP-4509, both overall ($P < 0.0001$) and within infection types (BSI-$P = 0.001$, DEEP-$P = 0.002$, SUP-$P < 0.0001$). Statistical significance, however, doesn't necessary imply a very big difference, as it can be seen from Table 1 and Figures 1 to 4. Table 1 shows identical values for the MIC Mode and the MIC_{50} across all groups. The MIC_{90} of MXP-4509 is always one order of magnitude higher and, consequently, its geometric mean is slightly higher in all cases. The MIC Range, however, is, with one exception, always narrower or, to be more precise, it extends less to the right in the case of MXP-4509.

Figures 1 to 4 show a large number of zero differences (the median of differences was also zero) and that the positive differences between MXP-4509 and VOR MICs never went past 0.5 mg/L and rarely past 0.0156 mg/L.

Although slightly inferior, the activity of MXP-4509 against *C. albicans* clinical isolates is very much comparable to that of VOR. The MIC Range and the MIC50 and MIC90 suggest a MIC distribution curve slightly shifted to the right but more "peaked", with more kurtosis and less skewness, for MXP-4509 in comparison with that of VOR, but more data is necessary to confirm such a supposition.

Given the comparable antifungal activity and the fact that propiconazole, the raw material for MXP-4509, is cheaper than voriconazole, MXP-4509 presents itself as an interesting compound that deserves further research regarding its *in vivo* antifungal activity as well as its pharmacokinetics and pharmacodinamics.

Table 1. MIC characteristics for the *C. albicans* clinical isolates tested

Infection Type	Compound	MIC (mg/L)				
		Range	Mode	MIC_{50}	MIC_{90}	Geometric Mean
Overall	VOR	≤0.0156 - 1.0	0.0156	0.0156	0.0156	0.0163
	MXP-4509	≤0.0156 - 0.25	0.0156	0.0156	0.0312	0.0193
BSI	VOR	≤0.0156 - 1.0	0.0156	0.0156	0.0156	0.0168
	MXP-4509	≤0.0156 - 0.25	0.0156	0.0156	0.0312	0.0187
DEEP	VOR	≤0.0156 - 0.0312	0.0156	0.0156	0.0156	0.0158
	MXP-4509	≤0.0156 - 0.0625	0.0156	0.0156	0.0312	0.0183
SUP	VOR	≤0.0156 - 0.5	0.0156	0.0156	0.0156	0.0164
	MXP-4509	≤0.0156 - 0.125	0.0156	0.0156	0.0312	0.0200

Conclusions

1. 278 clinical *C. albicans* isolates from three infection types (blood stream infections, deep seated infections and superficial infections) were tested
2. 57.56% of the isolates came from superficial infections, 21.94% from deep seated infections and 20.50% from blood stream infections
3. Two of the *C. albicans* isolates (one BSI and one SUP) showed voriconazole resistance
4. The activity of the two compounds did not differ with infection type
5. MXP-4509 had an antifungal activity comparable to that of voriconazole

Fig. 1 Differences between MXP and VOR MICs for all isolates

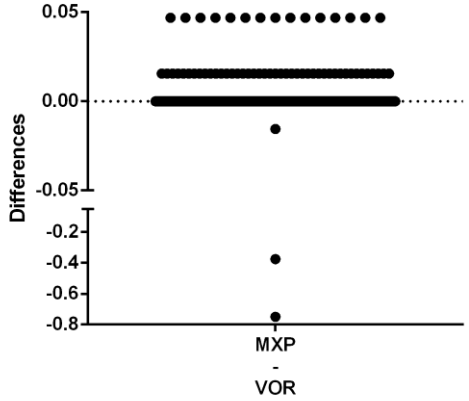


Fig. 2 Differences between MXP and VOR MICs for BSI isolates

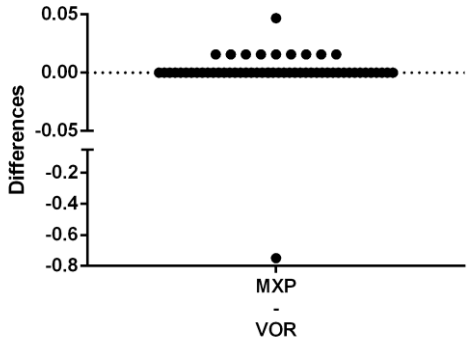


Fig. 3 Differences between MXP and VOR MICs for DEEP isolates

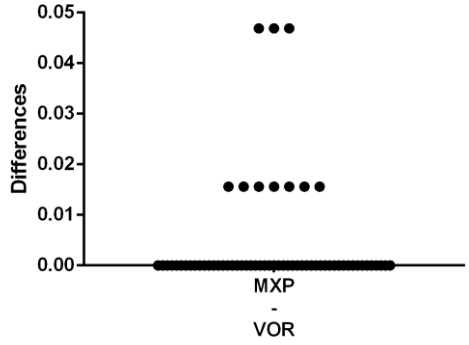
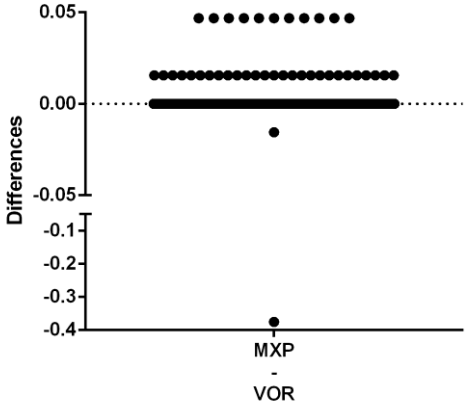


Fig. 4 Differences between MXP and VOR MICs for SUP isolates



Acknowledgements

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COMPARATIVE EVALUATION OF THE ANTIFUNGAL ACTIVITY OF VORICONAZOLE AND A NEW PROPICONAZOLE DERIVATIVE (MXP-4509) AGAINST FLUCONAZOLE-RESISTANT YEAST ISOLATES

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Abstract

57 resistant clinical yeast isolates, collected within the 2010-2011 time-frame from four Romanian tertiary hospitals located in different parts of Romania (Iaşi, Cluj, Tg. Mureş and Timişoara), were investigated. The EUCAST E. Def. 7.1 broth dilution method was used to determine susceptibility. Assigning isolates to the "resistant" category was done using specific or non-specific breakpoints or ECOFFs, as applicable. 18 isolates (31.58%) came from blood stream infections (BSI), 9 (15.79%) from deep seated infections (DEEP) and 30 (52.63%) from superficial infections (SUP). 53 isolates belong to Candida spp. 11 Candida spp. isolates and one non-Candida isolate also showed voriconazole resistance. In most cases MXP-4509 was superior to voriconazole (P < 0.05), except against C. krusei, in which case the new compound was slightly inferior but still comparable (P=0.041). Apparently MXP-4509 is especially efficient against C. glabrata; the differences, however, were not statistically significant (P=0.0625), which was to be expected with only 6 isolates tested. Nonetheless, P's close to 0.05 values show the need for further research in both cases.

Keywords*: candidiasis, resistance, fluconazole, voriconazole, MXP-4509*

Introduction

Fungal infections are a major health problem worldwide, especially for immunosuppressed or immunocompromised patients [1]. Among the various classes of antifungals, the triazoles are the most widely used and studied [2]. In the context of a worldwide increasing incidence of resistance to fluconazole (the oldest and most extensively used triazole), especially with isolates of *C. glabrata* and also rare *Candida* and non-*Candida* species [3], the necessity of adding new antifungal triazole agents to the therapeutic arsenal becomes evermore obvious.

Materials and methods

Antifungal agents: Pure powders of fluconazole and MXP-4509 were used. Voriconazole was used as VFEND. Fluconazole was purchased from Sigma (St. Louis, USA) while VFEND was purchased from Pfizer Ltd. (Sandwich, UK). MXP-4509 was synthesized at the "Petru Poni" Institute of Macromolecular Chemistry (Iaşi, Romania).

The new compound is a bionanoconjugate based on a propiconazole derivative and a ciclodextrin. Its synthesis and characterisation have been previously published [4]. Prior to use the antifungal agents were dissolved in distilled water.

Isolates: A number of 57 clinical yeast isolates were tested. The isolates were collected in the 2010-2011 time frame, from 4 tertiary hospitals located in various regions of Romania (Cluj, Iaşi, Tg. Mureş and Timişoara).

The strains used for quality control belong to the Romanian Type Culture Collection (RTCC) from the Laboratory of Antimicrobial Chemotherapy of Iaş i, Romania: *C. albicans*, *C. krusei*, and *C. parapsilosis*.

Yeast identification: the microorganisms were presumptively isolated using various techniques – germtube test, manual and automatic Vitek 2®, Api Candida®. The isolates were centralized by our laboratory where they were rechecked for purity and they were kept in a 20% glycerol aqueous solution, at -80°C, until analysis.

The identification was redone using ID 32 C® (BioMerieux). The isolates identified as *C. albicans* or *C. dubliniensis* were further analysed by PCR duplex [5]. In cases where the identity of the yeast remained unclear, MaldiTof was used for identification [6]. The rare species, for which even this method yielded doubtful profiles, were identified by DNA sequencing.

Prior to susceptibility testing, each *Candida* isolate was cultivated on YPD agar while the non-*Candida* isolates were cultivated on Sabouraud agar (Bio-Rad, France). All were incubated at 36±1°C for 24-48 h.

Antifungal susceptibility testing: MICs for the three compounds were assessed using the EUCAST (European Committe for Antimicrobial Susceptibility Testing, ESCMID) broth microdilution method described in the EUCAST Definitive Document EDef 7.1 [7]. Briefly, for each isolate, 100 µL of two-fold dilution series of the antifungal agents, at double the final strength, in a modified RPMI-1640 medium (containing 2% glucose), also at double the final strength and at a 7.0 pH, were 1:1 mixed with 100 µL of inoculum at double the final density, in 96 well microplates. This way, the final concentrations for each dilution and for the medium as well as the final density for each inoculum were obtained.

The ranges of final antifungal concentrations tested were 0.125 µg/mL - 64 µg/mL, for fluconazole, and 0.0156 µg/mL - 8 µg/mL, for voriconazole and MXP-4509. For the preparation of the inocula, a yeast suspension in sterile 0.9% saline solution, adjusted to a turbidity equivalent to the 0.5 McFarland standard, was made for each isolate. These initial suspensions were 1:10 diluted with distilled water and used for the inoculation of the microplates that contained the antifungal agents dissolved in culture medium. The final inoculum density was 0.5-2.5 × 10^5 CFU/mL (EUCAST E. Def. 7.1).

MICs for *Candida* spp. isolates were determined after a 24 h incubation period, at 36±1°C. The incubation for *Cryptococcus neoformans* isolates was longer, of 70-74 h [8,9]. MICs were established, according to EUCAST E. Def. 7.1, as the smallest concentrations that induced an obvious reduction of turbidity (spectrophotometrically measured 50% growth reduction) compared to the positive control.

Microbiological resistance: The isolates were classified as "resistant" according to the EUCAST Breakpoints, version 6.1, valid from 11/03/2013 [10]. In the case of species for which breakpoints have not yet been determined, the ECOFFs were applied, when available, otherwise two non-specific breakpoints were used. For fluconazole, the 4 µg/mL EUCAST non-specific breakpoint was used. For voriconazole, a 0.25 µg/mL value, mentioned in the "EUCAST Technical Note on voriconazole" [11] as a possible non-specific breakpoint, was used.

Data analysis: The comparison of MICs was done with the Wilcoxon matched-pairs signed-rank test [12] using a fully functional trial version of GraphPad Prism version 6.02 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. In case of ties, the method of Pratt for ties was used. Two-tailed *P* values were calculated. *P* values

smaller than 0.05 were considered as indicative of statistical significance. Right-censored values (greater than the maximum tested concentration) were considered as the next theoretical value (double of the maximum tested concentration).

Results and discussions

The 57 resistant yeasts were isolated from various sources: blood, bronchial aspirate, peritoneal fluid, sputum, urine, faeces, oral infection and vaginal infection. These sources were grouped into three infection types: blood stream infections - BSI (blood), deep seated infections - DEEP (bronchial aspirate, peritoneal fluid, sputum, urine) and superficial infections - SUP (faeces, oral infection and vaginal infection). The largest number of resistant isolates came from superficial infections (30 - 52.63%) seconded by blood stream infections with 18 isolates (31.58%) while deep seated infections came last with 9 isolates (15.79%) (Fig. 1).

Fig. 1 Distribution of FCA-resistant isolates according to infection type

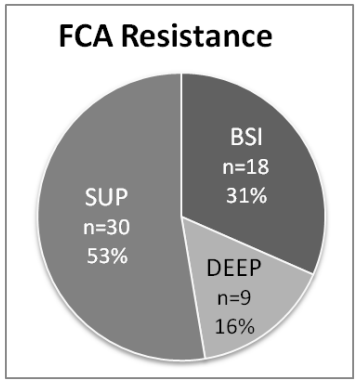
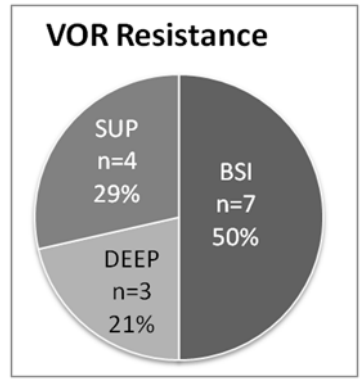


Fig. 2 Distribution of VOR-resistant isolates according to infection type



The distribution of isolates according to species and infection type is presented in Table 1. *Candida* spp. was the dominant group with 53 resistant isolates while the non-*Candida* group only had 4 resistant isolates. *C. krusei* accounted for almost half of all the resistant isolates, followed at a distance by *C. glabrata* and *C. albicans*. Only *C. krusei* and *C. tropicalis* had isolates in all three infections types. 12 of the 57 FCA-resistant isolates also showed VOR resistance. As Fig. 2 shows, in this case BSI was the most abundant infection class with 7 isolates (50%) followed by SUP with 4 isolates (29%) and DEEP with 3 isolates (21%).

Table 1. FCA-resistant isolates distribution according to species and infection type

Group	Species	BSI	DEEP	SUP	Total
Candida spp.	*C. albicans*	1	–	3	4
	C. famata	–	1	–	1
	C. glabrata	4	2	–	6
	C. haemulonii	1	–	–	1
	C. inconspicua	–	–	2	2
	C. krusei	5	5	17	27
	C. lambica	–	–	1	1
	C. norvegiensis	–	–	1	1
	C. parapsilosis	1	–	–	1
	C. pelliculosa	1	–	–	1
	C. robusta	2	–	1	3
	C. rugosa	–	–	2	2
	C. tropicalis	1	1	1	3
Non-*Candida* spp.	*Geotrichum candidum*	–	–	1	1
	Rhodotorula mucilaginosa	1	–	–	1
	Trichosporon asahii	1	–	–	1
	Trichosporon moniliiforme	–	–	1	1

The *Candida* sp. group was again dominant with 11 isolates (6 BSI, 3 DEEP and 2 SUP) while the non-*Candida* group had only one isolate (SUP). The distribution of VOR-resistant isolates according to species and infection type is presented in table 2.

In general, MXP-4509 had a better activity than VOR against *Candida* spp. FCA-resistant isolates (P=0.0058) (Fig. 3) as well as against all FCA-resistant isolates (P=0.0047).

