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ULTRASONOGRAPHIC ASPECTS DURING PREGNANCY IN SNAKES

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Abstract
Being relatively easy to maintain, snakes are increasingly common as pets. Therefore it is necessary to know the detailed characteristics and common anatomical aspects of these reptiles by veterinarians and breeders to identify the diseases that may be present in them. Snakes reproduction in captivity requires knowledge of normal reproductive behavior, and the ultrasound aspects encountered during pregnancy. Ultrasound examination is an important non-invasive method in identifying individuals who have reached sexual maturity and the stage of development of the egg (the oviparous snakes) or embryo (the ovivivіparous and viviparous snakes). Ultrasound provides a dynamic picture of the evolution of pregnancy in snake allowing identification of the number of eggs or embryos and their viability.
Keyword*: snake, ultrasound, oviparus, viviparus, snake pregnancy.*

Introduction

Increased interest in snakes as pets caused an increase in interest in breeding them in captivity. Therefore, for successful reproduction of snakes in captivity is needed a detailed knowledge of reproduction behavior and physiology of these animals. Although the snake breeding occurs naturally, biological and anatomical knowledge of reproduction help diagnose and treat clinical aspects quite common in captivity.

Sexual maturity of the snake is determined primarily by body size, age having a secondary role (Porter, 1972). In captivity, sexual maturity is influenced by mode of feeding and growing conditions, resulting in accelerated development, in some cases they could reach sexual maturity at 18 months. Under normal conditions, the snakes reach sexual maturity at 2-3 years of age (Mader, 2006).

Although most reptiles lay eggs (oviparous), some species give birth to already developed offspring. They are divided into ovoviviparous and viviparous, depending on the degree to which they share nutrients with embryo (Tinkle and Gibbons, 1977; Blackburn, 1992). Of oviparous snakes most common in captivity are certain species of the Colubridae family (Elaphe spp, Lampropeltis spp) and Pythonidae family (Burmese python, royal python) and as the viviparous snakes found in captivity is the Boidae family (boa).

Compared to other reptiles (turtles, cheloidians) in the diagnosis of pregnancy in snake radiographs is rarely used due to the thin eggs shell. Ultrasound is used not only for diagnosis of gestation and parturition time, but also to identify the optimal time for mating to increase the rate of fertility (Mader, 2006).

Material and method

The ultrasound examination was performed on a total of six females of different species (1 Pantherophis guttatus, 2 Python bivittatus and 3 Boa constrictor). The length of specimens was 50 cm (Pantherophis guttatus), 1.8-2.2 m (Python bivittatus) and 1 -2 m (Boa constrictor), body weight was between 1 kg - 40 kg.

Restraint can be done manually or by sedation. For large specimens, that present aggressivity is recommended to performed anesthesia or sedation. Sedation can be done with Ketamine 22-44 mg / kg sc, im (Bennett, 1996), and the anesthesia with ketamine 10-30 mg / kg + butorphanol 0.5-1.5 mg / kg im (Schumacher, 1996). The restraining of examined specimens was done manually without anesthesia or sedation. Restraint was to catch the animal with one hand on the back of the skull, vertebrae and immobilization of the body with the other hand (Fig. 1, Fig. 2). The restraint of large individuals was performed by two or three persons. When restraining the snake it is necessary to immobilize the head to avoid bites.

Fig. 1 Snake restraining for ultrasonographic examination

Fig. 2 Head restraining

The ultrasound examination was performed on a Logiq E9 and Mindray DC-6 devices, both equipped with linear and convex probes.

For examination of organs in coelomic cavity were used two approaches: lateral intercostals approach and ventral approach. Ultrasound linear probe frequency was between 7.5 to 20 MHz, respectively 7.5 to 10 MHz. B mode ultrasound was used to assess two-

dimensional aspects of the ovary and Doppler mode has been used to assess hemodynamic of the organ.

An important aspect of the ultrasound examination is the correct application of ultrasound gel, this had to be applied 10-15 minutes before beginning the examination, to be absorbed by the scales; otherwise the risk of getting artifacts increases. Ultrasound gel is indicated for use in high volume because it will remove the air that is present under the scales.

Results and discussions

To identify the ovaries the gallbladder was taken as benchmark, it is easy to identify the gallbladder in individuals below 1.5 m in size, in larger individuals it is difficult to observe it due to its mobility. Large individuals are hardly restrained; body is rich in muscles, arches continuously, which cause the gallbladder to change its position. Gallbladder shape is usually spherical, sometimes can have a pear shape aspect, anechoic and located to caudal pole of the liver (Fig. 3, Fig. 4). When it is visible can be evidenced both by intercostals and ventral approach. The same is true of the liver parenchyma.

Fig. 3 The liver (white arrow) and gallbladder (blue arrow) in *Pantherophis guttatus*, intercostals approach, 15 MHz frequency

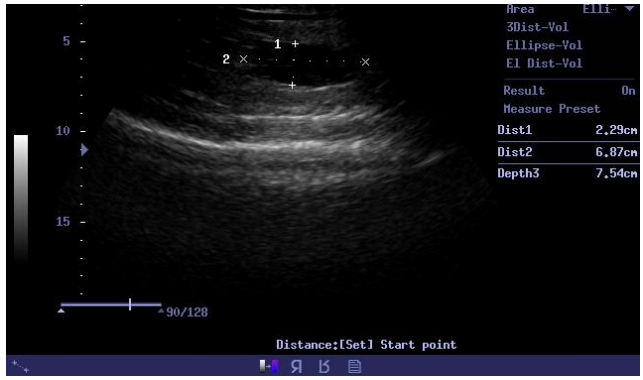

Fig. 4 Gallbladder in *Boa constrictor*, ventral approach, 10 MHz frequency

Ovarian follicles are visible caudal to gallbladder only in sexually active females, immature females ovary is small and not visible by ultrasound. Through ultrasound may show stages of follicular development. In oviparous snakes, half the egg is occupied by hypoechoic albumin, and the other half is occupied by echogenic yolk (Mannion, 2006). Time clutch may be determined by the disappearance in the egg yolk, which took place approximately one week after the yolk is no longer visible by ultrasound (Denardo, 2006).

In oviparous snakes during pregnancy, the ovary may be identified in previtelogenic state or vitelogenic state (Stetter, 2006). In previtelorgenic stage the ovary is organized as a cluster of follicles, anechoic, size below 1 mm.

In vitelogenic preovulatory stage follicles are organized more linear than as a cluster, having dimensions of about 1 cm, with the echogenity of central area higher than the peripheral area (Fig. 5, Fig. 6).

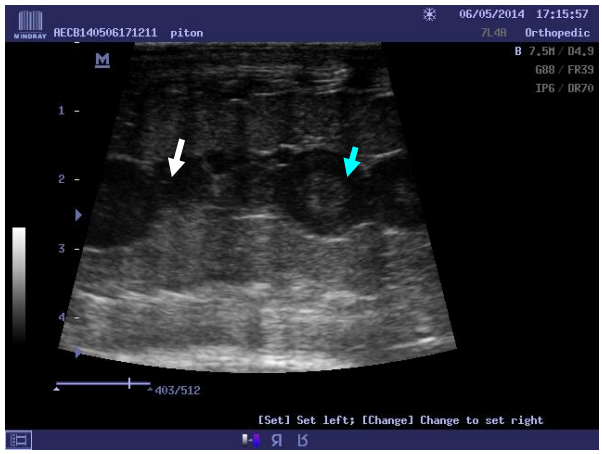

Fig. 5 Previtelogenic follicles (white arrow), vitelogenic preovulatory follicles (blue arrow), *Python bivittatus*, 7,5 MHz frequency, intercostals approach

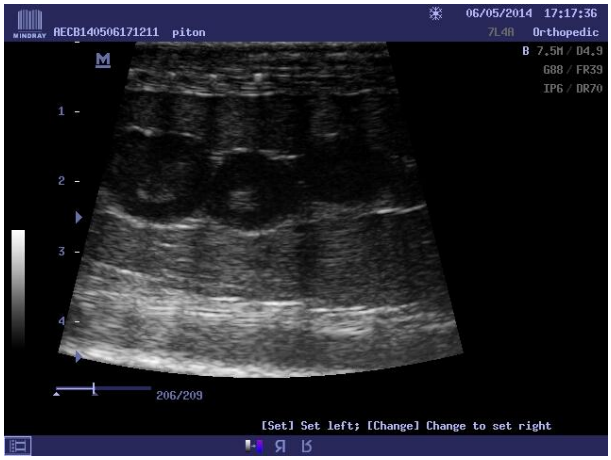

Fig. 6. Vitelogenic preovulatory follicles, *Python bivittatus*, 7,5 MHz frequency, intercostals approach

In the postovulatory stage ovas are arranged linearly, have larger sizes 1-2 cm, the center area is hypoechoic or anechoic, has a peripheral echogenic area and begins to form crust (shell) (Fig. 7). In advanced stages of the egg formation content is organized, the egg have an elongated appearance (Fig. 8, Fig. 9). Before time clutch, eggs migrate into the caudal portion of the oviducts in the posterior third of the body, having hyperechoic margins (Fig. 10).