Table 2. VOR-resistant isolates distribution according to species and infection type

Group	Species	BSI	DEEP	SUP	Total
Candida spp.	*C. albicans*	1	–	1	2
	C. famata	–	1	–	1
	C. glabrata	2	1	–	4
	C. haemulonii	1	–	–	1
	C. rugosa	–	–	1	1
	C. tropicalis	1	1	1	3
Non-Candida spp.	*Rhodotorula mucilaginosa*	1	–	–	1

Since *C. krusei* isolates account for almost half of all the resistant isolates, it made sense to separate them and then analyse two resulting groups, i.e. *C. krusei* and *C.* non-*krusei*. In this case, the superiority of MXP-4509 became even more obvious in the *C.* non-*krusei* group (P=0.0039) (Fig. 4). In the *C. krusei* group, however, MXP-4509 had a slightly inferior but still comparable activity to that of VOR (P=0.041).

MXP-4509 showed a very good antifungal activity against VOR-resistant isolates (Fig. 5) and it seems to be especially efficient against *C. glabrata* isolates, but the results were not statistically significant (P=0.1133 for VOR-resistant isolates and P=0.0625 for *C. glabrata* isolates).

The P values, show the need for further research in both cases. The same can be said about *C. krusei* where the P value, although it shows statistical significance, is very close to the 0.05 threshold.

Fig. 3 Differences between MXP and VOR MICs for *Candida* spp. FCA-resistant isolates

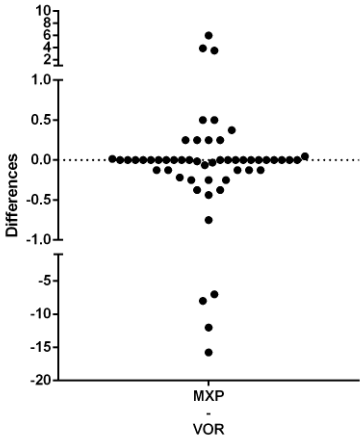
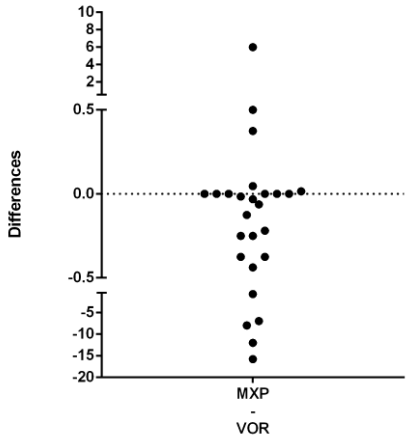


Fig. 4 Differences between MXP and VOR MICs for *C.* non-*krusei* FCA-resistant isolates



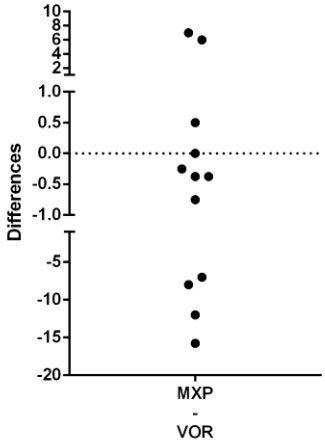


Fig. 5 Differences between MXP and VOR MICs for all VOR-resistant isolates

Conclusions

1. 57 FCA resistant yeasts isolated from three infection types (blood stream infections, deep seated infections and superficial infections) were tested
2. 52.63% of the FCA-resistant isolates came from superficial infections, 31.58% came from blood stream infections, and 15.79% came from deep seated infections
3. *C. krusei* accounted for almost half of all the FCA-resistant isolates
4. 12 of the 57 FCA-resistant isolates also showed VOR resistance
5. In general, MXP 4509 had a better activity than VOR against FCA-resistant isolates (P=0.0047)
6. Against *C. krusei*, MXP 4509 had a slightly inferior but still comparable activity to that of VOR (P=0.041)
7. MXP-4509 appeared to be very efficient against *C. glabrata*, but more data for further research is necessary.

Acknowledgements

This research was financially supported by the Ministry of National Education from Romania - CNCSIS-UEFISCDI, project numbers **PN II-RU 159/2010** - "Evaluation of the antifungal effect of nanoconjugates of a new propiconazole derivative with β-ciclodextrin" and **PN-II-ID-PCCE-2011-2-0028** - "Biologically inspired systems for engineered structural and functional entities"

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EPIDEMIOLOGICAL INVESTIGATIONS ON ANTIBODIES AGAINST BHV-1 IN FARMS FROM VASLUI COUNTY

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Abstract

Infectious bovine rhinotracheitis is a highly contagious infectious disease that affects both cattle and buffalos. The infection is produce by bovine herpesvirus type 1 (BHV-1) which belongs to the family Herpesviridae. Infectious bovine rhinotracheitis (IBR) is prevalent worldwide, and its epidemiology varies from sporadic to enzootic evolution in many countries in Europe, America, Asia, Africa and Australia, particularly in countries with intensive cattle farming. According to the OIE, infectious bovine rhinotracheitis is widespread throughout the world, but had been eradicated in some countries such as Austria, Denmark, Finland, Sweden, Italy (province of Bolzano), Switzerland and Norway, and control programs are implemented in countries such as Australia, Belgium, Canada, India, Poland, Turkey and USA. Epidemiological investigations have performed in two years 2011 and 2013 in Vaslui County. In the study were included six farms, with 227 animals tested. During this study, in the studied farms has not been made specific immunization against infection with BHV-1. All 227 cattle were serologically tested using HerdCheck IDEXX IBR gB ELISA kit, which reveal the presence of antibodies anti-gB of BHV-1. Of the 227 samples tested, 203 bovine had detected positive for anti BHV-1 antibodies, representing a seroprevalence of 89.42%.

Key words: infectious bovine rhinotracheitis, ELISA, seroprevalence

Introduction

The BHV1 infection is a specific type of viral disease in cattle which is caused by the Bovine Herpes Virus type 1 (BHV1). It manifests itself in many different ways, predominantly as an acute feverish respiratory disease, as well as in the sexual organs of both male and femail animals, frequently as a symptomless infection without any clear signs of disease.

Seasonal incidence is due to the management practice of assembling feedlot cattle rather than due to any true seasonal variance. In Europe, autumn and early winter are usually associated with a higher incidence of infectious respiratory diseases in calves where BHV-1 can be one of the etiological agents (Nuotio L., 2007).

The bovine herpesvirus type 1 (BHV-1) belongs to the subfamily *Alphaherpesvirinae* and is an important pathogen of cattle (Wyler et al., 1989). After the virus has entered the host through the membranes in the airways (droplet infection) or through the genital membranes it replicates itself and this leads to a general infection. If cattle form specific antibodies within 10 to 14 days then the virus can be forced back into the interior of the body (nerve cells), where it remains without reaction (M. Ackermann, R. Wyler, 1984). This is the reason why an animal which is infected once must be regared as a lifelong carrier of the virus. The latent infection may be reactivated periodically (e.g. changing stalls, transportation, giving birth), with or without clinical signs; the virus is transported back to the site of entry and is shed with potential transmission to other animals. Most, if not all, seropositive animals are latently infected and virus shedding can be reactivated following stress or corticosteroid treatment. Viremia is rarely detected, but does occur. Although a strong immune response is provoked during primary viral replication, these mechanisms help the herpesviruses to escape from immune surveillance during latency and to a lesser degree during reactivation.

BHV-1 infection is commonly diagnosed by detection of the host response to the virus (for example, antibodies in serum) or by direct detection of the agent. Serological tests are frequently used for the detection of BHV-1 infection. The types of serological tests commonly used for testing for BHV-1 antibody are: virus neutralisation (VN) test and ELISA.

Antibodies are detected in the serum of most animals within 2–3 weeks of infection. Maternally-derived antibodies may be detected for up to 7 months (Florent G., 1986), but usually disappear in about 4–5 months (Graham D.A., 1997). There is no known way of distinguishing passively transferred antibodies from those resulting from active infection.

A number of ELISAs have been developed and are now used extensively for certification where the antibody status of a large number of animals needs to be determined. A number of commercial ELISA kits are available. Most have very similar sensitivity and specificity when testing samples from individual animals. Some kits are designed to be used in a screening test format whereby any positive samples should be retested in a confirmatory test of a different format. The screening tests have all wells coated with viral antigen while the confirmatory tests have alternate wells coated with a 'negative control' antigen (usually an uninfected cell culture extract) or a viral antigen. Glycoprotein B-ELISAs are generally more sensitive and also highly specific. An alternative confirmatory test can be a monoclonal antibody based blocking ELISA.

Europe has a long history of fighting against BHV-1 infections (Ackermann et al., 1990a; Ackermann et al., 1990b). The first IBR outbreaks in Europe were observed in the 1970s (Metzler et al., 1985). Since that time most European countries reacted with a variety of control programs, the extent of which depended on economical considerations and interests. However, only a small number of countries have achieved the goal of IBR-eradication. The infection is endemic, worldwide in the cattle population. It not present in countries such as Austria, Denmark, Sweden, Bozen, Bavaria which have eradication programs as specified under article 10 of 64/432EWG.

The main objectives of our study were to estimate the herd level BHV1 antibodies among dairy cattle in farms from Vaslui County.

Materials and Methods

The study population that was used to estimate the prevalence of BHV1 anttibodies using an imunoessay method consisted of 8 farms. The survey was conducted during two years 2011 and 2013. In 2011 were colected and tested 168 bovine serums and in 2013 were tested 59 samples. In each of the selected herds a representative random sample of cows and youngstock older than 6 months was tested for BHV1 antibodies in serum. Calculations to determine the sample size used the actual number of the animals present in a farm.

Blood samples were collected from the mammary vein into 9 ml vacuum tubes containing a clotting activator, using disposable needles (0.9 mm × 38 mm). Serum samples were stored at room temperature for 24 h before the serums was collected. Serum was separated by centrifugation of blood at 3000 g for 10 min at room temperature; the aliquots were transferred into 1.5 µl sterile microtube and were kept at -20°C until analysis.

The serum samples were analysed for BHV-1 antibodies using a commercial HerdChek* IBR gB ELISA test kit (IDEXX). The experiment was carried out according to the kit protocol. OD of samples and controls were measured at 450nm by using ELISA reader (Tecan Sunrise, Switzerland) and recorded using a computer.

According to test instructions serum sample was considered to be "negative" if the blocking percentage was less than 45%, "suspect" between 45 and 55% and "positive" when over 55% (IDEXX).

Results and Discussion

The results of the present study demonstrate that the prevalence of antibodies to BHV1
on dairy farms in Vaslui County in both years (2011 and 2013). During the study in all farms have not been made vaccinations campaigns against BHV-1 infection.

The results of BHV-1 were plotted based on the ELISA-BHV-1 test was determined by using 277 sera. Six herds out of eight had at least one sero-positive animal. Of the 227 samples tested were found to be serologically positive 203 bovine sera, representing a prevalence of 89.43%. In five farms, representing 181 animals serologically tested, were identified antibodies against gB of BHV-1 in 100% of the animals studied.

In farm MF1 Bârlad, of 8 animals tested serologically, only one was identified with dubious response (12.5%), the remaining seven sera were negative for antibodies to gB of BHV-1. The farm F5, from Gura Idrici, all 14 samples serologically tested had identified as negative for antibodies to IBR.

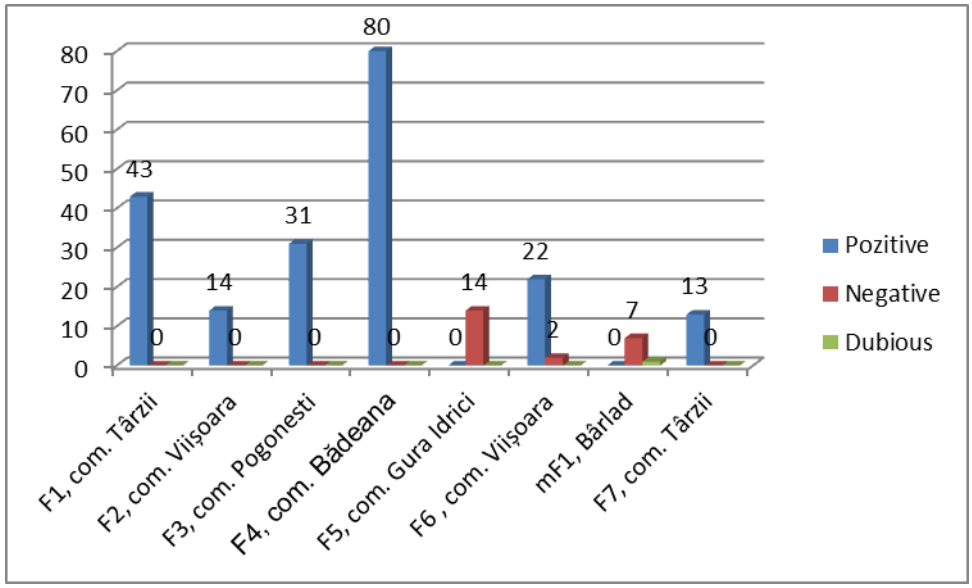

Fig. 1 Graphical representation of serologic results on the eight farms in Vaslui County

Type immune response is represented by the production of neutralizing antibodies by B lymphocytes. Antibodies can be detected in approximately 8-12 days after infection. First appears immunoglobulin M excretion that has a peak and then decreases during one month. IgA occurs several days later together with an increase and a decrease in excretion during a month. The IgG have a maximum of secretion in almost a month and the level still remains stable.

The presence of a large number of animals identified as seropositive for IBR, reveals the existence of subclinical infections. BHV-1 is able to remain latent, escaping the action of the immune response and animal organisms may persist for a long time so this form, possibly lifelong animal (Winkler et al 2000). The virus can remain latent after primary infection, a reinfection or after vaccination with a live attenuated vaccine (Nandi et al 2009).

Infection of cattle with BHV-1 impairs resistance to secondary bacterial infection such as *Mycoplasma haemolytica*, *Pasteurella multocida* and *Histophilus somnis* leading to fatality and depression of cell mediated immunity (Leite et al 2002).

The lack of a specific prevention program for infection with BHV-1, allow us to affirm that seropositivity of farm animals tested may be a consequence of the purchase of animals from herds that have either been previously vaccinated against infectious bovine rhinotracheitis virus or were naturally infected with BHV-1 and remained latent carriers of the virus.

These cattle can be incriminated in the spread of infectious bovine rhinotracheitis virus in the herd, knowing that the movement of animals from a herd to another as well as transportation itself is a major stress factors leading to reactivation of the latent virus. These animals do not show clinical signs but can eliminate increased quantities of virus infecting receptive animals from the herd.

Conclusions

The results of this study highlight that farmers should take into account the immune status of cattle related to BHV-1 before their introduction in herd.

The lack of a specific prevention program for bovine respiratory viral diseases can allow us to affirm that the percentage of seropositivity (89.43%) is a consequence of persistent latent infections clinically undetectable.

In genetically valuable herds that intend to market youth for reproduction, should be used deleted vaccine, being possible the differentiation between vaccinated and naturally infected animals.

Acknowledgment

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DENTAL MALOCCLUSION IN GUINEA PIG - CASE REPORT

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USAMV Iassy, Faculty of Veterinary Medicine**,** 8, Mihail Sadoveanu Alley

Abstract
Dental malocclusion represents a relatively common disease in companion rodents, pathology very difficult to treat. Pathology severity varies between individual of same species or from one species to other, (Reiter M. Alexander, 2008).The teeth grow curved, excessively, restraining animal from feeding. Causes of malocclusion are congenital disorders and feeding errors. Specific to rodents are two pairs of incisors on mandibula and maxillary, teeth with continuous growth, curved posteriorly with enamel only on anterior surface. They do not possess canines or premolars.This article is a case report of malocclusion in Guiney pig and its remedy. The treatment purpose was to reestablish the normal function of the dental arches, readjusting the teeth length and occlusion with a dental turbine.

Key words: *guinea pig, malocclusion, rodent*

Introduction

Guinea pig presents a feature generally common in all rodents (rabbit, capybara, chinchilla), in which the upper and lower incisors (frontal teeth, up and down) as well as the other teeth (totally of 20), grow continuously throughout the life. This is justified in rodents living in the wild environment, because they use their teeth to crunch, to smash, to mince andd to break twigs, fruit seeds, peanut shells and nuts, (Okuda A., and all., 2007). The rodents need a constant regeneration of the teeth, that otherwise would fret so much that they become unusable.

In Guinea pigs kept in captivity, this feature is preserved, but due to the life conditions, that sometimes are quite different from those of animals living in their natural environment, the teeth can grow excessively, putting in danger their correct feeding, and sometimes their own lifes.

This paper reports a case of dental malocclusion in guinea pig, raised as a pet.

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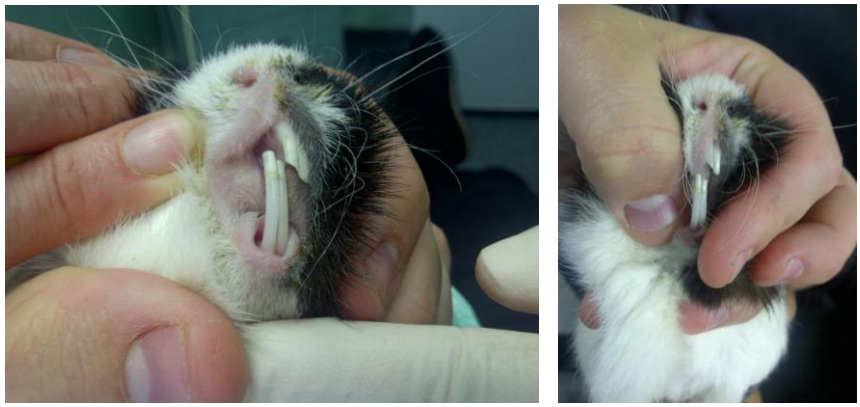
Materials and Methods

To the Infectious Diseases and Preventive Medicine Clinic, from the Faculty of Veterinary Medicine, Iasi, it was admitted for consultation a guinea pig, four years old, male, with the following clinical sings: excessive growh of teeth (lower incisors) and unequal fretted incisors (figure 1 A, B).

The animal suffered also a drop of the weight, weighing 500-600g, the appetite was absent for 2 weeks, with excessive tearing, dehydration and abundant salivation with gritting of teeth.

After observing the incisors changes, it was performed their shortening using a dental turbine.



A **B**

Fig. 1. Excessive growh and unequal fretted incisors

Results and discussion

Using the dental turbine, both upper and lower incisors were shorten and straighten, reaching the normal size (figure 2).

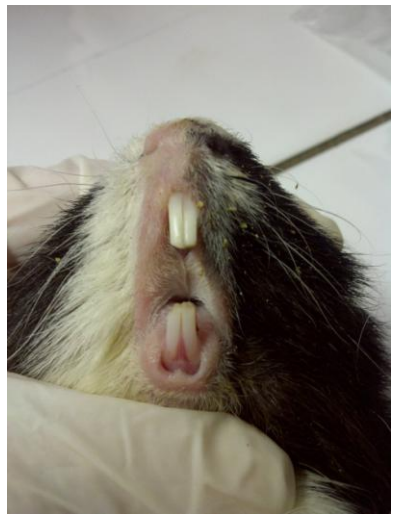


Fig. 2. Incisors appearance after shortening

After this workmanship it was observed that the animal could not shut his mouth, being noted a defective consolidation with the ankylosis of the temporomandibular joint and a masseter muscle atrophy (figure 3).

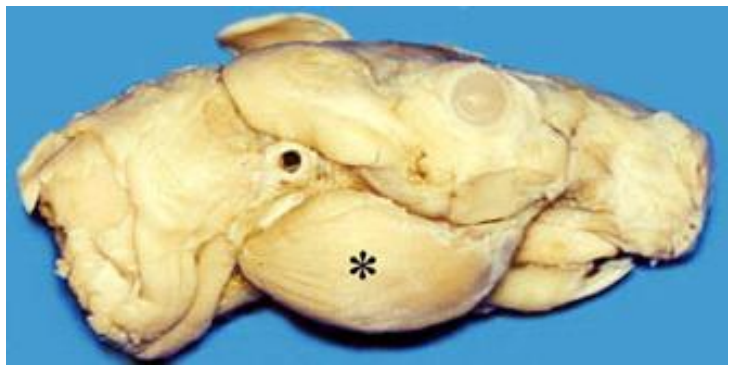


Fig. 3. Normal masseter muscle in guinea pig
(After Legendre L.F.J.)

After 24 hours, the guinea pig slowly began to shut his mouth, but because of the illness chronicity and of the complications occurred during the long evolution (weight loss, dehydration, joint and muscular irreversible modifications), the animal could not be recovered.

In guinea pigs, the excessive incisors growth is due to the low fiber diet, but sometimes there can exist a genetic predisposition, and even if is getting enough hay and root, the growth rate of the incisors is greater than the natural ability of the blunting. In such situations it is advisable the veterinarian intervention for a regular tooth shortening, (Perez de Freitas Elisângela and all., 2012).

If the pig is not genetically predisposed to an excessive growth of the teeth, a daily diet rich in food containing fiber, will help the animal to naturally blunt his teeth.

In the cases when the animal is not having the dental arches in contact, is manufactured an elastic band that supports the jaw (figure 4), which forces the teeth to come into contact. This band relaxes muscles and helps them recover (figure 5).

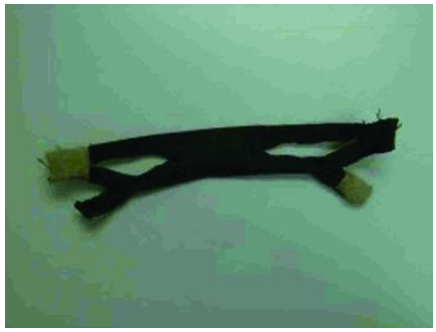


Fig. 4. Elastic band for jaw support (after **Legendre L.F.J.)**

Fig. 5. Mounting the elastic band for the masseter muscle recovery (after **Legendre L.F.J.)**

In case of dental anomalies suspicion, in order to obtain a correct diagnosis, in addition to a thorough medical examination, is recommended an X-ray examination or MRI scan.

Conclusions

There should be considered all the anatomical and raising features of the guinea pig, when is raised as pet.

The type of food consumed has an influence on the teeth blunting and in keeping them in good condition. To prevent the occurrence of excessive teeth growth, is recommended a diet rich in fiber and ensuring hay ad libitum.

If the incisors get longer, they must be regularly shorten; when the genetic side is criminalized, the animals will not be used for reproduction.

The specimens that suffer periodic episodes of dental malocclusion are recommended the euthanasia.

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DETECTION OF BOVINE PAPILLOMAVIRUS TYPE 1 IN CUTANEOUS FIBROPAPILLOMAS IN CATTLE

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Abstract

Bovine papillomavirus type 1 is associated with the development of hyper-proliferative lesions of the epithelial and dermal cells, in bovids and as well in equids. In cattle,the consequence of the infection with this virus is the occurrence of cutaneous fibropapillomas. The aim of this study was to detect by PCR the presence of BPV 1 in cutaneous fibropapillomas and normal skin in cattle. Four normal skin and ten bovine cutaneous fibropapillomas were tested by PCR using primers for the amplification of BPV-1 L1 gene. BPV-1 L1 DNA was amplified in all tested fibropapilloma samples (100%) and in three out of four normal skin samples. Consecutively, the sequencing of the amplified PCR product confirmed the presence of BPV-1 in the tested samples. These results are confirming the importance of the BPV-L1 gene in the detection of the BPV positive samples and the widespread of the BPV, even in apparently healthy cattle.

Key words: *Bovine fibropapilloma; BPV-1; PCR*

Introduction

Papillomaviruses (PV) are classified in *Papillomaviridae* family, divided in 16 genera. Generally, these viruses are causing hyper-proliferative lesions of the epithelial and dermal cells in humans and animals (5). Specifically, the lesions induced by PV are benign and usually with spontaneously regressing, although some lesions can undergo neoplastic transformation under the influence of environmental co-factors, such as consumption of the fern (3). PV are species-specific, the only case of cross-infection is noted on Bovine papillomasvirus (BPV) type 1 and 2, which can infect the horses, mules and donkeys (7). BPV-1 and -2 are classified in the *Deltapapillomavirus* genera along with BPV-13 (6) and are causing cutaneous and mucosal fibropapillomas in cattle (4). The genome of BPV-1 and -2 contains a double stranded DNA with 7900 bp, divided in early (E1, E2, E4, E5, E6, E7 genes) and late genes (L1 and L2) (8).

In cattle, the consequence of the infection with bovine papillomaviruses is the occurrence of cutaneous warts, which can be responsible for significant economic damages due to the retarded growth of the animals, loss of weight and decrease in milk production. Although bovine cutaneous warts are occurring in cattle of different age and breed from Romania, there are no studies concerning the detection of the BPVs involved in the occurrence of the bovine cutaneous fibropapillomas.

The aim of this study was to detect the presence of BPV-1 DNA in cutaneous fibropapillomas and normal skin in cattle by PCR.

Materials and methods

Four normal skin samples and ten bovine cutaneous fibropapillomas were collected from cows of different breeds and ranging in age between 6 months and two years. The samples were immediately snap frozen in liquid azote and then conditionated at -80^{o} C until the molecular analysis. The DNA was extracted from the fibropapillomas F1-F10 and from skin samples S1-S4 using the Dneasy Blood and Tissue kit (Qiagen), according to manufacturer instructions. PCR for the detection of BPV- 1 was performed using a single set of primers for the detection of BPV -1 L1 gene, nucleotides 5721-6021. The amplification was conducted in a final volume of 50 μL, containing 10 μL of sample DNA, 3 mM $MgCl_2$, 0.02 U/μL Platinum Taq (Invitrogen), 0.5 pmol/μL each oligonucleotide primer, 200 μM each dNTP (F – 5' GGA GCG CCT GCT AAC TAT AGG 3' and R – 5' ATC TGT TGT TTG GGT GGT GAC 3'). For BPV-1 L1 gene, the reaction conditions were denaturation for 3 minutes at 95 ^{0}C, followed by 35 cycles of denaturation at 94 ^{0}C for 40 seconds, annealing at 68 ^{0}C for 40 seconds and extension at 72 ^{0}C for 1 minute. Subsequently, the PCR products were separated by electrophoresis in 2% agarose gels with Tris acetate ethylene diamine tetraacetic acid (EDTA) buffer (TAE; 40 mM Tris, 1 mM Na_2EDTA, 20 mM acetic acid), at a constant voltage (100 V) for approximately 35 minutes, then stained with ethidium bromide and visualised under ultraviolet light.

The PCR products were purified using the Wizard SV Gel and PCR Clean-Up System Kit (Promega) and directly sequenced using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Results and discussions

BPV-1 L1 DNA was amplified in all tested fibropapilloma samples (100%) and in three out of four normal skin samples (figure 1). Consecutively, the sequencing of the amplified PCR product confirmed the presence of BPV-1 in the tested samples. The score of the samples positivity is presented in table 1.

In accordance with other authors, in this study was identified the presence of BPV-1 DNA both in normal skin samples collected from healthy cows and fibropapillomas.

In accordance with other authors (9, 10), in this study was identified the presence of BPV-1 DNA both in normal skin samples collected from healthy cows and fibropapillomas. Using these type specific set of primers, PCR assay may be a successful method to detect BPV DNA, but its sensibility and specificity may be affected by the concentration and the purity of sample DNA. Moreover, the DNA of human papillomavirus was detected by PCR assay in healthy skin, suggesting a subclinical or latent infection with HPVs (1, 2).

Table 1. Score of the samples positivity

Samples	BPV-1 L1
F1	+
F2	+
F3	+
F4	+
F5	+
F6	+
F7	+
F8	+
F9	+
F10	+
S1	+
S2	+
S3	+
S4	-

Legend: F1-F10 fibropapilloma samples;
S1-S4 normal skin samples;
+ Positive samples; - negative samples.

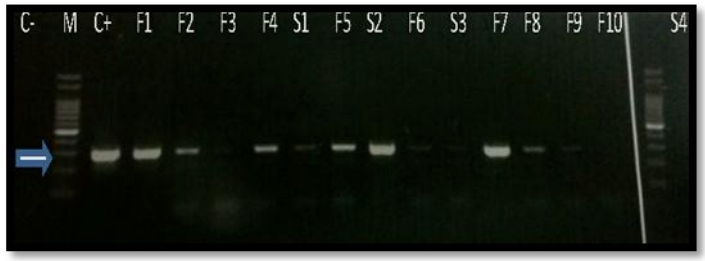

Fig. 1. The amplified fragment of BPV-1 L1 gene and the sequence of 300 bp. C-: negative control; C+: positive control; F1-F10: fibropapillomas; S1-S4: normal skin

Conclusions

These results confirm the efficacy of PCR targeting BPV-1 L1 gene and reveals the importance of BPV epidemiological studies in apparently healthy and papillomatosis-affected cattle to understand the spreading of this virus even in apparently healthy cattle.

Acknowledgments

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SEROLOGICAL REACTIVITY TO *RICKETTSIA CONORII*, THE ETIOLOGIC AGENT OF MEDITERRANEAN SPOTTED FEVER IN DOGS FROM ROMANIA

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Abstract

The Rickettsiae is a group of bacteria that causes diseases in both animals and humans. Rickettsia conorii is the predominant etiologic agent of Mediterranean spotted fever (MSF), and it is reported to be transmitted by Rhipicephalus sanguineus, the brown dog tick. Rhipicephalus sanguineus is the main vector and reservoir of Rickettsia conorii, and dogs are the principal host in the life cycle of this ticks species. In the areas where MSF is endemic, dogs have high prevalence (26% - 60%) of Rickettsia spp. – neutralizing antibodies and proximity to these seroreactive dogs represents a risk factor for MSF in humans. The aim of the present study is to assess the seroreactivity against Rickettsia conorii in dogs from 3 counties of Romania. A total of 92 samples from healty dogs were analyzed by ELISA (Euroclone Spa – Life Sciences Division, Italy). The imunoenzimatic test revealed that 40 (43.47%) of the 92 dogs had IgG antibodies reactive with Rickettsia conorii. This is the first serological survey on antibodies to Rickettsia conorii in Romanian dogs.

Key words: *Mediterranean spotted fever, Boutonneuse fever, Rickettsia conorii, dogs, ELISA*

Introduction

Mediterranean spotted fever (MSF), also called Marseille fever or Boutonneuse fever is a zoonosis caused by *Rickettsia conorii*, an obligately intracellular, slow-growing Gram-negative bacterium, transmitted by the bites of the brown dog tick *Rhipicephalus sanguineus* (Raoult D. and Roux V., 1997). Although this tick can feed on a variety of mammalian and even avian animals, it is most closely associated with canines and primarily with the domestic dog, *Canis familiaris* (Levin M.L. et al., 2012). For that reason alone, dogs stand to play an important role in the epidemiology of MSF. Geographically, *Rickettsia conorii* is widely distributed and can be found in southern Europe, Africa, the Middle East, and India (Walker D.H. and Raoult D., 1995).