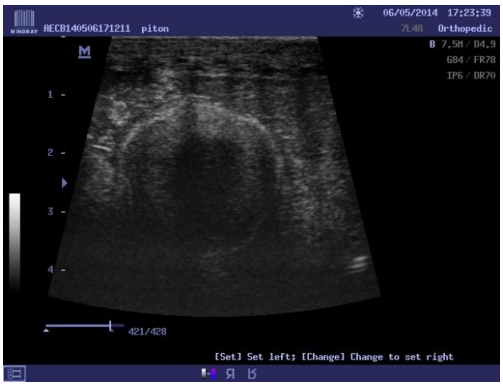

Fig. 7. Postovulatory stage, *Python bivittatus*, 7,5 MHz frequency, intercostals approach

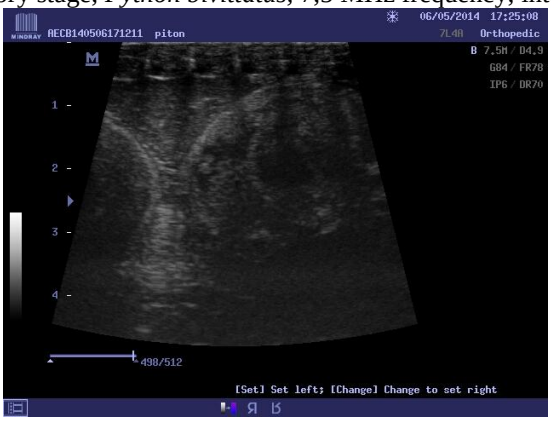

Fig. 8. Postovulatory stage, *Python bivittatus*, 7,5 MHz frequency, intercostals approach

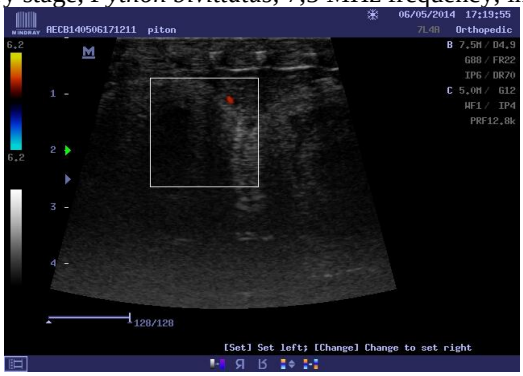

Fig. 9. Ova aspects in the postovulatory stage, *Python bivittatus*, 7,5 MHz frequency, intercostals approach

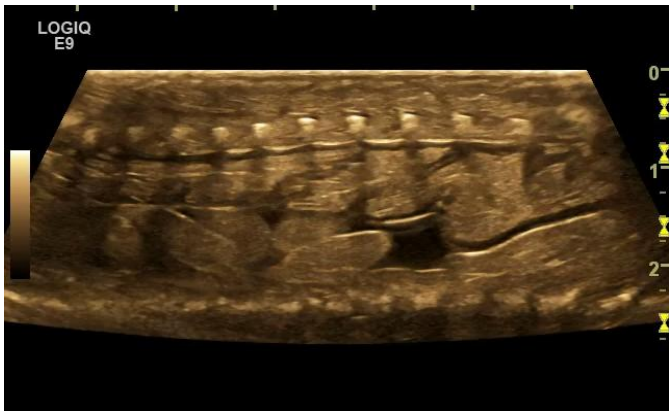

Fig. 10. Eggs in the oviduct, *Pantherophis guttatus*, 12 MHz frequency, intercostals approach

In viviparous snakes ultrasound appearance of the follicles is similar to that encountered in oviparous snakes until ovulation, after ovulation embryo begins to develop, having an echogenic appearance. In advanced stages embryo and its movement is visible (Fig. 11). The viability of embryos is assesed by Doppler examination of the heart which is evident (Fig. 12). If the embryo does not develop, the egg will be similar with those found in oviparous snakes, with echogenic appearance, inhomogeneous aspect, called "slugs" (Mader, 2006) (fig. 13).

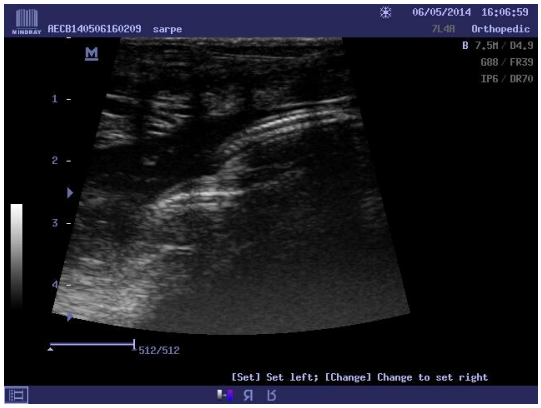

Fig. 11. Embryo in *Boa costrictor*, 7,5 MHz frequency, intercostals approach

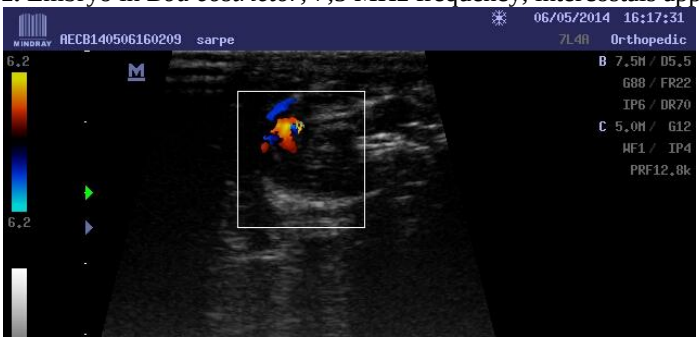

Fig. 12. Cardiac activity in embryo, *Boa costrictor*, 7,5 MHz frequency, intercostals approach

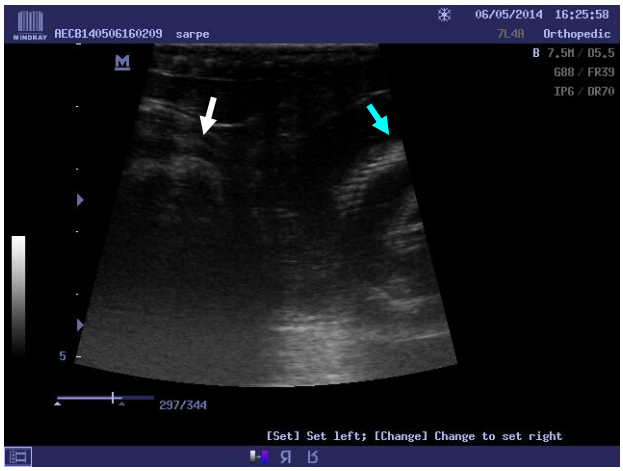

Fig. 13 Slugs (white arrow), ambryo and amniotic sac (blue arrow), *Boa costrictor*, 7,5 MHz frequency, intercostals approach

Conclusions

Ultrasound is a non-invasive diagnostic method and can be routinely used for monitoring the reproductive status of snakes, the evaluation of ovarian activity, the ovulation and the stages of ovarian follicles, as well as determining the presence of eggs in the oviduct, playing an important role in the management of reproduction in this species.

In oviparous snakes Doppler examination can not determine fertility of eggs in the oviduct, unlike in viviparous snakes where Doppler allows the assessment of embryo viability by highlighting the cardiac circulation.

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THE UTILITY OF TOLUIDINE BLUE STAIN IN ASSESSING CHROMATIN INTEGRITY OF HUMAN AND BULL SPERM

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Abstract

Background: Aim of the study: The purpose of this preliminary study was to observe the possible relationship between sperm chromatin integrity as assessed by toluidine blue stain and other semen parameters such as motility and morphology, in order to establish the utility of this analysis in routine semen evaluation.
Material and Methods: In experiment 1, frozen-thaw semen straws from a total of 5bulls which were routinely used for A.I underwent semen analysis, including chromatin integrity. In experiment 2, semen samples from 5 men attending a fertility clinic were also analyzed according to WHO criteria. In both cases, sperm chromatin integrity was assessed in both cases using toluidine blue stain. Results: In experiment 1, the mean motility was 64.54 (± 3.72%), while the percentage of sperms with normal morphology was 81.8 (±4.65). The percentage of sperm cells with damaged chromatin was 5.28 (± 0.85%). Normal morphology was positively correlated with motility (r=0.950, p=0.013), and negatively correlated with damaged chromatin sperms (r= -0.892, p=0.021). Furthermore, motility percentage was also negatively correlated with the percentage of sperms with damaged chromatin (r=-0.772, p=0.049) In experiment 2, semen was analyzed according to the WHO criteria, the mean motility being 38,33 (± 5.34). The tested samples had a mean strict morphology of 5.9% ± 3.6%, and a mean abnormal chromatin structure of 24.1% ± 14.5%. The percentage of sperms with abnormal chromatin structure was negatively correlated with the strict morphology (r= -0.738, p= 0.047) and motility (r= -0.483, p= 0.329. A positive relationship was observed between strict morphology and motility (r=0.412, p=0.035). Conclusions: the TB stain could be introduced as additionally assay in the routine semen analysis for the couples with infertility history. In the case of bulls, where the sperm chromatin damaged is lower than in humans, the sperm chromatin integrity assay could detect subfertility, condition which usually passes beneath the radar in the routine semen analysis. Acknowledgments: The current survey was supported by the National Authority of Scientific Research of Romania (POSCCE, Grant no. 1357, entitled "Infertility – a three-piece puzzle: couple investigation, infertility diagnostic, possible therapy").