Mediterranean spotted fever in humans beings is an acute disease, characterized by a short onset of fever, maculo-papular erythematous rash usually involving palms and soles (Fig.1), inoculation eschar `tache noire` (Fig.2) at the tick-bite site and myalgias (Raoult D. and Roux V., 1997). Severe forms may include multiorgan involvement, major neurological manifestations, and can be fatal (Amaro M. et al., 2003). In dogs illness has been associated with *R. conorii* natural infection in only 2 dogs since human MSF was described in 1932. Clinical signs observed in experimentally infected dogs were pain, erythema and edema at the incoulation site, and regional lymphadenopathy (Kelly P.J. et al., 1992).

In Romania, Mediterranean spotted fever has been reported since 1948 and it is endemic in the southern region of the country. Between May and October 2009, a serological survey was conducted on 300 people from 3 counties from the south of Romania. The highest

seropositivity was recorded in Constanța (32%) followed by Tulcea (21.1%) and Bucureşti (18.2%), (CNCSBT).

The aim of this study was to investigate the prevalence of *Rickettsia conorii* antibodies in the blood of healthy dogs from three counties in Romania where human clinical cases have been diagnosed and to look for a possible association between human and canine exposure.

Materials and methods

A total of 92 dogs with no clinical disease were examined for MSF from November 2010 (n=12, Tulcea) to September – October 2011 (n=80, Tulcea, Buzau, Braila). Of the 92 dogs, 47 were male and 45 female. Ages ranged from 3 month to 10 years. For each dog, data concerning the dog characteristics were recorded. Blood samples were collected from common breed dogs from shelters (Tulcea, Buzău) and private homes (Brăila). Samples were collected in sterile tubes without anticoagulant. Serum was then separated after centrifugation and stored at -20^0C.

All dogs serum samples were analyzed for anti-*Rickettsia conorii* antibodies using a commercial enzyme-linked immunosorbent assay (Rickettsia conorii Canine IgG – ELISA; (Euroclone Spa – Life Sciences Division, Italy), according to the manufacturer`s instructions, with sera diluted 1:100. Absorbance values were measured using a MicroplateReader (Bio-Rad) at 450 nm wavelength. Interpretation was done by comparing both: the absorbance values of the samples to that of the provided cutoff and the final color reaction with positive and negative controls.

Results and discussions

The imunoenzimatic test revealed that 40 (43.47%) of the 92 dogs had IgG antibodies reactive with *R. conorii*. The highest seroprevalence was recorded in Buzău county 47.5% (19/40), followed by Tulcea county 46.87% (15/32) and Brăila county 30% (6/20).

The high seroprevalence of dogs (43.47%) in this study indicates that dogs were heavily exposed to *R. conorii*. Seroprevalence recorded in our study was higher than figures reported in Sardinia (26.1%) (Segura-Porta F. et al., 1998) and in Portugal (38.5%) (Levin M.L. et al., 2012), but lower than values from Israel (81%) (Harrus S. et al., 2007). A significant difference in seroprevalence was found between Buzău and Brăila. This may reflect difference in the abundance of tick vectors or variations in the infection rates of ticks.

The rate infection in males 46.8% (22/47) was greater as the rate in females 40% (18/45). It has been suggested that male dogs and men may be at increased risk for infection and may develop more severe illness with *R. rickettsii* and *R. conorii* (Solano-Gallego L. et al., 2008).

The highest prevalence, 48.3% was recorded in age group ``> 5 years`` (Table 1).

Serological studies, conducted in various countries endemic for MSF, have demonstrated a correlation between human disease and *R. conorii* antibody prevalence in canines (Levin M.L. et al., 2012). Seroprevalence of dogs is considered a good marker of infection with *R. conorii*. Dogs have a very short rickettsaemia and do not present clinical signs of disease, - infection is only detected by humoral immune response. Although the main epidemiological role of dogs is probably as carriers of ticks, they can occasionally act as a reservoir of the infection (Segura-Porta F. et al., 1998). Owing to high levels of *Rh.*

sanguineus exposure, dogs have been used in epidemiological studies as sentinels for human MSF and proximity to seroreactive dogs is a risk factor for MSF in humans (Solano-Gallego L. et al., 2008).

Table 1. Distribution of Seroreactive Dogs Regarding Age Group

Age / years	Tulcea (n%)	Brăila (n%)	Buzău (n%)	Total (n%)
0 - 1	8 / 17 (47.1)	1 / 4 (25)	2 / 6 (33.3)	11 / 27 (40.7)
2 - 4	3 / 8 (37.5)	3 / 8 (37.5)	9 / 20 (45)	15 / 36 (41.6)
> 5	4 / 7 (57.1)	2 / 8 (25)	8 / 14 (57.1)	14 / 29 (48.3)

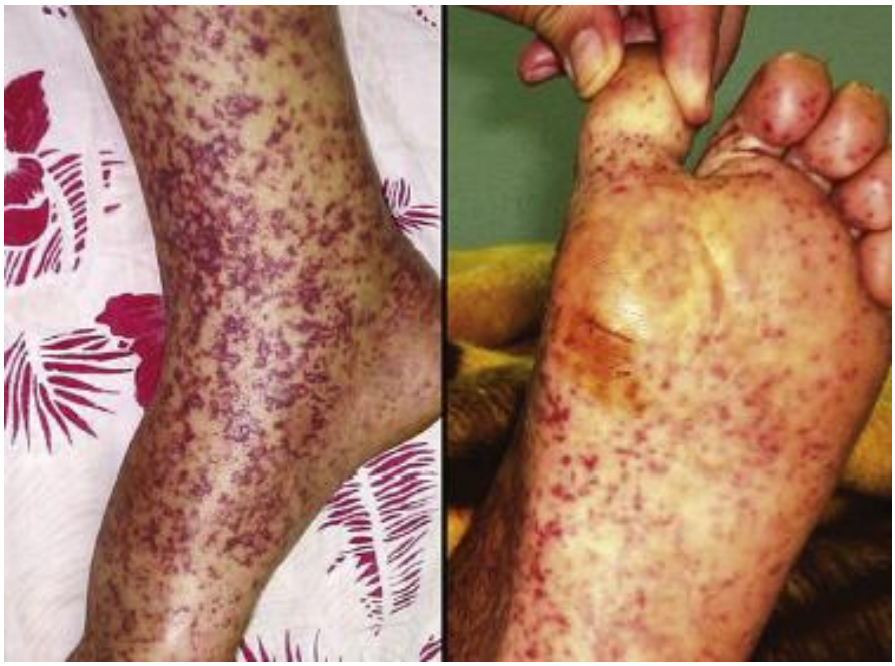

Fig. 1. Purpuric rash with vasculitis in a severe form of Mediterranean spotted fever (MSF) caused by Rickettsia conorii in Oran, Algeria
(Mouffck N. et al., 2009)

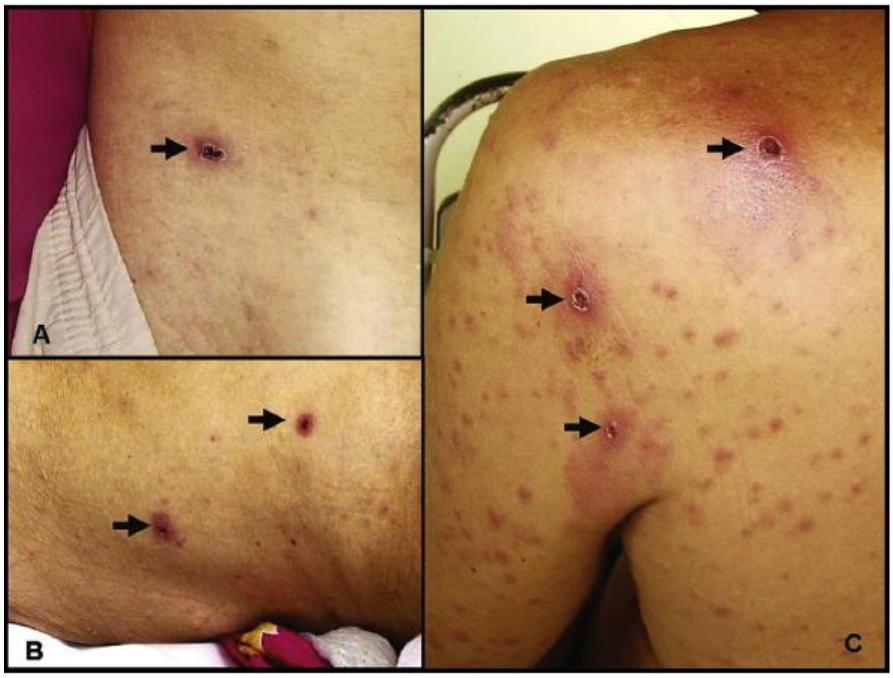

Fig. 2. Inoculation eschars (tache noire) in three Mediterranean spotted fever. (A) Single eschar, the most common feature in MSF; (B) two eschars; (C) three eschars, a rare finding in MSF, accompanied with maculopapular rash
(Mouffck N. et al., 2009)

Conclussions

Of the 92 serum samples from 3 counties, 40 (43.47%) samples have been identified as seropositive for *Rickettsia conorii* infection.

Our results suggest that canine serology could be a useful and sensitive indicator for the presence and extent of MSF in endemic regions.

Because the major route of infection with *R. conorii* is transmission by a vector tick, the presence of infected ticks in the vicinity of humans is probably the most important risk factor related to human infection. This must be borne in mind by public health officials and veterianry practitiones when advising dog owners on the importance of prophylactic treatments against ticks.

Futher studies are needed to investigate the risk factors regarding infection with *R. conorii* in humans and dogs.

Acknowledgments

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OXIDATIVE STRESS INDUCES THE PRODUCTION OF SPECIFIC MICROPARTICLES AND PROMOTES CELL DYSFUNCTION IN EXOCRINE PANCREAS

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Abstract

One of the major factors leading to pancreatic exocrine dysfunction is oxidative stress. The metabolism efficiency is essential for cell response. CFTR is a transmembrane protein found in many epithelial tissues and is responsible for the chloride ion exchange across the plasma membrane. This function is crucial for the cell balance. Microparticles (MPs) are cell membrane fragments released by stimulated or apoptotic cells. They represent veritable markers for cell alteration, also acting as pro-inflammatory mediators. To study the effect of oxidative stress on exocrine pancreas and to explore the properties of exocrine cell-derived MPs in two epithelial cell line models (normal or CFTR deficient). Microparticles were produced from two exocrine cell lines, PANC-1 (normal) and CFPAC-1 (CFTR deficient) respectively after H2O2 treatment. The two-type MP effects were compared into an autologous cell communication model. The cell response to autocrin action was assessed by hypodiploid DNA measurement and cell viability. Pharmacological modulation was achieved by Cyclosporine (CsA) and Vitamin C (VitC). CFPAC-1 cells were characterized by an increased susceptibility to oxidative stress compared to normal PANC-1, doubled by the release of deleterious MPs. Pharmacological modulation by CsA and VitC confirmed the MP-mediated dissemination of inflammatory signal in an autologous manner. Oxidative stress may promote exocrine dysfunction and the liberation of deleterious MPs. CFTR protein plays a key role for the cell stability and it could be involved in the defense activity of epithelial cells against oxidative stress.

Keywords: *Oxidative stress, CFTR, Microparticles, Pancreatic dysfunction*

Introduction

Le stress oxydant e la dysfonction cellulaire

Dans une cellule eucaryote normale, l'énergie nécessaire à son fonctionnement est produite de façon aérobie par des réactions d'oxydo-réduction, entre oxydants ou accepteurs d'électrons, et réducteurs ou donneurs d'électrons. La chaine respiratoire de la mitochondrie, fournit ainsi 90% de l'énergie nécessaire (Rolfe and Brown, 1997). Dans cet organite intracellulaire, l'oxygène est l'accepteur final d'électron après une cascade de réactions

d'oxydo-réduction, faisant intervenir quatre complexes protéiques. Lorsque l'oxygène est transformé en molécule d'eau, cela permet de générer de l'ATP, molécule à haut potentiel énergétique. Cependant 2 à 3% de l'oxygène n'est pas réduit en eau : il forme des radicaux libres ou des espèces dérivées de l'oxygène très réactives, espèces chimiques possédant un électron libre non apparié sur la dernière couche électronique (Koppenol, 2001). L'équilibre redox est maintenu par de nombreux systèmes antioxydants (Sies, 1991). Un déséquilibre entre molécules pro-oxydantes et antioxydantes, en faveur des entités oxydantes, conduit au stress oxydant. Il peut être la cause de système antioxydant défectueux ou d'une quantité d'entités oxydantes produites trop importante. Les entités oxydantes étant très réactives, elles réagissent avec les premières molécules en contact. Elles ont comme cibles potentiels : les lipides, les acides nucléiques, les protéines et les sucres. De façon physiologique, les espèces réactives radicalaires (OH^-) ou non (H_2O_2), existent dans les cellules et dans les tissus à des concentrations faibles mais mesurables (Halliwell and Gutteridge, 1986; Sies, 1993). Elles permettent de maintenir une certaine homéostasie de l'état redox de l'organisme. Lorsqu'elles sont produites dans un compartiment cellulaire spécifique, elles peuvent participer au fonctionnement de certaines enzymes, intervenir dans la défense immunitaire, agir en tant que second messager cellulaire, intervenir dans les voies de transduction du signal et ainsi réguler les fonctions et le devenir cellulaires (Dikalov et al., 2007).

Les microparticules cellulaires

La membrane plasmique constitue une plateforme d'échange bidirectionnelle avec le milieu environnant facilitant par le biais de ses récepteurs, transporteurs et canaux, le maintien de l'homéostasie cellulaire et la régulation autocrine ou paracrine. La membrane au repos présente une asymétrie de répartition des composants lipidiques et protéiques. Les aminophospholipides (phosphatidyléthanolamine et phosphatidylsérine) sont séquestrés dans le feuillet interne. Après activation cellulaire, la membrane plasmique est remaniée (flip-flop), les aminophospholipides sont externalisés et simultanément, le cytosquelette est dégradé. Le feuillet externe déstabilisé par la surcharge phospholipidiques transitoire bourgeonne et libère des microparticules (MP) qui portent alors les caractéristiques de la cellule émettrice : elles exposent la phosphatidylsérine (PhtdSer) et des protéines membranaires identitaires, utilisées pour leur dosage et leur identification. L'émission des MPs par les cellules parentales peut être considérée comme l'expression d'une réponse cellulaire privée à la stimulation ou au stress et sont des indicateurs quantitatifs (Aupeix et al., 1997). Le transfert d'information trans-cellulaire sous la forme des MPs a montré qu'elles sont des effecteurs cellulaires (Morel et al., 2004) capables de transmettre un signal bioloique notamment apoptotique. Les mécanismes moléculaires qui sous-tendent ce transfert sont mal connus.

Le déséquilibre ionique alter la cellule

La protéine CFTR (*Cystic Fibrosis Transmembrane conductance Regulator*) est exprimée à la membrane plasmique des cellules exocrines où elle assure les fonctions d'un canal chlore. Le canal CFTR est exprimé dans de nombreux organes comme le poumon, le foie, le pancréas, le conduit gastro-intestinal et les glandes sudoripares. À l'échelon cellulaire, l'absence de protéine CFTR fonctionnelle dans la membrane se traduit par un déséquilibre ionique modifiant l'équilibre hydrique, expliquant les sécrétions cellulaires épaisses et concentrées (Hubert, 2003).