Keywords: *DNA damage; sperm chromatin; bull semen; human semen;*

Introduction

Fertilization is the result of the of male and female gamete genome union, therefore, the success of this process and subsequently embryo development depends highly on the integrity and the quality of the sperm DNA (Ahmadi et al., 1999). Unlike the somatic cells, in which the chromatin has a loose structure, in sperm cells the chromatin is characterized by a tightly compact structure due to the unique associations between the DNA and sperm nuclear proteins also known as protamines (Ward et al., 1991). During spermatogenesis, the spermatid nucleus is remodeled and condensed, the histones being replaced (Steger et al., 2000). The DNA compaction is the result of disulfide bonds which are originated by the protamines. These bonds have the purpose to protect the genetic material from stressing agents such as reactive oxygen species (ROS) and high temperatures during the transit

through the male and female reproductive tract (Evenson *et al*., 2002; Oliva, 2006; Zini *et al*., 2001). There are also other factors that might affect chromatin integrity such as cryopreservation, although the precise underlying mechanism it is not completely understood. A study conducted by Thomson et al (2009), reported that the sperm DNA-damage induced by cryopreservation is generated mainly by oxidative stress and less by apoptosis. According to Mukhopadhyay *et al.* (2011), the percentage of sperms with DNA fragmentation is consecutive cryopreservation is relatively higher in bulls with poor semen quality.

The semen quality evaluation includes routinely assessed parameters such as motility, viability, morphology but recent studies suggest that markers of sperm DNA integrity may offer a better perspective of male fertility potential as compared to conventional parameters (*Tandara et al, 2013)*. The study of sperm DNA integrity is particularly relevant in an era where advanced forms of assisted reproductive biotechnologies are frequently used in both human and animal reproductive programs. According to Cho et al, (2003), there appears to be a solid relationship among sperm DNA damage (i.e., DNA fragmentation, abnormal chromatin condensation) and embryo development. In bulls, the level of sperm with damaged chromatin has been negatively correlated with motility and viability (Januskauskas *et al.*, 2003; Khalifa *et al.*, 2008). Sperm chromatin integrity has been shown to affect the reproductive potential in several studies (Dobrinski *et al*., 1994; Madrid-Bury *et al*., 2005).

Over the past decade, there has been an explosive development of in vitro assays for the determination of sperm intactness and measurement of sperm function. These assays improved the andrological diagnosis and contribute to the optimization of semen processing methods, as summarized in multiple reviews (Petrunkina et al., 2007; Rodriguez-Martinez and Barth, 2007).

Sperm DNA damage can be assessed either directly (fragmentation, oxidation) or indirectly (sperm chromatin compaction). Direct assessment of DNA damage can be made by single-cell gel electrophoresis assay, by Comet assay, or TUNEL assay (Aranvindan et al., 1997). Indirectly, the DNA damage may be measured by means of sperm chromatin integrity assays and by evaluation of nuclear protein levels (Erenpreiss et al., 2001).

Toluidine blue (TB) is a classic nuclear dye that has been used to evaluate the sperm chromatin integrity. The principle of the method is based on the detection of rupture or absent disulfide bonds. It has been used to determine sperm chromatin integrity in several species, such as bulls, horses, rabbits, buffaloes and humans (Mello and Beletti, 2002; Beletti and Mello, 2004; Erenpreisa *et al*., 2004; Beletti *et al*., 2005; Sardoy *et al*., 2008) but it is not introduced as a routine analysis in semen quality evaluation. TB stain was high correlated with SCSA, TUNEL and AO (Erenpreisa *et al*., 2003; Erenpreiss *et al*., 2004) in human sperm; while in rabbits, a study conducted by Beletti and Mello, (2004) showed a high correlation with Feulgen reaction

Material and Methods

The study was conducted thorough two individual experiments (Experiment 1 and Experiment 2), using human and bull semen.

Experiment 1

Semen samples

Ejaculates were collected using an artificial vagina, from bulls used for artificial insemination (A.I). After routine evaluation, only ejaculates that reached the minimum quality standards established by the Bull Center were processed and loaded into commercial straws of 0.25 mL, which were then frozen in liquid nitrogen.

Motility and morphology in thawed bull semen

The straws were thawed in a water bath at 37 °C for 30 seconds, motility being subjectively estimated immediately, under light microscope at x 400 magnification. For the sperm morphology evaluation, eosin-nigrosin stain was used as described by Tamuli and Watson, 1994. Briefly, 15 µL of semen were mixed with 10 µL of eosin-nigrosin stain for 30 seconds. Wet smears were prepared onto a pre-warmed glass slide and the percentage of sperm cell with normal morphology was determined under light microscope at x 1000 magnification by counting at least 200 spermatozoa. The mean motility and mean morphology was determined.

Chromatin integrity in thawed bull semen

Sperm chromatin integrity was assessed using the toluidine blue stain according to the protocol described by Agarwal and Said, 2004. Semen smears were air dried and fixed in an freshly prepared mixture of ethanol:acetone (1:1 v/v) solution for 30 minutes and then hydrolyzed for 5 minutes in 0.1 N HCl. Consecutively, the smears were washed in distilled water two times, each time for 2 minutes. The air-dried smears were stained for 10 minutes with a 0.05% toluidine blue (pH 4) prepared using McIlvain buffer. The final step consisted in evaluation under a light microscope at x 1000 magnification. Sperm cells with normal chromatin were stained light blue or green, whereas sperms with damaged chromatin were stained dark blue or violet. The percentage of sperm with abnormal chromatin was establish after counting 300 spermatozoa. Additionally, the mean percentage of sperms with abnormal chromatin was determined.

Experiment 2

Semen samples

The subjects of the present study were 5 male partners, under 35 years old, from couples attending a fertility clinic. Semen samples were collected at the clinic site, by masturbation, into sterile containers, after a period of sexual abstinence of 2-7 days. Semen was collected by masturbation. After liquefaction for 30 minutes at room temperature, each sample was routinely assessed using light microscopy.

Motility and strict morphology in human semen

Complete semen analysis was carried out after sample liquefaction at 37°C. The following parameters were taken into account: semen volume, total motility, morphology and sperm chromatin integrity. All parameters, except motility, were determined according to WHO.

Chromatin integrity in human semen

Sperm chromatin integrity of human sperm was assessed according to the same protocol as described previously for frozen thaw bull semen.

Results

In experiment 1, the mean motility was 64.54 (± 3.72%), while the percentage of sperms with normal morphology was 81.8 (±4.65). The percentage of sperm cells with

damaged chromatin was 5.28 (± 0.85%). Normal morphology was positively correlated with motility (r=0.950, p=0.013), and negatively correlated with damaged chromatin sperms (r= -0.892, p=0.021). Furthermore, motility percentage was also negatively correlated with the percentage of sperms with damaged chromatin (r=-0.772, p=0.049) (Table 1).

In experiment 2, semen was analyzed according to the WHO criteria, the mean motility being 38,33 (± 5.34). The tested samples had a mean strict morphology of 5.9% ± 3.6%, and a mean abnormal chromatin structure of 24.1% ± 14.5%. The percentage of sperms with abnormal chromatin structure was negatively correlated with the strict morphology (r= -0.738, p= 0.047) and motility (r= -0.483, p= 0.329. A positive relationship was observed between strict morphology and motility (r=0.412, p=0.035) (Table 2).

Table 1. Percentage of motility, normal morphology and abnormal sperm chromatin in frozen thaw bull sperm

Bulls	Motility	Normal Morphology	Abnormal sperm chromatin
1	59.66a	75a	6.7a
2	64.5b	82b	5.44b
3	65.25c	85b	4.9c
4	63.33a	80ab	4.6b
5	70d	87d	3.77d

Values with superscript in the same column differ significantly ($p<0.05$)

Table 2. Percentage of motility, normal morphology and abnormal sperm chromatin in human sperm

Patient	Motility	Normal Morphology	Abnormal sperm chromatin
1	23a	4.78	34.2
2	59	5.31	20.1
3	16	5.52	23.7
4	20	4.58	28.5
5	67	5.87	22.3

Discussions

Sperm quality has been routinely assessed upon certain parameters such us concentration, motility or morphology, however these classical parameters may not reveal subtle defects such as sperm DNA damage or offer a clear view over the male's fertility potential. Evenson (1999), revealed, that at least in humans, the chromatin damage may be responsible for embryo death in the early stages of embryonic development.

The results in our study showed that the overall level of sperm chromatin damage in the examined bulls was relatively low (5.28 ± 0.85%), whereas in men, the degree of sperm cell with damaged chromatin was significantly higher (24.1% ± 14.5%). One of the possible explanation for these results might be the sperm chromatin protamination. Studies conducted by Gatewood et al. (1987), Bench et al., (1996) showed that bull, stallion, hamster and mouse sperm nuclei contain significantly higher amounts of protamines (about 95%) compared to human sperm nuclei (about 85%). As a consequence, in animals, the degree of sperm chromatin condensation is higher, thus chromatin resistance to fragmentation would be higher (Irvine et al., 2000).

In contrast to human sperm, which contains protamines P1 and P2, bull, boar, ram and cat spermatozoa contain only P1 protamines which are more abundant in cysteine residues, contributing to the formation of disulfide bridges and the stabilization of sperm chromatin (Corzett et al., 2002). These differences between human and animal sperm may suggest that bull sperm chromatin is more resistant to damage than human sperm chromatin.

There are studies that either confirm the relationship between sperm chromatin integrity and other sperm characteristics (Zini et al., 2001, Kim et al, 2013).) or contradict this (Bochenek et al., 2001). However, studies conducted by Bochenek et al., (2001) on animal semen, as well as those conducted on human semen (Evenson, 1999; Larson et al., 2000; Zini et al., 2001) revealed a close relationship between the extent of sperm chromatin damage and fertility potential.

In the present study, the sample size was not statistically significant and neither of the experiments included data regarding the clinical outcomes such as IUI or pregnancy rate. Although the results are preliminary, we may conclude that TB stain proved to be a rapid, useful and nonetheless cheap method for assessing sperm chromatin integrity.

In conclusion, the TB stain could be introduced as additionally assay in the routine semen analysis for the couples with infertility history. In the case of bulls, where the sperm chromatin damaged is lower than in humans, the sperm chromatin integrity assay could detect subfertility, condition which usually passes beneath the radar in the routine semen analysis.

Acknowledments: The current survey was supported by the National Authority of Scientific Research of Romania (POSCCE, Grant no. 1357, entitled "Infertility – a three-piece puzzle: couple investigation, infertility diagnostic, possible therapy").

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HORMONAL MODULATION OF THE SPERM ACROSOME REACTION

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Abstract
Spermatozoa of both animal species and humans, must undergo a Ca^{2+}-dependent exocytotic process known as the acrosome reaction (AR) in order to fertilize the oocytes. This reaction is characterized by multiple fusions of the outer acrosomal membrane with the overlying plasma membrane and as a consequence, the content of the acrosome is released to the outside of the sperm cell. Current evidence shows that acrosome reaction (AR) might be regulated by the action of different compounds such as metabolites, neurotransmitters and hormones. The aim of the present review is to describe the modulating effect of several hormones that have been classified as inductors or inhibitors of acrosome reaction. The AR inductors category includes hormones such as progesterone, angiotensin II, atrial natriuretic peptide, cathecolamines, insulin, leptin, relaxin, while among inhibitor, estradiol and epidermal growth factor are the most important. The hormones which are located in the fluids of the female reproductive tract may also act as regulators of AR, having an essential role in the success of fertilization.

Key words: capacitation, hormone modulation, steroids, sperm acrosome reaction.

Introduction

Human and mammalian reproduction is a very complex process, based on a series of synchronized physiological events (Colombo, 2006). The success of fertilization depends on different factors such as physical (i.e mechanical), biochemical, endocrine and of course environmental. In both humans and mammals, semen is deposited mainly in the vagina and then the sperm cells ascend through the female reproductive tract.The reproductive tract ensures a microenvironment that supplies the optimum conditions for sperm survival, capacitation and migration, stages preceding the fusion with the oocyte. The fertilization process is also influenced by the morphological characteristics of both sperm cells and oocytes (Familiari et al., 2006), any alteration of the gametes having an important impact.

Spermatozoa are produced in the testicles during a process of differentiation known as spermatogenesis. The sperm cell is composed of a head, neck, mid-piece and flagellum (tail and end-piece) (Fawcett, 1975), the genetic material being located in the head, along with the acrosome (Figure 1). The flagellum contains axoneme and a set of mitochondria that supply energy required for sperm cell motility. The acrosome is located in the apical region of the spermatozoon covering the anterior extremity of the nucleus (Figure 1.) and has been described by Moreno and Alvarado, 2006, as a secretory vesicle, that produces acrosin, acrogranin, hyaluronidase as well as other enzymes present in classic organelles, such as peroxisome, lysosome (Zhao et al., 2007).

The objective of the present review is to describe the role of certain hormones in the process of human and mammal spermatozoon acrosome reaction (AR).

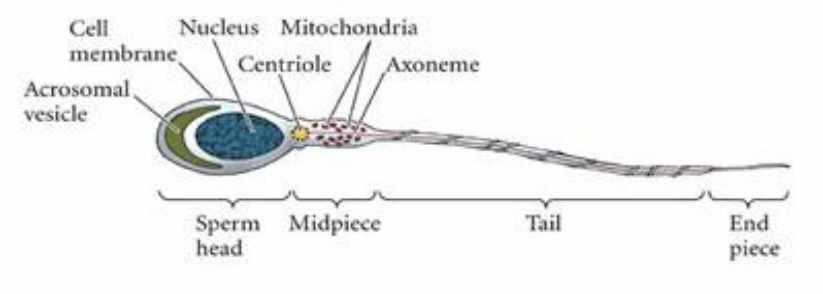

Figure 1. Mammalian spermatozoa structure
(www.courses.stu.qmul.ac.uk/SMD/Kb/microanatomy)

ACROSOME REACTION – Definition, requirements

The acrosome reaction is a process during which the content of the acrosome suffers a process of exocytosis in which the outer acrosome membrane will merge with the sperm cell plasma membrane and as consequence of this process, the acrosome content is released to the external medium (Moreno and Alvarado, 2006). In order to reach the final goal, which is fertilization, there are several events that take place during the acrosome reaction process:

a) The acrosome enzyme is released, favoring the passing of the spermatozoon through the zona pellucida;

b) The inner acrosome membrane is exposed

c) The plasma membrane located in the equatorial segment of the spermatozoa acquires fusogenic ability

The reproductive process depends on a series of events that ensure proper physical and chemical conditions to reach the final goal- oocyte fertilization (Colombo, 2006). Spermatozoa capacitation, is on these important events (Fraser, 1995) and is characterized by a number of structural and biochemical modifications that take place when the sperm cells travel along the female reproductive tract. Some of the most important changes are increased plasma membrane fluidity, decreased cholesterol content (Cross, 1998), and increased intracellular concentrations of calcium and cAMP and phosphorylation of tyrosine residues into proteins (Leclerc et al., 1996). The patterns of spermatozoon movement and motility are also modified consecutively capacitation, a process known as capacitation. These events are critical for AR since only capacitated spermatozoa can experience acrosome exocytosis (DeLamirande et al., 1997). During capacitation, the sensitivity of the male gamete is modified, triggering certain physiological changes in the spermatozoa, thus increasing the probability of oocyte fertilization.

HORMONAL MODULATOR OF AR – inducers and inhibitors

There is a high variety of compounds that have been reported to modulate AR by exerting their effect on the receptors located at the level of the spermatozoa plasma membrane (Llanos et al., 1995; DelRío et al., 2007; Vigil et al., 2008). Some of these ligands

exert an in situ AR control in specific sectors of the female reproductive tract (DelRío et al., 2007). The hormones that affect the AR will be described below (Figure 2).

Progesterone – a steroid hormone that ensures the binding with nuclear receptors and consequently the transcription of several genes possesses through a signaling pathway. In the case of the human and mammalian spermatozoon, this hormone participates in a range of processes such as: induction of AR (Vigil et al., 2008), hyperactivation and increasing the percentage of spermatozoon penetration into hamster oocytes. Usually these effects are mediated by a non-genomic signaling pathway that operates through receptors present in the spermatozoon membrane (Shah et al., 2003), but the action of progesterone is possible due to an increase in phosphorylation of cytoplasmic proteins, alongside a rise in intracellular calcium concentration (Rathi et al., 2002). Since the cumulus oophorus secretes progesterone, important levels of this hormone may be detected in the periovulatory follicular fluid (Morales et al., 1992) thus playing a stimulating effect on AR when spermatozoa are in the proximity of the oocyte. A study conducted by Cross and Razy-Faulkner, 1997 showed that a decline of plasma membrane cholesterol during capacitation would influence the degree of response the human spermatozoon to progesterone.