La réponse apoptotique des cellules et la ciclosporine

En transplantation, la ciclosporine, est un immunosuppresseur qui agit par inhibition de la sécrétion d'IL2 par les lymphocytes immunocompétents. Récemment, la ciclosporine CsA s'est vue attribuer des propriétés anti-apoptotiques sur les cellules neuronales. Elle agirait par inhibition de l'ouverture des pores mitochondriaux responsable de la libération du cytochrome C favorisant l'apoptose. Les effets anti-apoptotiques de la ciclosporine sont suspectés sur l'endothélium vasculaire, et les plaquettes seraient moins sensibles au remodelage membranaire en présence de CsA (Leytin et al., 2009).

Nous faisons l'hypothèse qu'au cours des infections chroniques chez les mammifères, les cellules pancréatiques exocrines qui présentent sont soumises à une oxydation et une réaction apoptotique, menant à la dysfonction pancréatique. Le déséquilibre ionique dans la cellule augmente l'effet délétère.

Materielles et methodes

Culture et numération cellulaire

Les lignées pancréatiques PANC-1, et CFPAC-1, présentant la mutation CFTRΔF508, sont des cellules humaines exocrines et procurées de chez ATCC®. Elles sont cultivées respectivement en DMEM et IMDM complet (Glucose à 4,5 g/l) supplémenté par 10 % de SVF, et contenant 100mg/mL de streptomycine et 100 U/mL de pénicilline.

Les cellules sont cultivées à 37°C sous atmosphère enrichie à 5% en CO_2. Elles sont trypsinées à l'aide de trypsine EDTA à 0,05% (Sigma) lorsque elles sont à pré-confluence (80% de confluence), et réensemencées à 30% de confluence pour les expandre. Le milieu est changé toutes les 48 heures. La numération des cellules vivantes est déterminée par test de viabilité cellulaire à 10% (v/v). Les cellules sont ensemencées à 30% de confluence 48h-72h avant induction, afin qu'elles atteignent 70% de confluence. Après élimination du surnageant de culture, et lavage, l'inducteur de stress est ajouté dans un milieu complet frais.

Stress inducteurs

L'induction cellulaire par un agent oxydant, le peroxyde d'hydrogène (H_2O_2), pendant 20 h est réalisée lorsque les cellules atteignent 70 % de confluence. Un contrôle non stimulé est obtenu par addition d'un volume de solvant ou tampon identique à celui utilisé pour réaliser la solution d'inducteur.

Traitement pharmacologique

Traitement des cellules par la vitamine C

Les cellules ont été traitées à 70% de confluence par la vitamine C (100 µM) et les échantillons récoltés après 20 h d'incubation en présence ou non du stress inducteur.

Traitement des cellules par la CsA

Les cellules PANC-1 et CFPAC-1 (CFTRΔF508) ont été prétraitées à 70% de confluence pendant 4 h par la ciclosporine A (10 µM) et les échantillons récoltés après 20 h d'induction par le stress oxydant. Seule la CsA a été appliquée tout au long de l'expérience en tant que modulateur pharmacologique, contrairement à la vitamine C.

Quantification de l'apoptose par mesure du taux d'ADN hypodiploïde

Les culots cellulaires de cellules adhérentes et détachées obtenus par centrifugation des cellules trypsinisées et du surnageant (200 g, 5minutes) sont rassemblés et les cellules sont perméabilisées par une solution d'éthanol à 70% (v/v) pendant au moins 4 h à 4°C. Après lavage avec du HBSS, les cellules sont traitées par de la RNase de type I-A (10 µg/ml) à 37°C pendant 15 min afin d'éliminer toute trace de RNA cytoplasmique. Après une étape

de lavage en HBSS, les cellules ($5x10^5$ cellules/ml) sont incubées dans l'obscurité en présence d'iodure de propidium (100 µg/ml) 10 min à 22°C. L'iodure de propidium est un intercalant de l'ADN. Le taux d'ADN hypodiploïde est évalué par les basses intensités de fluorescence, il témoigne de la dégradation de l'ADN par les endonucléases activées par le processus apoptotique et est proportionnel au taux d'apoptose cellulaire dans la suspension. L'intensité de fluorescence des cellules est mesurée par cytométrie en flux pour 10000 cellules.

Dosage des microparticules totales et leur phénotypage

Capture des MP procoagulantes

Les cellules apoptotiques ainsi que les débris cellulaires sont éliminées par centrifugation du surnageant. Le système de capture des MP utilise la très haute affinité de l'Annexine-5 pour la PhtdSer (Kd 10^{-10} M) et celle de la streptavidine pour la biotine (Kd 10^{-14} M). Les MPs sont capturées au fond de puits recouverts de streptavidine covalemment fixée (Roche, France), et passivés par incubation d'une solution d'albumine humaine (5g/L) en tampon TBS (Tris 50 mM, NaCl 120 mM, KCl 2.7 mM, $CaCl_2$ 1 mM, pH 7.5). L'Annexine-5 biotinylée (600 ng/mL) est fixée après 1h d'incubation à 37°C. Les MPs émises dans le surnageant sont dosées directement ou après lavage en HBSS et concentration par centrifugation (12000 g, 45 min). Les échantillons contenant les MPs sont déposés (100 µL/puits) et incubés 30 min à 37°C. Après trois lavages, la quantité de MP insolubilisées est mesurée par dosage prothrombinase mis au point au laboratoire.

Quantification des microparticules par test prothrombinase

Dans ce test, le degré d'exposition des phospholipides anioniques à la surface des MPs est le facteur limitant de la réaction d'assemblage du complexe de la coagulation prothrombinase qui conduit à la libération de thrombine soluble révélée à l'aide du substrat chromogénique pNAPEP. Les mesures sont effectuées en mode cinétique, dans un spectrophotomètre thermostaté (VERSAmax, Molecular Device, USA) et en milieu réactionnel standardisé contenant des facteurs de la coagulation d'origine humaine (FII 1.2 µM, FVa 33.3 pM, FXa 11.2 pM, $CaCl_2$ 2.2 mM, 30 min d'incubation à 37°C). Les absorbances sont converties en « équivalent phosphatidylsérine » (éq PhtdSer) par référence à une courbe de calibration obtenue à l'aide d'une suspension de vésicules synthétiques (33% (p/p) de PhtdSer et 67% (p/p) de PhtdChol). La détermination du phénotype des MPs est réalisée en remplaçant l'Annexine-5 par les anticorps d'intérêt.

Test de viabilité cellulaire au Neutral Red

La mesure de la viabilité cellulaire a été faite grâce à un kit Neutral Red (Sigma) : les cellules viables intègrent le colorant (Neutral Red 0,33% dilué dans du tampon salin phosphate de Dulbecco (DPBS) par transport actif et l'incorporent dans les lysosomes. Les cellules sont ensuite fixées (formaldéhyde 0,5% et chlorure de calcium 0,1%), et le colorant initialement incorporé est libéré des cellules par addition d'une solution d'acide acétique 1% diluée dans de l'éthanol 50%. La plaque est lue au spectrophotomètre à 540 nm. Les résultats sont rapportés à 50 000 cellules.

Resultats

Effet du stress oxydant sur les cellules pancreatiques exocrines

L'effet du stress oxydant sur la réponse apoptotique des cellules exocrines a été evalué aprés application du peroxyde d'hydrogene, pendant 20h. Le degré d'apoptose basale est similaire entre les deux lignées car on ne constate aucune différence significative dans les cellules non traitées (Fig. 1). Par contre, les CFPAC-1 montrent une susceptibilité supérieure avec un doublement de la proportion de cellules apoptotiques par rapport à l'apoptose retrouvée dans les cellules PANC-1, dès 50 µM de peroxyde d'hydrogène. La courbe dose-réponse montre que l'apoptose induite est minime dans les cellules PANC-1, et que le plateau est atteint à 75 µM de peroxyde d'hydrogène. La mesure de la viabilité confirme cette observation avec une baisse importante et dose-dépendante dans les CFPAC-1 (Fig. 2).

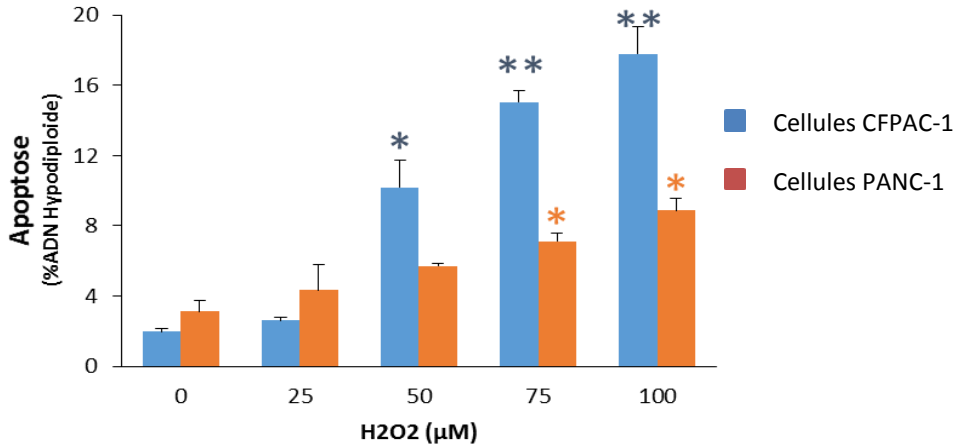

Figure 1: Effet de l'H_2O_2 sur l'induction de l'apoptose des cellules CFPAC-1 et PANC-1.
Les cellules normales PANC-1 et mutées CFPAC-1 sont traitées à 70% de confluence pendant 20 h à des concentrations croissantes de H_2O_2 (25-50-75-100 µM). Après perméabilisation à l'éthanol froid (70%), l'ADN est marqué par l'iodure de propidium (0,5 µg/mL). La population hypodiploïde est mesurée par cytométrie en flux.
Le H_2O_2 provoquent une apoptose dose-dépendante.
Les cellules mutées CFPAC-1 présentent les plus forts taux d'apoptose.
* $p < 0,01$, ** $p < 0,001$ comparés avec leur contrôles respectifs. n = 3

L'ensemble de ces résultats suggère une susceptibilité particulière des CFPAC-1 au stress oxydant. Dans les manipulations pharmacologiques ultérieures, nous avons retenu 50 µM de H_2O_2 pour la stimulation des cellules. En effet, cette concentration, qui induit une apoptose significative des CFPAC-1 (10% versus 2% dans le contrôle $p < 0,001$), permet de comparer la susceptibilité au stress des deux lignées CFPAC-1 et PANC-1 (10% versus 5% d'apoptose respectivement).

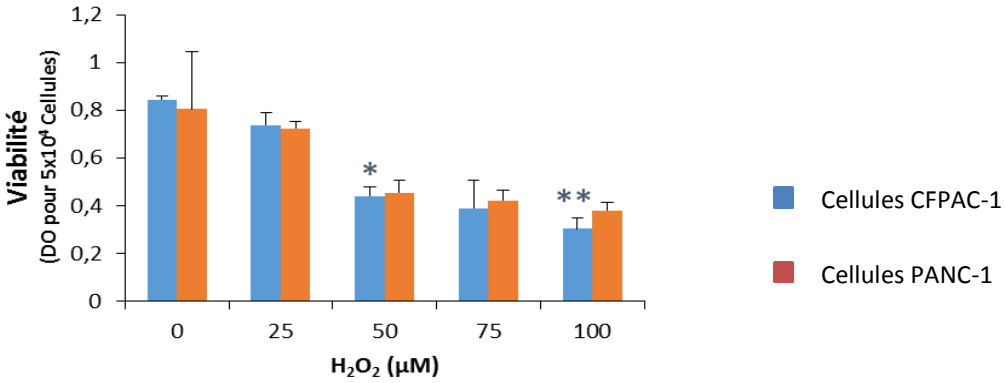

Figure 2: Effet de l'H_2O_2 sur la viabilité des cellules CFPAC-1 et PANC-1.
Les cellules normales PANC-1 et mutées CFPAC-1 sont traitées à 70% de confluence pendant 20 h à des concentrations croissantes de H_2O_2 (25-50-75-100 µM).
Le H_2O_2 diminue de manière dose-dépendante la viabilité Les cellules mutées CFPAC-1 présentent la plus forte perte de viabilité.
* $p < 0,01$, ** $p < 0,001$ comparés avec leur contrôles respectifs. n = 3

Effet des MPs issues du stress oxydant sur les cellules exocrines

Afin d'établir le rôle des MPs dans la propagation du stress oxydant, nous avons récolté des MPs produites *in vitro,* et isolées après lavage, à partir des deux lignées traitées par le peroxyde d'hydrogène. Ces MPs ont été appliquées en système autologue (Fig. 3).
La courbe dose-réponse montre que les MPs de CFPAC-1 induisent une réponse apoptotique dès la concentration de 10 nM avec un effet plateau (10 nM-20 nM) et le doublement de l'apoptose par rapport aux cellules non traitées. Au contraire aucune différence significative n'est observée après application des MPs de PANC-1.

L'ensemble de ces résultats suggère que les MPs générées au cours du stress oxydant contribuent à l'apoptose des cellules CFPAC-1.

Effet des MPs sur l'apoptose induite par le stress oxydant en système autologue

Après traitement par 50 µM de peroxyde d'hydrogène, nous avons observé 10% de cellules CFPAC-1 apoptotiques après 20 heures. Afin d'évaluer le rôle précoce des MPs dans la propagation du stress, nous avons prétraité les cellules par des MPs pendant 4h. Après lavage, les cellules cibles ont été soumises au stress oxydant pendant 20 heures selon le protocole décrit plus haut (Fig. 4).

De façon étonnante, nous avons observé un effet protecteur et concentration-dépendant des MPs issues de CFPAC-1 oxydées avec une baisse de l'apoptose induite par le peroxyde d'hydrogène de 50% dans les cellules CFPAC-1 prétraitées par 10 nM de MPs. Au contraire, l'effet protecteur des MPs issues des PANC-1 est quasi total sur les cellules PANC-1, avec un degré d'apoptose identique à celui des cellules non stressées. Cet effet ne semble pas dépendre de la concentration de MPs dans la gamme explorée.

L'ensemble de ces données en système autologue, montre un effet cyto-protecteur précoce des MPs (4 h), et délétère après 20 h, suggérant que les MPs orientent la réponse cellulaire quel que soit la lignée.

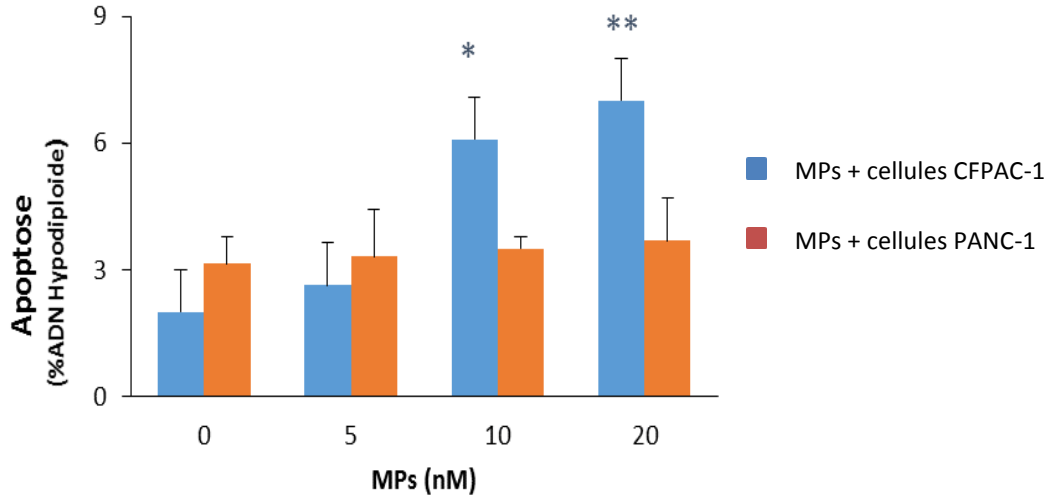

Figure 3: Effet des MPs exocrines sur l'induction de l'apoptose des cellules CFPAC-1 et PANC-1 en systéme autologue après 20 h.