Estradiol- Hess et al, (1997) showed that estradion exerts an important role in male reproductive system not only in the female reproductive events as it has been known. Estrogens usually act through the union with nuclear/cytoplasmic receptors, but investigations conducted by Luconi et al., (2004), Baldi et al., (2009) revealed another mechanism of action based on a faster/non-genomic pathway. The binding of estradiol to the human spermatozoon plasma membrane is realized though out membrane receptors, a and p (Solakidi et al., 2005). This non-genomic mechanism of action is dependent on a calcium influx (Luconi et al., 2004; Aquila et al., 2004). Estradiol has been described as an AR inhibitor by Vigil et al., (2008), a lower percentage of reacted spermatozoa being observed in the samples incubated with estradiol, compared to the samples incubated without estradiol.
High concentration of estradiol were observed in the cervical mucus of some species such as rabbits, ruminants, primates and humans during female fertile period. Since the spermatozoa have to migrate though the cervical mucus in order to fertilize the egg, the estrogens from the mucus prevent the premature occurrence of the reaction during the passage, ensuring that AR will take place in a proper place for fertilization to take place.This fluid is subjected to endocrine regulation, therefore, in pathological situation, the entire fertilization process may be affected (Ceric et al., 2005; Vigil et al., 2009a).

Catecholamines (Adrenalin, Noradrenalin, Dopamine) have an important effect on the nervous system. In addition, high levels of these hormones have been determined in the fluid from the mammalian oviduct (Way et al., 2001). According to Way and Killian (2002), noradrenalin may induce acrosome reaction in bovine spermatozoa by generating higher rates of capacitation, as compared to control incubations samples. A similar effect, but more weak effect has been reported for the addition of adrenalin. During the same study, Way and Killian, (2002) did not observed any effect of dopamine on the characteristics of the bull spermatozoa.

Angiotensin II - has many physiological functions, plasma volume regulation and arterial blood control being the most important of them. Its mechanism of action is based on its union to surface receptors AT1 and AT2 (Griendling et al., 1996). Studies conducted by

Gur et al., 1998; Kóhn et al., 1998 showed that in both bovine and humans, this hormone may induce AR by binding to AT1 receptor, which is located on the tail of the spermatozoon. However, in capacitated bovine spermatozoa, the AT1 receptor is located on the head (Gur et al., 1998). The binding to the receptor is mediated by extracellular calcium concentration and may be inhibited by the administration of selective AT1 receptor inhibitor such as losartan (Gur et al., 1998). A study conducted by Sabeur et al. (2000) on equines revealed that after a 20 minutes incubation of capacitated sperm cells with a dose of angiotensin II ranging from 1 to 100 nmol/L determines a significant increase in live reacted acrosome spermatozoa Certain levels of angiotensin II have been reported in follicular fluid (Heimler et al., 1995), which supports the idea that this hormone can have a physiological role in *in vivo* induction of AR.

Atrial natriuretic peptide (ANP) - also known as atrial natriuretic hormone, is produced by heart muscle cells (Potter et al., 2009) and has a vasodilator role. ANP has been found in both oviducts (Zhang et al., 2006), and ovarian follicular fluid (Anderson et al., 1994). Zhang et al. (2006) observed that the sperm cell possesses ANP receptors and consequently, ANP may induce the AR in human spermatozoa (Anderson et al., 1994;), bovine (Zamir et al., 1995), giant panda (Zhang et al., 2005) and boar spermatozoa (Zhang et al., 2006). It has also been suggested that ANP may be involved in the regulation of the acrosome exocytosis and the fertilizing ability of mammalian spermatozoa (Zhang et al., 2006).

Insulin- is produced by the pancreas and plays an essential role in the glycemic homeostasis. Additionally, insulin participates in the processes of differentiation, growth, development and cell metabolism (Brüning et al., 2000; Saltiel and Kahn, 2001). A study conducted by Beccetti et al (2002), showed that insulin exerts an important role in the biology of human spermatozoa, sperm cells originating from men with diabetes mellitus type 2, presenting severe structural and morphological defects, reduced motility and lower ability to penetrate hamster oocytes. Lampiano and du Plessis (2008) showed that by treating human spermatozoa with insulin, a significant increase in spontaneous AR and progressive motility appeared compared to the control samples.

Leptin - is a peptide hormone produced by the gene LEP. Leptin plays an important role in glucose and lipid homeostasis, by regulating the food intake and thermogenesis (Farooqi and O'Rahilly, 2009). Lampiao and du Plessis (2008) designed a study to determine the in vitro effects of leptin on some variables of the human spermatozoon since this hormone has been shown to affect acrosome exocytosis. Treating spermatozoa with leptin determined a significantly increases in spontaneous AR as compared to control, and an increase in spermatozoa motility (Lampiao and du Plessis, 2008). Lampiao et al. (2009) showed that leptin is involved in the regulation of several processes related to mammalian reproduction. According to Riyahi et al (2011), insulin and leptin improves sperm capacitation/acrosomal reaction and viability while their effects on in vitro embryo production were not significant. There are also studies that contradict these results, according to which leptin does not have a significant effect on motility and capacitation/acrosome reaction in human ejaculated mature spermatozoa (Li et al, 2009).

Prolactin (PRL) - is synthesized and released by the adenohypophyzis lactotroph cells and its main purpose is stimulation and production of milk in the mammary glands. The hormone exerts functions which are related to mammalian reproduction (Smith, 1980), its

receptors being identified in mammalian spermatozoa (Hashimoto et al., 1988). In mice, PRL shortens the optimal preincubation period for spermatozoa to acquire capacitation (Fukuda et al., 1989), but studies conducted on human sperm showed that prolactin does not play an important role in human sperm capacitation or acrosome reaction (Stovall and Shabanowitz, 1991).

Relaxin- is a peptide hormone that plays an essential role in mammalian and human pregnancy, being responsible for the relaxation and softening of the uterus, as well as pubic symphysis distention during childbirth in humans. Essig et al., (1982), Lessing et al., (1986) noticed the presence of this hormone in human seminal plasma and Kohsaka et al., (2003) revealed the physiological effects that this hormone has on spermatozoon motility in certain species of domestic animals, such as bulls. According to Miah et al. (2006), boar spermatozoa incubated with relaxin significantly stimulates motility, the percentage of AR and glucose use. Similar results were observed on another study conducted on bovine sperm (Miah et al., 2007).

Epidermal growth factor (EGF) - known as epidermal growth hormone, is responsible for cellular growth, proliferation and differentiation in various tissues. According to Tsutsumi et al. (1986), EGF exerts a role in the proliferation of human testicles, EGF receptors being observed in both human and mammal spermatozoa (Naz and Ahmed, 1992). In human spermatozoa, EGF inhibits the AR, causing a dose-dependent decrease in the percentage of reacted spermatozoa, consequently leading to a reduction in the rate of penetrated oocytes. Naz and Kaplan (1993) reported that EGF influences the sperm cell kinetic variables, determining a decrease in velocity and flagellar beat frequency.

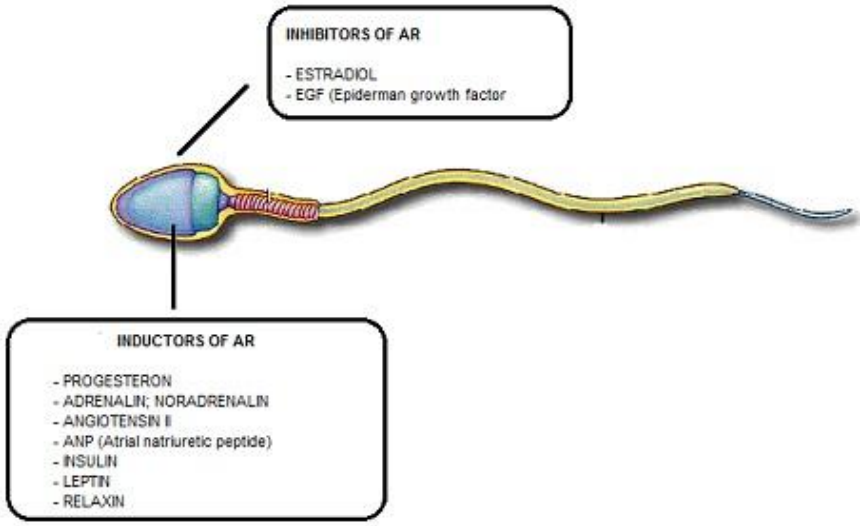

Figure 2. Hormonal modulation of the sperm acrosome reaction

Conclusions

The sperm acrosome reaction (AR) is affected by several chemical substances and metabolites, as well as a series of hormones. Although the effects of these hormones upon the

AR is a subject of active research (Vigil et al., 2009c; Baldi et al., 2009), the mechanism of action is far from being completely understood. This review was focused upon the hormones that are known to be important modulators of AR. Nevertheless, there may be other hormones acting as modulators of the AR.

Considering the effects of this hormones, it is probable safely to assume that the dynamic of hormonal levels that appears in vivo in the female reproductive tract during the reproductive cycle may regulate not only the AR, but also the timing of the events that take place before and after AR.

Due to their importance, it is worth concluding the essential role that the steroid hormones play in the fertilization process. At the level of the cervix, the estrogens which are present in the periovulatory cervical mucus exert inhibitor role over the AR since fertilization would not be possible in this region of the female tract (Vigil et al., 2008; Vigil et al., 2009b). On the contrary, in the distal third of the Fallopian tube, where the spermatozoon and the oocyte are more likely to encounter, there are high levels of progesterone in the follicular fluid, which will promote the AR, thus favoring successful fertilization (Vigil et al., 2008; Vigil et al., 2009c).

We may conclude that due to their implications in the entire process of fertilization, the effect of this hormones and their interactions should be taken into consideration when investigation possible causes of subfertility, infertility in both human and mammalian andrology.

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A STUDY ON THE PREVALENCE OF *DIROFILARIA IMMITIS* INFESTATION ON STRAY DOGS IN NORTHERN PART OF REGION MOLDOVA

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Abstract

The aim of this study is to asses the prevalence of heartworm disease at stray dogs in northern part of the region Moldova, Romania:Botoşani, Suceava, Iaşi, Piatra Neamţ. The blood samples from stray dogs were randomly selected from shelters. Dogs were screened for presence of heartworm antigen using the PetCheck® ELISA. A total of 238 blood samples were collected from 125 males and 113 female dogs between March2013 and July 2014. 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. Dogs less than 9 months of age were excluded because of extremely low probability of patent infections. For detection of microfilariae in the canine peripheral blood, it was performed also Knott test. Only five dogs were heartworm antigen positive in shelter of Iaşi County. The rest of blood samples colected from Suceava, Botosani, Piatra-Neamt cities were negative for heartworm infection. No differences in seroprevalence were observed by sex (male: 3; female 2). All dogs have not received any sort of heartworm preventive medication and the time spent outdoors was 100%. The length of positive dog's coat was: 4 short-haired (N=42), 1 long-haired (N=19).

***Keywords**:* Heartworm, microfilariae, PetCheck Canine Antigen Test.

Introduction

Heartworm canine is an important infection not only because of its severe pathology but also because of the risk of spread into previously non endemic areas. For example data obtained by several authors confirm the distribution patterns changes of canine hearworm disease. *Dirofilaria immitis* has expanded now into other territory in southern regions of Italy where is more frequently diagnosed (Traversa et al., 2010) than in northern Italy which was endemic. Furthermore heartworm infection has now spread all over the country (Otranto et al., 2009). The available information indicates an increasing distribution in the geographical range of *Dirofilaria* infections due climatic changes, and animal movement (Genchi et. al., 2011).

Cardiopulmonary dirofilariosis (heartworm disease, HWD) caused by the filarial nematode Dirofilaria immitis is a vector borne disease, affecting domestic and wild carnivores from temperate and tropical areas of the world. The intermediate hosts and vectors are different species of culicid mosquitoes of the genera *Culex, Aedes*, and *Anopheles* (Simon et al., 2009 a,b).

The global spread of heartworm infection includes all continents except Antarctica. Cases of heartworm positive dogs has been frequently reported in the USA, South America, Europe, Japan, Africa and Australia (Miller and Crosbie, 2011). Genchi and associates (2011), provided the heartworm spreading from the Mediterranean countries with the highest prevalence reported, towards previously *Dirofilaria* free central and Northern countries in Europe (Simon et al., 2012) using the Fortin and Slocombe q/model (1981) and geographical

information system (GIS) to estimate the number of Dirofilaria generations. Models predict that heartworm transmission risk in Romania increases dramatically from June to August.

In Romania, epidemiological studies were carried out in only a few counties (Popescu, 1933, 1935; Ciocan et al., 2009, Tudor et al., 2009, Paşca et al., 2008). Recent studies revealed the distribution cases of hearworm infection with the highest prevalence ranged between 3.6% and 14% in Tulcea County and 3.3% in the south, southwest and southeast parts on the country (Mircean, et al., 2012). Another study conducted in different areas from the southeastern part of the country showed 23.7-35% seroprevalence for *Dirofilaria immitis* (Coman et al., 2007). The existence of *Dirofilaria immitis* in the Moldova region, Romania with 60% prevalence on stray dogs in Galați County (data reported in the year 2012) and also in Iași County with an increase of positive owned dogs represent a constant threatening for animal and public health. To continue the monitoring of heartworm dirofilariosis, the current study provides data on the prevalence in northern part of Moldova, Romania.

Materials and metod

The study was carried out from March 2013 to July 2014. Blood samples (N=238) from stray dogs in northern part of the region Moldova, Romania:Botoşani, Suceava, Iaşi, Piatra Neamţ, were randomly selected from local shelters and tested for presence of heartworm antigen using ELISA (PetCheck®). For each individual animal were recorded data about the potential exposure factors.

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. 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 (43) and long (22) during the time of sample collection. None of the dogs in the shelter have been receiving anti-heartworm medication. From the total of 238 canine blood samples 113 were female and 125 male. 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 (98%) and specifity (100%) 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. 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

The number of dogs serologically positive for heartworm infection in this study was five (2.1% /N=238), older than 2 years with the prevalence 7.6%/N=65 recorded in Iași county. There was no difference in heartworm infection rates between female (N=32), 2

positive dogs, prevalence 6.2 %) and male (N=33, 3 positive dogs, prevalence 9%). All positive dogs have not received any sort of heartworm preventive medication and the time spent outdoors was 100%. The length of positive dog's coat was: 4 short-haired (N=43), prevalence 9.3%, 1 long-haired (N=22) prevalence 4.5%. Microfilarial infection evidenced a pattern similar to that of adult worms. So it was noticed the presence of microfilariae in all positive adult worm antigens. Regarding the geographical distribution of positive cases, we obtained a low prevalence of heartworm on stray dogs in northern part of region Moldova, Romania: a few cases in Iași county towards *Dirofilaria immitis* free on Suceava, Botoșani and Neamț counties (Table 2).

Table 1. Epidemiological variable on positive dogs from Iași County, Romania

	No. Samples	Di Ag	P. rate (%)
Age	9 months-12 years	older than 2 years	
Sex female/male	32/33	2/3	6.2/9
Coat length short/long	43/22	4/1	9.3/4.5
Antifilarial prevention		Absent	

Di. Ag –*Dirofilaria immitis* antigen; P. rate –positive rate

This study, carried out in northern region of Moldova has shown low values for the prevalence of *D. immitis* (2.1%) in the stray dog population examined using antigen detecting ELISA kit.

Table 2. Heartworm positive dogs

Areas	Number of samples	Di Ag	P. rate (%)
Iasi	65	5	7.6
Botosani	40	0	0
Suceava	100	0	0
Neamt	33	0	0
Total	238	5	7.6

Di. Ag –*Dirofilaria immitis* antigen; P. rate –positive rate

Dirofilaria immitis has been reported by other researchers in dogs in Iasi county. In 2008, was reported the first case of combined forms of dirofilariosis (Pașca S. et al., 2008). Since then 27 new cases were diagnosed owned by Iași citizens and 41 in stray dogs from shelters and public kennels. (Acatrinei D., et al., 2010). Moreover last year we reported another nine cases of *Dirofilaria immitis* in owned dogs.

The distribution of positive cases in this area could be due to favorable climatic conditions in correlation with vector competent species for *Dirofilaria immitis*. The presence of mosquitoes species of the genera *Anopheles: Anopheles maculipennis, A. atroparvus* was revealed by molecular biology techniques in Iași county (Parasca-Ivănescu et al., 2012).

These species were recognized as vectors for *Dirofilaria immitis* (Cancrini and Gabrielli 2007).

A previous study from Romania showed no positive cases for heartworm disease in Iaşi county. All serum samples used in their study were tested by Snap® 4Dx® (IDEXX Laboratories, Inc., Westbrook, ME), for *Dirofilaria immitis* antigen and other tick-borne disease: *Anaplasma phagocytophilium, Borrelia burgdorferi and Erlichia canis* antibodies (Mircean et al., 2012). By contrast, our study found that the prevalence of heartworm infection in Iaşi county was 7.6%.