Les MPs sont appliquées pendant 20 h sur des cellules naïves de même origine et l'apoptose des cellules cibles est mesurée par le taux d'ADN hypodiploïde dans les cellules perméabilisées.

Les MPs de cellules CFPAC-1 provoquent une apoptose dose-dépendante (5-20nM) après 20 h d'incubation qui n'est pas retrouvée dans les cellules PANC-1

* p < 0,05, ** p < 0,01 comparés avec leur contrôles respectifs. n = 3

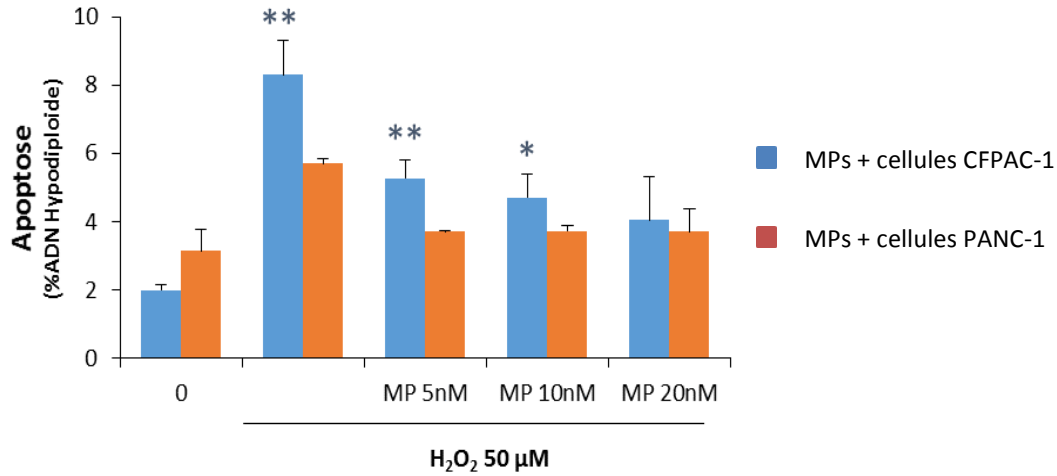

Figure 4: Effet des MPs exocrines sur l'induction de l'apoptose des cellules CFPAC-1 et PANC-1 induite par H_2O_2 en système autologue après 4 h.

Les cellules normales PANC-1 et mutées CFPAC-1 sont prétraitées à 70% de confluence pendant 4 h par des concentrations croissantes (5-10-20 nM) de MPs, puis traitées pendant 20 h par 50 µM de H_2O_2. La population avec ADN hypodiploïde est quantifiée par cytométrie en flux.

* p < 0,01, ** p < 0,001 comparés avec leur contrôles respectifs. n = 3

Modulation pharmacologique et effet autocrine des MPs

En présence de 100 µM de vitamine C aux propriétés antioxydantes, l'apoptose cellulaire induite par H_2O_2 50 µM est partiellement inhibée de façon beaucoup plus importante pour les CFPAC-1 avec 37% d'inhibition ($p < 0,05$) par rapport aux cellules traitées. La vitamine C n'agit pas de façon significative sur l'apoptose basale. L'effet protecteur de la vitamine C est retrouvé dans les cellules PANC-1 avec 27,4% d'inhibition en réponse au traitement par H_2O_2. L'effet protecteur de la vitamine C sur l'action des MPs est plus marqué dans les cellules CFPAC-1 avec une diminution de 46%. Les cellules PANC-1 sont insensibles à l'action des MPs et très peu sensibles à H_2O_2 dans ces conditions (Fig. 5).

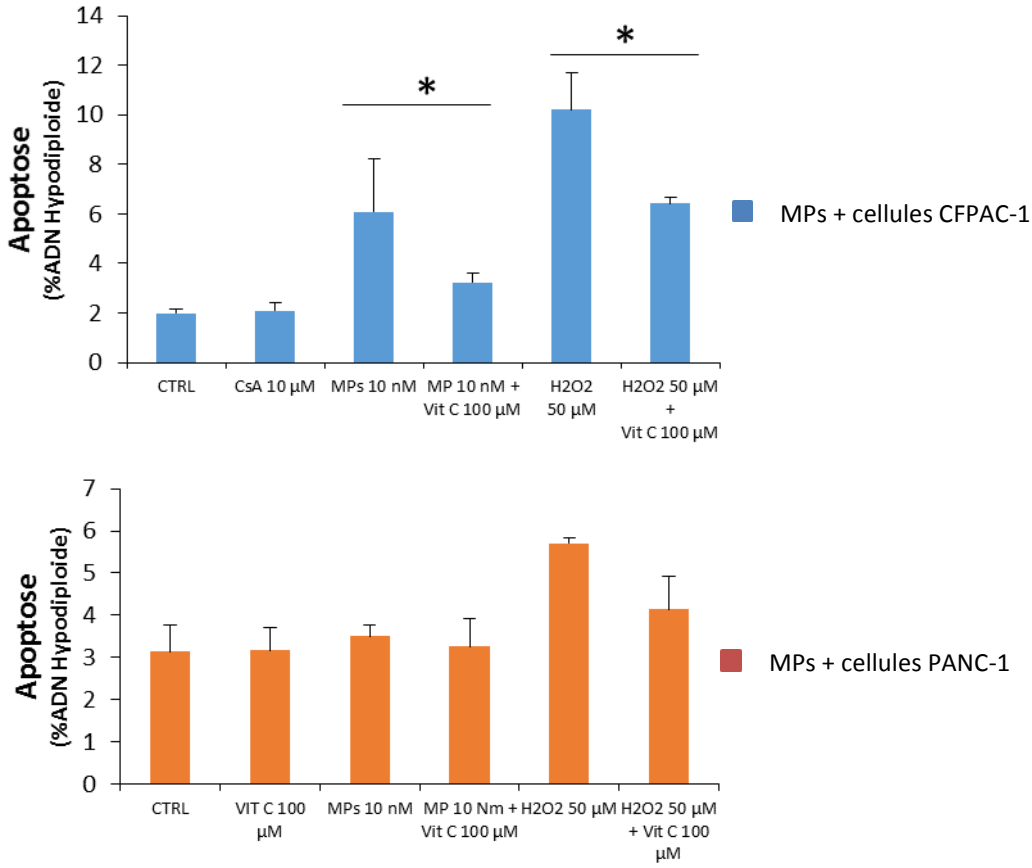

Figure 5: Effet de la vitamine C sur l'apoptose induite par H_2O_2 et sur l'effet protecteur des MPs en situation de stress.

Les cellules normales PANC-1 et mutées CFPAC-1 sont prétraitées à 70% de confluence pendant 4h par 100 µM de vitamine C et/ou 10 nM de MPs lavés, puis traitées pendant 20 h par 50 µM de H_2O_2. L'ADN hypodiploïde est mesuré par cytométrie en flux.

* $p < 0,05$. n = 3

La CsA se révèle efficace également dans la protection des cellules au stress oxydant. Au contraire de la vitamine C, la CsA protège de l'apoptose basale de 45% dans les CFPAC-1. La CsA permet également d'améliorer la survie cellulaire comparativement à la vitamine C. De façon intéressante la CsA semble contrôler plus efficacement l'apoptose des cellules PANC-1 soumises à 50 µM de H_2O_2 en présence ou en absence de MPs (Fig. 6).

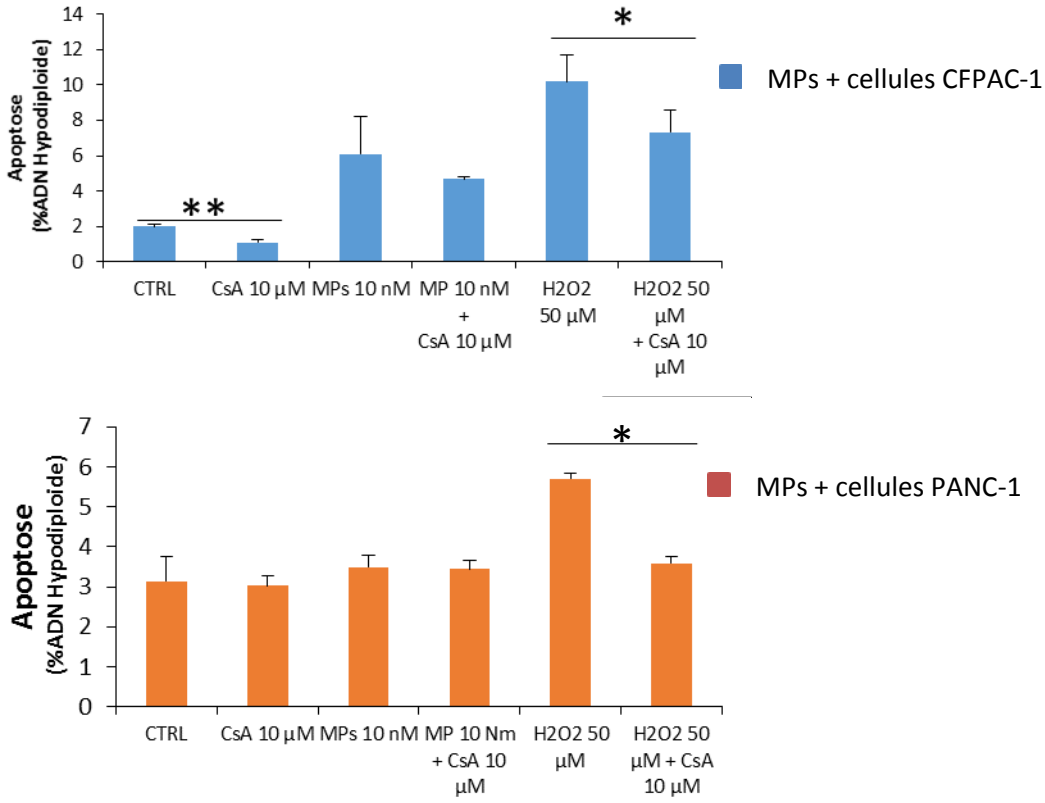

Figure 6: Effet de la ciclosporine CsA sur l'apoptose induite par H_2O_2 et sur l'effet protecteur des MPs en situation de stress.

Les cellules normales PANC-1 et mutées CFPAC-1 sont prétraitées à 70% de confluence pendant 4 h par 10 µM de CsA et/ou 10 nM de MPs lavés, puis traitées pendant 20 h par 50 µM de H_2O_2. L'ADN hypodiploïde est mesuré par cytométrie en flux.

* $p < 0,05$, ** $p < 0,01$. $n = 3$

Discussions

Les cellules porteuses de la mutation CFTRΔF508, dont qui présentent un déséquilibre ionique, présentent une rétention du canal CFTR dans le RE, ce qui nous a conduit à envisager que la topologie de la membrane des cellules mutées pouvait influer sur la réponse au stress oxydant, la libération des MPs et leurs caractéristiques fonctionnelles orientant ainsi que le message qu'elles véhiculent. Ce travail nous a permis de déterminer les conditions optimales pour l'observation des réponses au stress oxydant dans les cellules pancréatiques exocrines normales (PANC-1) ou porteuses de la mutation CFTRΔF508 (CFPAC-1). Nous avons évalué l'effet des MPs sur la réponse des cellules cibles exocrines au stress oxydant en mesurant l'apoptose, la viabilité et la sécrétion d'insuline.

L'observation des effets autocrines des MPs exocrines des deux lignées (CFPAC-1 et PANC-1) sur les cellules cibles naïves en système autologue, montre une perte cellulaire plus importante des cellules CFPAC-1 cibles. Une des explications de cette différence est mécanique, les CFPAC-1 sont plus grandes, ce qui limiterait leur plasticité membranaire et donc leur capacité à émettre des MPs à l'état basal. Cependant, les CFPAC-1 émettent plus de MPs que les cellules PANC-1 après traitement par 100 µM de H_2O_2, ce qui suggère une susceptibilité particulière au stress que nous avons confirmée.

Nos résultats indiquent que les MPs de CFPAC-1 appliquées à des concentrations d'environ 10 nM éq.Phtdser stimulent l'apoptose des cellules naïves. Dans le contexte de l'altération pancréatique ces MPs auraient ainsi des propriétés effectrices importantes en amplifiant l'apoptose des cellules voisines encore préservées.

L'effet autocrine des MPs en condition de stress oxydant montre un effet cyto-protecteur précoce des MPs (4 h), puis délétère après 20 h, suggérant que les MPs orientent la réponse cellulaire en fonction du temps de contact. Dans le cas des MPs issues des cellules CFPAC-1, la cyto-protection pourrait s'expliquer par le transfert d'annexine-a1 aux propriétés anti inflammatoires et dont la concentration est élevée dans les MPs de cellules dépourvues de CFTR (Dalli et al., 2010).

L'effet spécifique du stress oxydant est démontré par l'inhibition pharmacologique par la vitamine C appliquée à la concentration de 100 µM.

Nous avons mis en évidence que cet effet spécifique des MPs issues des cellules CFPAC-1 était réversible après addition de vitamine C et de CsA. Ces données suggèrent que les MPs exercent un pouvoir oxydant propre dont les mécanismes d'action restent inconnus.

Conclusions

Nos résultats montrent que les cellules exocrines porteuses de la dysfonction ionique sont particulièrement sensibles au stress oxydant induit par H2O2. Les MPs issues de ces cellules mutées modulent la réponse apoptotique au stress oxydant dans les cellules cibles de façon autocrine.

Dans ce contexte, le stress oxydant peut représenter un facteur important pour la dysfonction pancréatique chez les mammifères. L'équilibre ionique et la stabilité métabolique sont essentiels pour la réponse cellulaire en défense contre le stress oxydant. La vitamine C et la cyclosporine possèdent un effet protecteur contre le stress oxydant. Ces résultats sont très utiles pour comprendre la physiopathologie des cellules exocrines mutées et son rôle dans la dégénérescence pancréatique.

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EXOCRINE MICROPARTICLES PRODUCED IN RESPONSE TO OXIDATIVE STRESS PROMOTE CELL ALTERATION IN ENDOCRINE PANCREAS

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Abstract

One of the major factors leading to pancreatic endocrine dysfunction and insulin impairment is oxidative stress. Like any other stimulus, oxidative stress is able to produce microparticles (MPs) that are cell membrane fragments released by stimulated or apoptotic cells. They carry bioactive molecules from parent cells and can mediate intercellular cross-talks. To study the effect of oxidative stress on endocrine pancreas and identify the role of exocrine cell-derived MPs on insulin secreting cells. Microparticles were produced from two exocrine cell lines, PANC-1 (normal) and CFPAC-1 (CFTR deficient) respectively after H2O2 treatment. The two-type MP effects were compared into aparacrine cell communication model, using endocrine RIN-m5f as target cells. The cell response to MP action was assessed by hypodiploid DNA measurement, cell viability and insulin secretion. Pharmacological modulation was achieved by Cyclosporine (CsA) and Vitamin C (VitC). Endocrine cells submitted to H2O2 presented a dose-dependent decrease of viability correlated with increase of apoptosis. Exocrine MPs fromCFTR deficient exocrine cells induced a decrease of viability and impaired insulin secretion in endocrine target cells. Oxidative stress may promote endocrine dysfunction and can sustain a deleterious MP-mediated cross-talk between exocrine and endocrine cells with effects on insulin secretion.

Keywords: *oxidative stress, exocrine microparticles, insulin secretion*

Introduction

Dans la dysfonction endocrine, le stress oxydant a été décrit dans les cellules du muscle squelettique comme responsable de la dégénérescence des mitochondries, avec une accumulation intracellulaire de glucose et de lipides contribuant à un cercle vicieux favorisant l'insulino-résistance (Bonnard et al., 2008). Les processus inflammatoires sont associés à une production de ROS et à l'installation d'un stress oxydant dont les effets pourraient affecter la survenue du diabète (Ntimbane et al., 2008). La mise en évidence et l'importance de la manifestation d'un stress oxydant chronique, laisse suggérer un lien potentiel entre les évènements oxydants et la destruction des cellules β dans (Zirbes and Milla, 2009). Dans ce

contexte, la perte graduelle de la masse et/ou du nombre des cellules β ainsi que leur dysfonctionnement pourraient s'expliquer par l'activation de la voie de signalisation impliquant JNK (Kaneto et al., 2005; Robertson et al., 2003) et celle impliquant NF-κB (Karin and Ben-Neriah, 2000; Rahman et al., 2004). La résistance à l'insuline s'expliquerait quant à elle par un effet des cytokines pro-inflammatoires (IL-1, IL-6 et TNF-α) au niveau du récepteurs IRS-1 (Avogaro et al., 2008; Evans et al., 2003; Evans et al., 2005; Sun and Liu, 2009).

Le contenu des MPs en molécules actives de l'apoptose (caspase-3, Bcl etc.) ou de l'inflammation (RANTES, IL-1β) a orienté ainsi la recherche sur leur implication dans les mécanismes transcellulaires d'activation vasculaire et tissulaire (Al-Massarani et al., 2009). En effet, la libération des MPs augmente la surface d'échange avec le milieu et favorise l'amplification des réactions à la surface de la membrane plasmique (Morel et al., 2006). Bien que les MPs portent la PhtdSer qui est marqueur de senescence, leur élimination est retardée du fait de leur petite taille autorisant la diffusion rapide dans les tissus et limitant la reconnaissance par les macrophages. *In vivo*, les MPs circulantes constituent un marqueur d'altération cellulaire accessible par simple ponction veineuse périphérique. Elles sont un témoin circulant d'une apoptose tissulaire alors même que la cellule n'est plus accessible notamment en situation d'altération du greffon (Freyssinet, 2003). Des MPs portant le facteur tissulaire (FT) ont été mises en évidence comme des éléments séquestrées dans des îlots pancréatiques au cours de la réaction inflammatoire IBMIR associée au rejet de greffe.

Le travail a vise à explorer les mécanismes impliqués dans les modifications des fonctions de la cellule ß à insuline par les MPs de cellules exocrines et soumises à des stress mimant les conditions physiopathologiques de la dysfonction pancréatique (stress oxydant). Les mécanismes apoptotiques seront explorés à l'aide de la ciclosporine CsA, inhibiteur pharmacologique de l'ouverture des pores de transition mitochondriaux (PTP). Dans ce contexte, les cellules non mutées (PANC-1) et les cellules (CFPAC-1) cultivées constituent des outils de référence.

Materiels et methodes

Culture cellulaire

Les cellules PANC-1, CFPAC-1 (CFTRΔF508) et RIN-m5f ont été achetées auprès de l'American Type Culture Collection (Rockville, MD- Manassas, USA). Les milieux DMEM, IMDM, et RPMI 1640, le SVF qui provient du Sérum de Veau Fœtal, sont fabriqués par PAN BIOTECH. Le tampon HEPES a été fourni par Cambrex. Le peroxyde d'hydrogène (H_2O_2), la ribonucléase bovine A1 (RNAse A), l'iodure de propidium (IP), le bleu trypan et le kit Neutral Red provienne de Sigma-Aldrich (l'isle d'Abeau Chesnes, France). La Ciclosporine A CsA (ampoule injectable) a été fourni par par Novartis Pharma.

Les lignées pancréatiques PANC-1, et CFPAC-1, présentant la mutation CFTRΔF508, sont des cellules épithéliales exocrines. Elles sont cultivées respectivement en DMEM et IMDM complet (Glucose à 4,5 g/l, et 2 g/l) supplémenté par 10 % de SVF, et contenant 100mg/mL de streptomycine et 100 U/mL de pénicilline.

Les cellules d'insulinôme de rat clone m5F ou RIN-m5F, sont des cellules β endocrines, qui produisent et sécrètent de l'insuline. Les cellules sont cultivées en milieu RPMI-1640 (glucose à 4,5 g/L), supplémenté de 10% de SVF et contenant 20µg/mL gentamycine (Biowitthaker).

Les cellules sont cultivées à 37°C sous atmosphère enrichie à 5% en CO_2. Elles sont trypsinées à l'aide de trypsine EDTA à 0,05% (Sigma) lorsque elles sont à pré-confluence (80% de confluence), et réensemencées à 30% de confluence pour les expandre. Le milieu est changé toutes les 48 heures. La numération des cellules vivantes est déterminée par test de viabilité cellulaire à 10% (v/v). Les cellules sont ensemencées à 30% de confluence 48h-72h avant induction, afin qu'elles atteignent 70% de confluence. Après élimination du surnageant de culture, et lavage, l'inducteur de stress est ajouté dans un milieu complet frais.

Stress inducteurs

L'induction cellulaire par un agent oxydant, le peroxyde d'hydrogène (H_2O_2), pendant 20 h est réalisée lorsque les cellules atteignent 70 % de confluence. Un contrôle non stimulé est obtenu par addition d'un volume de solvant ou tampon identique à celui utilisé pour réaliser la solution d'inducteur.

Traitement pharmacologique

Les cellules ont été traitées à 70% de confluence par la vitamine C (100 µM) et les échantillons récoltés après 20 h d'incubation en présence ou non du stress inducteur. Les cellules RIN-m5f ont été prétraitées à 70% de confluence pendant 4 h par la ciclosporine A (10 µM) et les échantillons récoltés après 20 h d'induction par le stress oxydant. Seule la CsA a été appliquée tout au long de l'expérience en tant que modulateur pharmacologique, contrairement à la vitamine C.

Quantification de l'apoptose par mesure du taux d'ADN hypodiploïde

Les culots cellulaires de cellules adhérentes et détachées obtenus par centrifugation des cellules trypsinisées et du surnageant (200 g, 5minutes) sont rassemblés et les cellules sont perméabilisées par une solution d'éthanol à 70% (v/v) pendant au moins 4 h à 4°C. Après lavage avec du HBSS, les cellules sont traitées par de la RNase de type I-A (10 µg/ml) à 37°C pendant 15 min afin d'éliminer toute trace de RNA cytoplasmique. Après une étape de lavage en HBSS, les cellules ($5x10^5$ cellules/ml) sont incubées dans l'obscurité en présence d'iodure de propidium (100 µg/ml) 10 min à 22°C. L'iodure de propidium est un intercalant de l'ADN. Le taux d'ADN hypodiploïde est évalué par les basses intensités de fluorescence, il témoigne de la dégradation de l'ADN par les endonucléases activées par le processus apoptotique et est proportionnel au taux d'apoptose cellulaire dans la suspension. L'intensité de fluorescence des cellules est mesurée par cytométrie en flux pour 10000 cellules.

Dosage des MPs

Les cellules apoptotiques ainsi que les débris cellulaires sont éliminées par centrifugation du surnageant. Le système de capture des MP utilise la très haute affinité de l'Annexine-5 pour la PhtdSer (Kd 10^{-10} M) et celle de la streptavidine pour la biotine (Kd 10^{-14} M). Les MPs sont capturées au fond de puits recouverts de streptavidine covalemment fixée (Roche, France), et passivés par incubation d'une solution d'albumine humaine (5g/L) en tampon TBS (Tris 50 mM, NaCl 120 mM, KCl 2.7 mM, $CaCl_2$ 1 mM, pH 7.5). L'Annexine-5 biotinylée (600 ng/mL) est fixée après 1h d'incubation à 37°C. Les MPs émises dans le surnageant sont dosées directement ou après lavage en HBSS et concentration par centrifugation (12000 g, 45 min). Les échantillons contenant les MPs sont déposés (100 µL/puits) et incubés 30 min à 37°C. Après trois lavages, la quantité de MP insolubilisées est mesurée par dosage prothrombinase mis au point au laboratoire.

Dans ce test, le degré d'exposition des phospholipides anioniques à la surface des MPs est le facteur limitant de la réaction d'assemblage du complexe de la coagulation

prothrombinase qui conduit à la libération de thrombine soluble révélée à l'aide du substrat chromogénique pNAPEP. Les mesures sont effectuées en mode cinétique, dans un spectrophotomètre thermostaté (VERSAmax, Molecular Device, USA) et en milieu réactionnel standardisé contenant des facteurs de la coagulation d'origine humaine (FII 1.2 μM, FVa 33.3 pM, FXa 11.2 pM, $CaCl_2$ 2.2 mM, 30 min d'incubation à 37°C). Les absorbances sont converties en « équivalent phosphatidylsérine » (éq PhtdSer) par référence à une courbe de calibration obtenue à l'aide d'une suspension de vésicules synthétiques (33% (p/p) de PhtdSer et 67% (p/p) de PhtdChol). La détermination du phénotype des MPs est réalisée en remplaçant l'Annexine-5 par les anticorps d'intérêt.

Test de viabilité cellulaire au Neutral Red

La mesure de la viabilité cellulaire a été faite grâce à un kit Neutral Red (Sigma) : les cellules viables intègrent le colorant (Neutral Red 0,33% dilué dans du tampon salin phosphate de Dulbecco (DPBS) par transport actif et l'incorporent dans les lysosomes. Les cellules sont ensuite fixées (formaldéhyde 0,5% et chlorure de calcium 0,1%), et le colorant initialement incorporé est libéré des cellules par addition d'une solution d'acide acétique 1% diluée dans de l'éthanol 50%. La plaque est lue au spectrophotomètre à 540 nm. Les résultats sont rapportés à 50 000 cellules.

Dosage de l'insuline dans le surnageant des Rin-m5f

Le dosage de l'insuline a été réalisé à l'aide d'un kit ELISA double sandwich en suivant les constructions du fabriquant Millipore. Nous avons opté pour le dosage utilisant une courbe de calibration réalisée en tampon contenant une proportion de sérum (calibration dite « matrix »). Les surnageants des cellules ont été dilués au $10^{ème}$ ouau $20^{éme}$.

Marquage cellulaire des cellules RIN-m5f par cytométrie en flux

Des anticorps monoclonaux biothynilés anti CFTR ont été testés.Le marquage est réalisé dans des conditions standardisées et en maintenant un rapport constant entre concentration cellulaire (500000 cellules/mL), concentration des anticorps, la strepatavidine-FITC. Les concentrations d'anticorps primaires ont été optimisées (6,4 μg). Les anticorps contrôles isotypiques (IgG-kappa) sont appliqués à la même concentration dans les échantillons contrôles.

Resultats

Sensibilité des cellules endocrines au stress oxydant et modèle de communication cellulaire paracrine

Effet du stress oxydant sur les cellules RIN-m5f

L'effet du stress oxydant par application de H_2O_2 sur la réponse apoptotique des cellules β, ainsi que la viabilité, a été evalué après20 heures (Fig. 1).Les cellules RIN-m5f montrent une susceptibilité importante avec un doublement de la proportion de cellules apoptotiques mis en évidence par le pourcentage d'ADN hypodiploïde dès 50 μM de H_2O_2 (6,5%) parrapport à l'apoptose basale (2,5%). La courbe dose-réponse montre que l'apoptose induite est concentration-dépendante jusqu'à 100 μM, avec une apoptose significative à partir de 50 μM (Fig. 1A). Symétriquement à des doses croissantes de H_2O_2, une perte significative de viabilité est observé dès 50 μM (31,8%, $p<0,05$) Fig. 1B). Le stress oxydant croissant, provoque la libération dose-dépendante des MPs par les cellules endocrines cibles, et atteint un pic à 100 μM de H_2O_2 avec une concentration de 28,8 nM Phtdser (5 fois supérieure au contrôle non traité) rapportée à $5x10^4$ cellules (Fig. 1C). La dose choisie pour

l'étude des effets pharmacologiques est 75 µmol/L, correspondant à une viabilité cellulaire résiduelle de 40% ($p<0,01$).

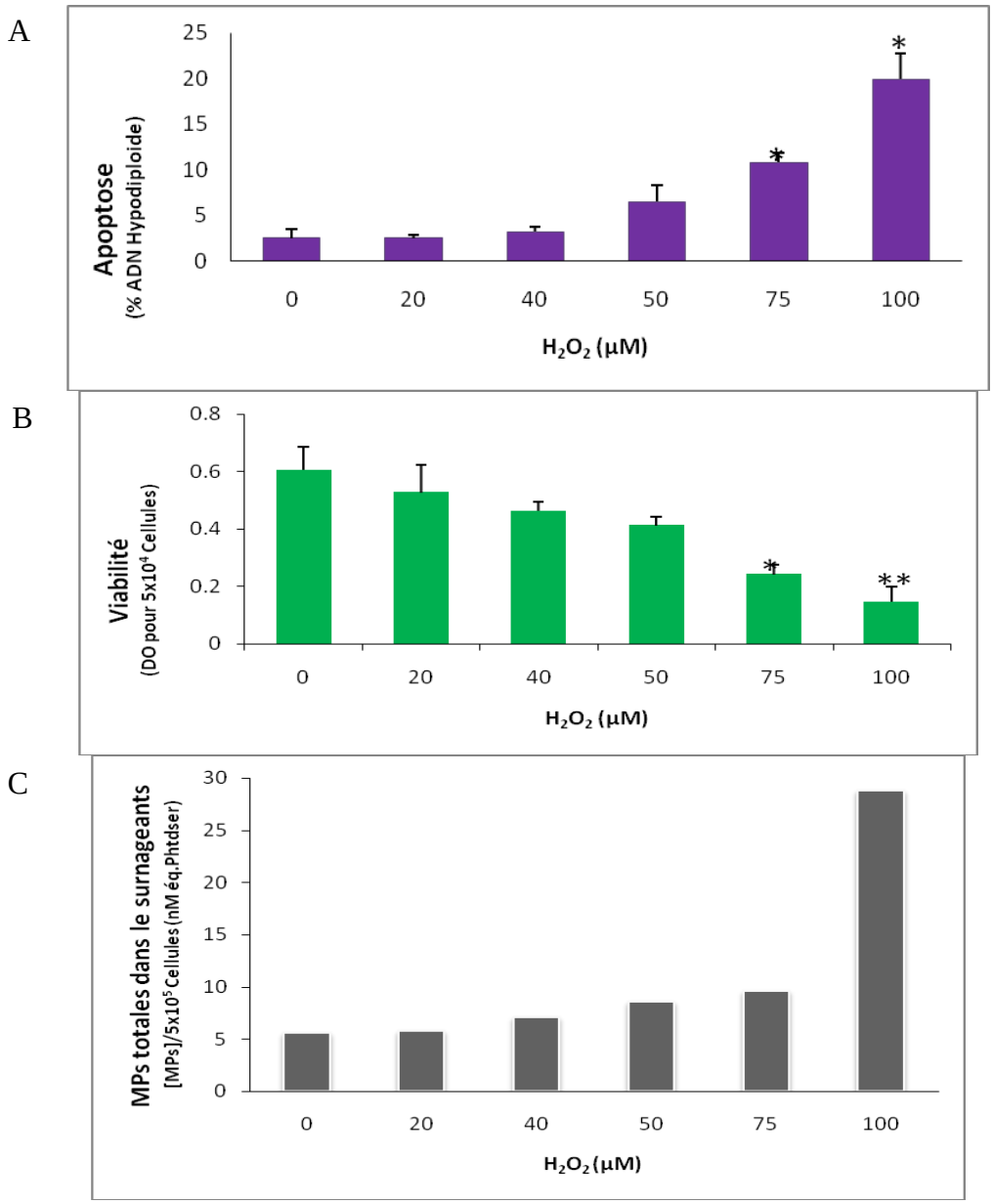


Figure 1: Effet de l'H_2O_2 sur l'induction de l'apoptose (A), la perte de la viabilité (B) des cellules endocrines RIN-m5f, et la libération des MPs (C) par les cellules RIN-m5f.