There are some notable differences between our study and that of Mircean and associates (2012), regarding the number of dogs tested and the specificity and sensitivity of the antigen tests used. The most accurate test currently available is the Petcheck assay used in this study (Roemer et al., 2000, Courtney and Zeng, 2001, Pantchev et al., 2009b). A possible explanation for the fail to detect positive cases in Iaşi county is that the antigen test could give false negatives in dogs with low heartworm burdens or in blood samples from those infected only by male worms. According to a study conducted on shelter dogs in the southeastern United States reported a rate of 7.1% of the false negative antigen test results (A.H.A., 2014).

Thus in our study we found a low prevalence in Iaşi county, acorrding to Miro (2013), in low incidence areas, *Dirofilaria immitis* may serve as a reservoir of infection for other animals.

The presence of *Dirofilaria immitis* in the northeastern part of the country achieve an extension of the areas positive for the parasite from the south, southwest and southeast parts of the country.

On the other hand, our data regarding the results of heartworm infection assesed in Botosani, Suceava and Neamt counties were negatives. Given the fact that transmission of dirofilarial infections by mosquitoes is strictly dependant on a suitable climate which enables development of the larvae in the vectors (Kalluri et al., 2007; Medlock et al., 2007), these areas inserted in our study, are characterized by a temperate-continental climate with eastern influences aridity and baltic-scandinavian influences in the north, having a cool and moist character.

Evaluation of canine heartworm prevalence by sex yielded no significant differences: the results provided two positive female (N=32) and three positive male (N=33). This is in concordance with some studies with the same results reported by many authors (Song et al., 2003); Montoya et al., 2006; Rapti and Rehbein, 2010). Montoya and collegues (1998) also indicated that the generally higher infection rate in male dogs could be due to their stronger attractiveness to vectors. Other studies (Yldrim et al., 2007; Traversa D., et al., 2010) revealed significantly higher prevalence rates in males.

Older dogs show an incresead risk for infection with D. immitis, according to several studies in which dogs younger than 6 months were accepted (Song et al., 2013; Tudor et al., 2009; Lim et al., 2010). In our study, *D. immitis* prevalence was higher in dogs over 2 years of age. For many authors, age represents an important risk factor. Some authors concluded that dogs are more receptive to the infestation with *Dirofilaria spp*. compared to the young ones (Vieira, et al., 2014).

The subjects identified with *D. immitis* were stray dogs from shelters, the way of life was mainly in open spaces (Otranto D., 2013) and, concerning the fur length, the prevalence showed no significant differences. Moreover literature data correlation disproves evolution and emergence of heartworm canine in relation to hair length (Montoya et al., 2007).

In the present study the heartworm prevalence was seen in dogs that are living in areas with favourable environmental temperatures and humidity (Genchi et al.,2009) factors required by the life cycle of this parasite. Thus the infection seems to expand in the northeast areas of the country where the climate conditions support the transmission of heartworm.

Conclusions

Finally periodic epidemiological studies are required to assess changes in the prevalence of heartworm canine and to evaluate and perform an elective procedure of the preventive measures in each specific area. The benefits of doing an adequate treatment in microfilaremic reservoirs of infection in the dog population have been confirmed by many authors (A.H.A., 2014) therefore we can oversee the spreading of heartworms in non-endemic areas.

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ADVANCEMENT FLAPS TECHNIQUE TO CLOSE DEFECT AFTER TOTAL EXCISION OF A THORACIC WALL MASS IN A MALE DOG

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Abstract
A 12-year-old male mixed breed not neutered was attended presenting a large mass, 20X20 cm in diameter, located in left dorsal thoracic wall, with progressive growth over three months. Preoperative fine needle aspirates of the mass were consistent with a liposarcoma. Paraclinical exams did not show any evidence of metastasis. Surgical wide resection of the mass was performed and the tumor was submitted for histolopathology. The mass was removed according to the principles of oncological surgery (wide excision) and the defect was closed using advancement flaps. No recurrence of the tumor was noted within 2 months after surgery. According to the literature, a median survival time of 1188 days after a wide excision of liposarcomas in dogs is expected.
Keywords: *advancement flap, dog, liposarcoma, histopathology.*

Introduction

Liposarcoma, consisting of neoplastic lipoblasts (Gross et al., 2005), is a rare tumor of older dogs (<0,5% to 1% of all skin tumors (Goldschmidt & Shofer, 1992; Pakhrin et al., 2007)). In dogs and cats, soft tissue sarcomas are usually located subcutaneously and secondarily involve the dermis (Goldschmidt & Shofer, 1992), although development in extracutaneous sites may occur (Chang & Liao, 2008; Frase et al., 2009; Galofaro et al., 2008, Rodenas et al., 2006). Cases of liposarcoma have also been associated with with a foreign body (glass and microchip) in two dogs (McCarthy et al., 1996; Vascellari et al., 2004) and 1% to 2% of feline injection site-associated sarcomas were liposarcomas (Dillon et al., 2005; Esplin et al., 1996; Shaw et al., 2009). Surgical resection is the treatment of choice.

Material and methods

A 12-year-old male mixed breed not neutered was attended presenting a clinical history of an approximately 20X20 cm in diameter soft and painless large mass, located in left dorsal thoracic wall (Fig. 1), with progressive growth over three months. The X-rays showed no presence of bone changes. Preoperative fine needle aspirates of the mass were consistent with a liposarcoma. Paraclinical exams did not show any evidence of metastasis. The decision was made to remove the tumor surgically. Intravenous administration of cefazolin (22 mg/kg) was performed at induction of general anesthesia and repeated after 2 hours during surgery. Perioperative pain control was realised using tramadol intravenously. The dog was induced with propofol and maintained on inhalatory anesthesia using isoflurane in oxygen. The mass was treated with wide excision (Fig. 2), according to the principles of oncologic surgery (Withrow, 2007). Skin around the defect was undermined deep to the panniculus muscle layer to preserve subdermal plexus and direct cutaneous vessels that run parallel to the skin surface. Tissue layers were separated using Metzenbaum scissors with the

blades closed, than opening the blades, and then withdrawing the scissors in an open position. Because of the large defect (Fig. 3), advancement flaps were used to close the wound, with on two sides of the defect (Fig. 4 and 5). Tension relief was periodically evaluated attempting to approximate the skin edges (Fig. 6). Dog ears resulted were excised (Fig. 7). Intradermal and skin sutures completed the surgery (Fig. 8) .

For the histological exam, tumor bioptic samples were fixed in 10% buffered neutral formalin, embedded in paraffin, sections were made at 4 micrometers and the slides were stained by Haematoxiline–Eosine (HE) method. The slides were examined using an Olympus BX51 light microscope. The images were taken with Olympus DP 25 digital camera and processed by a special image acquisition and processing program: Olympus Cell B. Cytological exam, using Wright stain was also performed.