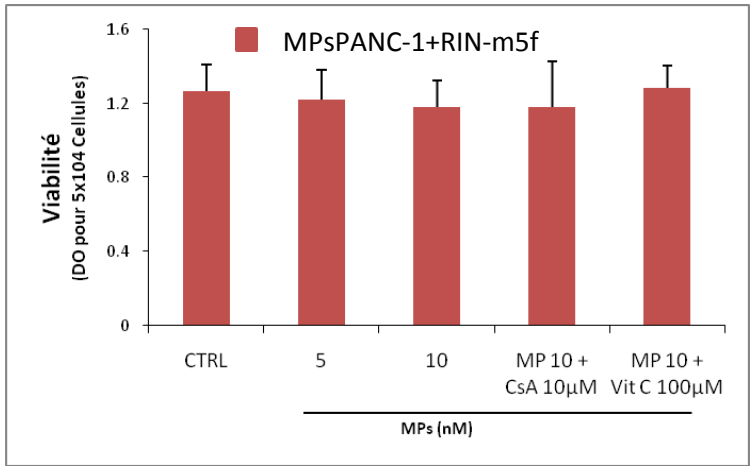
Les cellules RIN-m5f sont traitées à 70% de confluence pendant 20 h à des concentrations croissantes (20-40-50-75-100 µM)de H_2O_2.

Les cellules RIN-m5f présentent un taux d'apoptose et une perte de viabilité dose-dépendant.

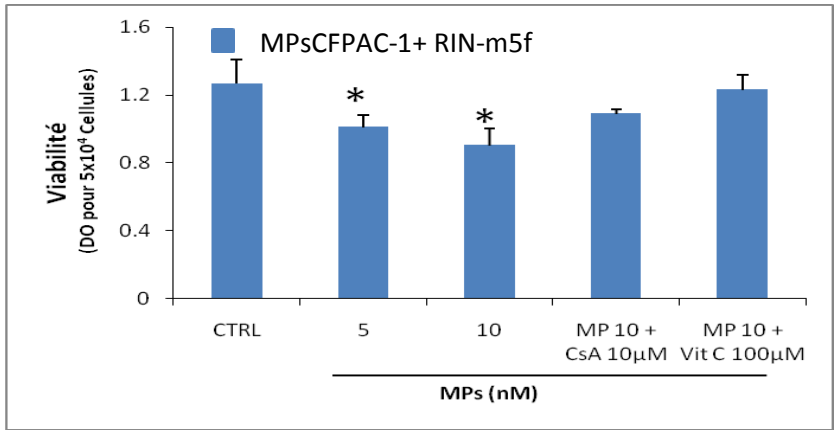
* $p < 0,01$, ** $p < 0,005$ comparés avec leur contrôles respectifs. n = 3pour A, B et n=1 en duplicat pour C

Modulation pharmacologique et effet paracrine des MPs issues de PANC-1 et CFPAC-1

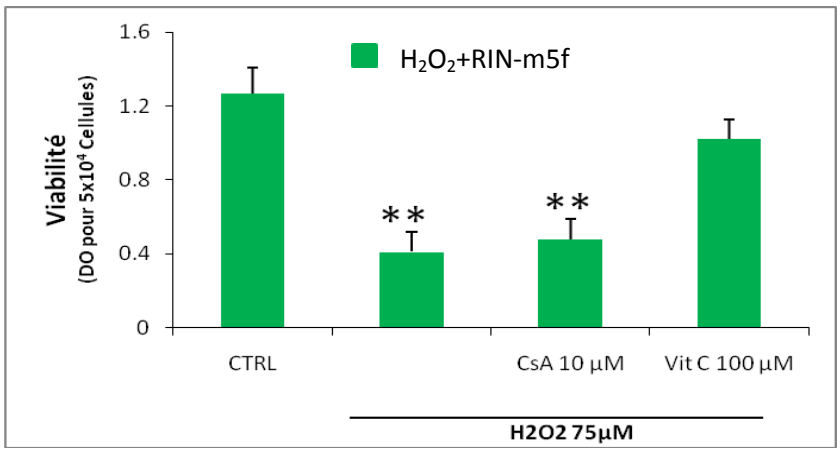
Les MPs produites dans les deux lignées ont été appliquées aux cellules endocrines RIN-m5f pour évaluer leurs effets paracrines sur la viabilité et la réponse apoptotique (Fig. 2). On n'observe pas de diminution significative de la viabilité des cellules RIN-m5f après un traitement de 20 h à une concentration de 5 nM ou 10 nM de MPs issues des PANC-1 (Fig. 2A). Les MPs de cellules CFPAC-1 ont un effet délétère sur la viabilité des cellules endocrines avec une baisse significative de la viabilité de 21%, et 29% en présence de 5 nM et 10 nM de MPs, respectivement et de façon significative ($p<0,05$) (Fig. 2B). En présence de 75 µM de peroxyde d'hydrogène, ou de 10 nM MPs CFPAC-1, la viabilité cellulaire diminue significativement de 40%, et 28%; ($p<0,05$), respectivement. En l'application de 100 µM de vitamine C, elle réduit la perte de la viabilité cellulaire. Avec restauration de 40% ($p<0,001$), et 24% ($p<0,05$) de la viabilité des RIN-m5f soumises au traitement par 75 µM de peroxyde d'hydrogène et 10 nM MPs CFPAC-1 respectivement. Le traitement par la CsA n'est pas plus efficace que celle par la vitamine C (Fig. 2C).



A



B



C

Figure 2 : Viabilité des cellules endocrines RIN-m5f en présence des MPs de cellules CFPAC-1 (A),MPs de cellules PANC-1 (B), et de 75 µM de H_2O_2 (C).

Les MPs ou l'H_2O_2 sont appliquées pendant 20 h sur des cellules RIN-m5f naïves, et la viabilité des cellules cibles est mesurée avec le kit Neutral Red.

B

*Les MPs de cellules CFPAC-1 ont un effet délétère sur la viabilité des cellules endocrines.** $p < 0,05$,** $p < 0,01$ comparés avec leurs contrôles respectifs. $n = 3$

Effet des MPs porteuses de CFTR normal ou muté sur la sécrétion d'insuline par les cellules RIN-m5f

La concentration d'insuline secrétée dans le surnageant des cellules endocrines après 18 h d'incubation en présence de MPs issues de cellules CFPAC-1 est diminuée de 60%. Les MPs de cellules PANC-1 ont un effet inverse car la sécrétion d'insuline est doublée en 18 h (Fig. 3). L'effet des MPs exocrines sur la sécrétion d'insuline cumulée au bout de 18h est très net si on le compare aux effets modeste de l'actinomycine D, agent apoptotique directement appliqué sur les cellules RIN-m5f.

Expression de CFTR à la surface des cellules endocrines RIN-m5f

Nous avons pu mettre en évidence par cytométrie en flux l'exposition de CFTR par les cellules RIN-m5f fixées en utilisant l'anticorps MATG1031 fluorescent. L'absence de CFTR n'est donc pas responsable de la sensibilité des cellules au stress apoptotique (Fig. 4).

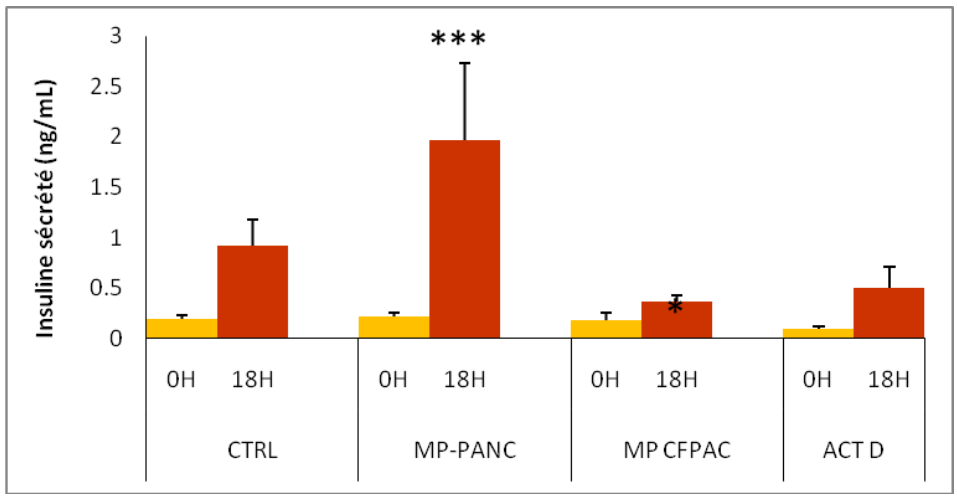


Fig. 3: Effet paracrine des MPs exocrinessur la sécrétion d'insuline par les cellules endocrines RIN-m5f pendant 18 h.

Traitement de $5x10^4$ cellules RIN-m5f par des MPs (10 nM éq.Phtdser) isolées des surnageants de cellules CFPAC-1 et PANC-1 soumises au stress oxydant par application de H_2O_2. L'insuline secretée dans le surnageant est mesurée après 18 h d'incubation. A titre de comparaison la secretion d'insuline cumulée dans les surnageants des cellules RIN-m5f traitées par l'actinomycine D est représentée (ACT D)

* $p < 0,05$, ** $p < 0,001$ comparés avec leur contrôles respectifs. n = 3

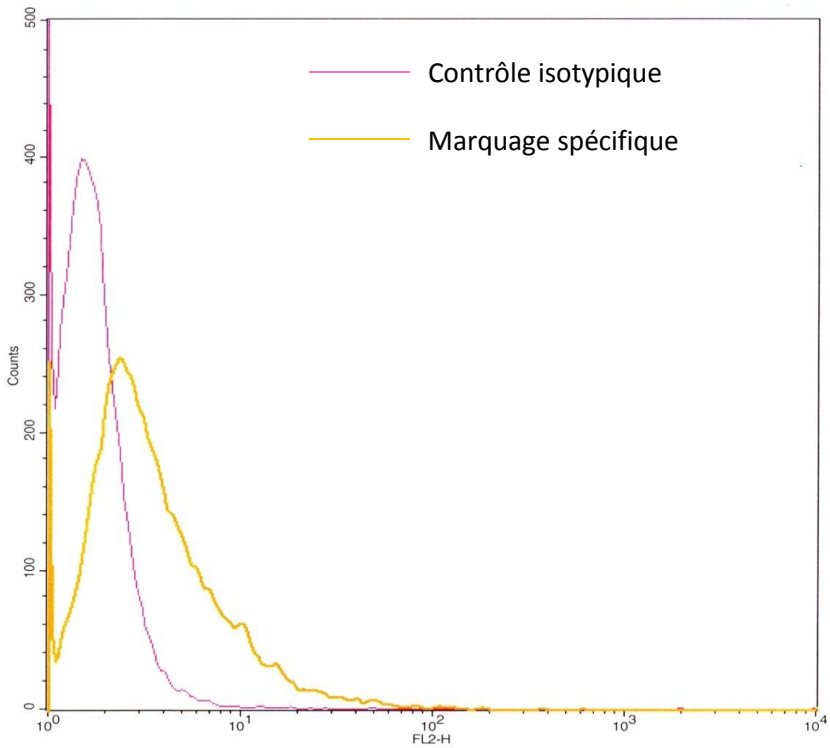


Fig. 4 : Mise en évidence de l'expression de CFTR à la surface des cellules RIN-m5f.

Discutions

Nos observations démontrent la sensibilité des cellules endocrines RIN-m5f au stress oxydant provoqué par application de H_2O_2, avec une apoptose et une perte de viabilité cellulaire dose-dépendante ainsi qu'une libération de MPs. Nos résultats sont en cohérence avec la vulnérabilité des cellules β au stress oxydant, résultant d'une part de leur pauvreté en Cu/Zn superoxyde dismutase, catalase et glutathion peroxydase, d'autre part de leur faible contenu en glutathion réduit (Lortz et al., 2000). Dans la littérature, l'action du peroxyde d'hydrogène est décrite comme de courte durée du fait de son instabilité dans le milieu. Cependant, nous avons observé qu'un temps d'incubation de 6h avec H_2O_2 était insuffisant pour déclencher l'apoptose des RIN-m5f. Nous avons retenu une exposition chronique à H_2O_2 de 20 h générant un stress modulable pharmacologiquement. Nos résultats confirment la différence de propriétés effectrices entre MPs de PANC-1 et de CFPAC-1 observées sur les cellules exocrines. En effet seules les MPs issues des cellules porteuses de la mutation CFTRΔF508 diminuent la viabilité des cellules cibles et la sécrétion d'insuline.

Les résultats de viabilité des cellules RINm5F en présence de MPs exocrines suggèrent que l'apport de molécules oxydantes peut être soit direct, via H_2O_2, ou indirect, via les MPs, probablement porteuses de lipides oxydés. En effet l'action des MPs est également inhibée par la vitamine C, même en absence de H_2O_2.

La baisse considérable de la sécrétion d'insuline provoquée par les MPs de CFPAC-1 suggère que les voies métaboliques régulant la production d'insuline sont sensibles aux MPs. De plus, cette diminution est spécifique aux MPs issues des cellules CFPAC-1. Des resultats similaires avec l'actinomycine D comme agent apoptotique ont été obtenus au laboratoire (M2 A.Kobiita 2009, UDS). Plusieurs hypothèses peuvent expliquer la diminution de la sécrétion d'insuline. La rétention de l'ARN_m de cette hormone ou la modification de sa production. Dans la littérature, on a pu déterminer deux micro-ARN, miR103 et miR107, différant seulement de quelques bases, qui régulent négativement l'expression de l'insuline (Trajkovski et al., 2011).

Dans une étude précédente, on a montré que les cellules exocrines sont détruites par le stress oxydant, notamment celles qui présentent une dysfonction de la protéine CFTR (Constantinescu et al. 2013). Dans la littérature, l'expression de CFTR par les cellules endocrines reste débattue, probablement en raison de l'insertion profonde de la molécule dans la membrane. Nous avons mis en évidence CFTR à la surface des cellules endocrine RIN-m5f.

Conclusions

Le stress oxydant induise une altération forte dans le pancréas endocrine. En plus, le stress oxydant stimule la production de microparticules délétères pour les cellules endocrines. La molécule CFTR, qui joue un rôle essentiel pour la stabilité métabolique des cellules, présente une protection pour les cellules à insuline contre le stress oxydant et pour le stress induit par les microparticules exocrines.

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