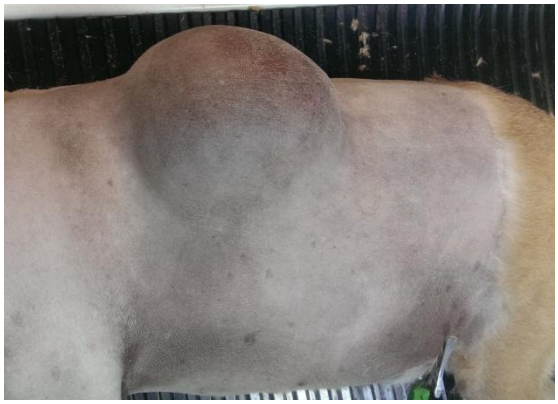

Fig. 1. Large mass of the left thoracic wall

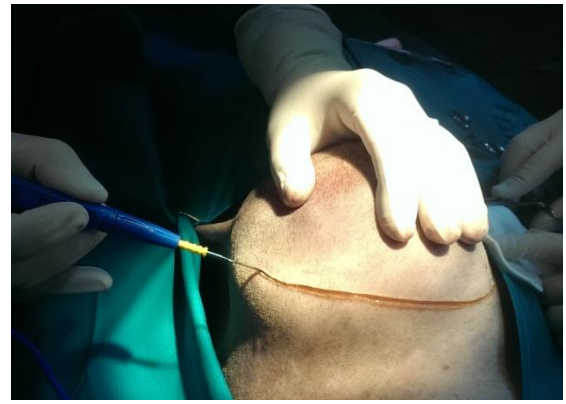

Fig. 2. Tumor excision

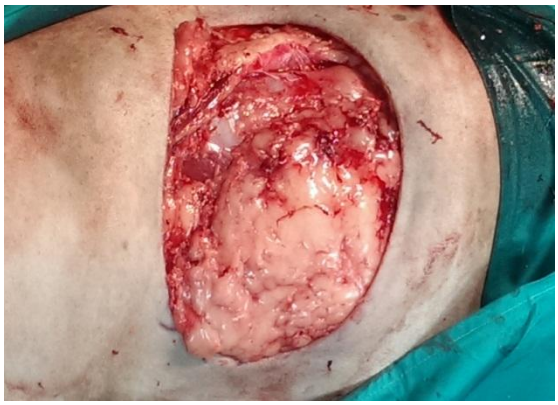

Fig. 3. The defect after tumor excision

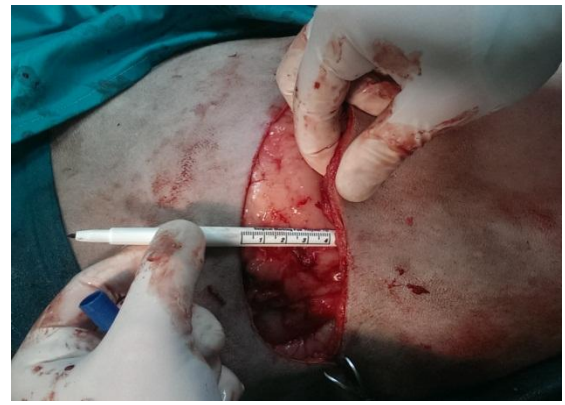

Fig. 4. Measurements for defect closure

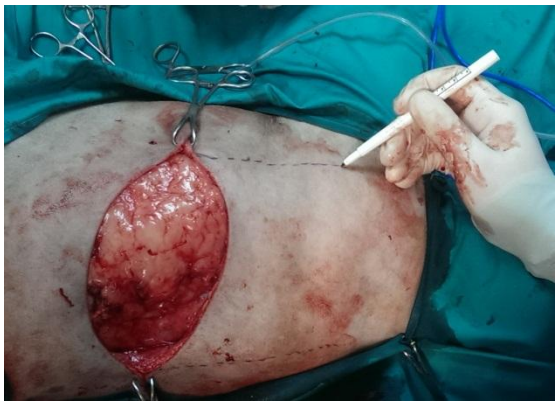

Fig. 5. Measurements for defect closure

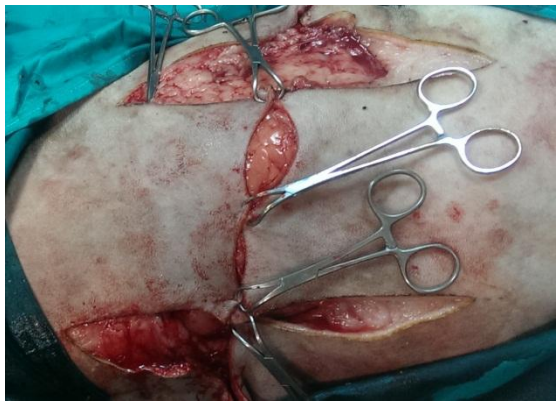

Fig. 6. Evaluation of the tension relief using Backhaus towel clamps

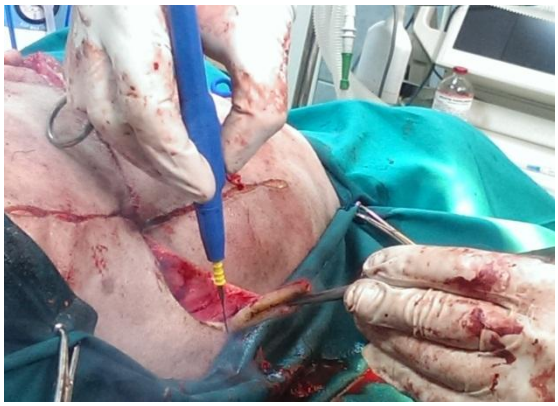

Fig. 7. Excision of "dog ears"

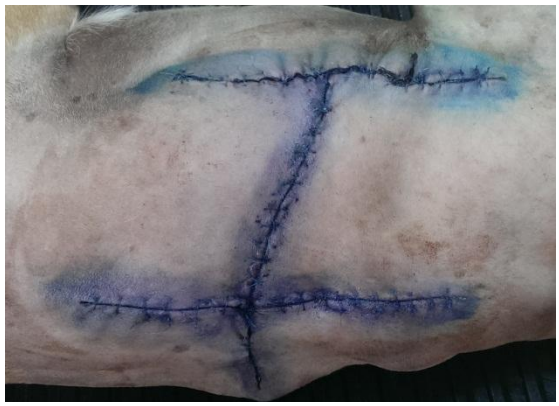

Fig. 8. Final aspect of the wound

Results and disscutions

Histological exam allowed us to establish the diagnosis of myxoid liposarcoma. Microscopically the tumor was consisted of proliferative lipoblasts at various stages of differentiation, embedded in a mucoid (myxoid) matrix seen as a basophilic ground substance (Fig. 9). Many of the lipoblasts had a single large lipid droplet that displaced the nucleus. A prominent, anastomosing capillary network was present. The tumor was not encapsulated.

Multifocally stellate to spindle shaped cells were present many of them without lipid storage, but some cells contained small lipid droplets. These cells were less differentiated than those present in the other areas (Fig. 9). In the tumor mass there were also areas of round cell liposarcoma characterized by the presence of small, round cells with an acidophilic cytoplasm and rare well differentiated lipoblasts. The mitotic index in these areas was much higher than in the rest of the tumor (Fig. 9).

Cytologic exam revealed the presence of moderately pleomorphic lipoblasts with abundant cytoplasm and the presence of various number of lipid droplets. A redish granular background material was prominent in all cases (Fig. 10).

The biologic behavior of this tumor is that it is locally aggressive, with a low incidence of metastasis (Baez et al., 2004). In a retrospective study of 56 dogs with liposarcoma, 23 of 56 were found in appendicular sites. Overall median survival time was 694 days. The only factor significantly associated with survival time was the type of excision performed, with a median survival time of 1188 days with a wide excision and 649 days with a marginal excision (Baez et al., 2004). Patients with liposarcoma have an excellent long-term prognosis if adequate local control can be attained (Tobias and Johnson, 2011). Metastasis may be common for pleomorphic liposarcomas (Baez et al., 2004).

On the follow-up examination in our case, 14 days later, the wound had healed normally. No signs of local swelling were observed. After 1 month postoperatively the dog was presented for a routine exam, having a normal quality of life without any signs of recurrence.

Conclusion

Complete surgical excision of the large mass was curative in our case, leading to an improvement of the quality of life, according to the discussions with the owner.

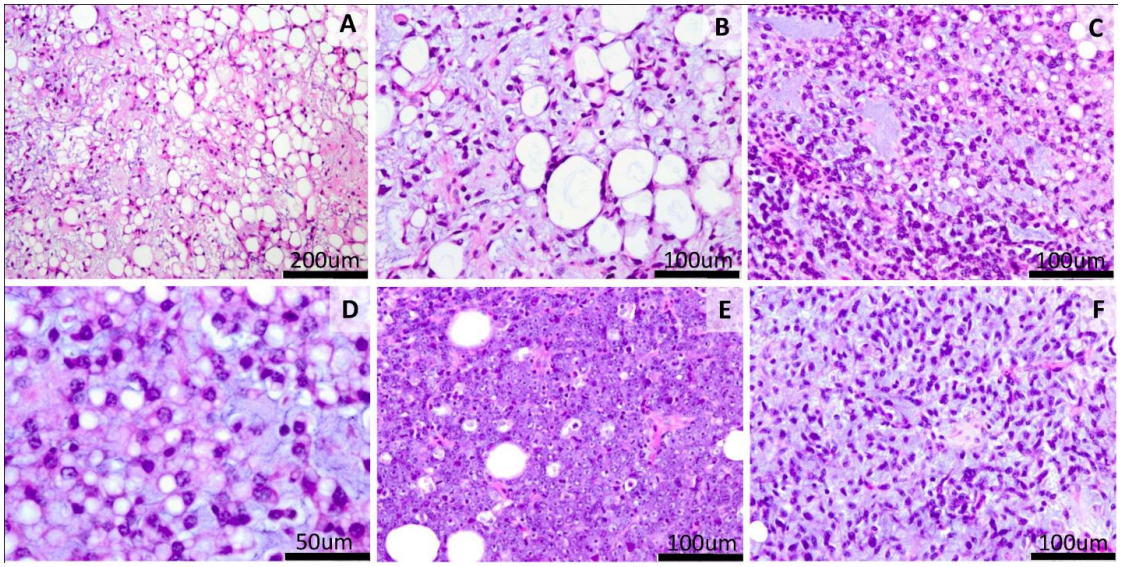

Fig. 9. Microscopical aspect of the tumor. Well differentiated lipoblast containing one, large lipid droplet with the presence of basophilic mucoid matrix (A,B); Areas of poorly differentiated round shaped lipoblasts containing small lipid droplets, or without lipid storage, multifocal areas of myxoid matrix (C,D); A poorly differentiated area, consisted of pleomorphic, immature lipoblasts with high nuclear to cellular ratio and high mitotic index, occasional mature adipocytes are present (E); Area with spindle shaped cells without lipid storage (F); HE stain, Scale bar = 200 µm (A), Scale bar = 100 µm (B,C,E,F), Scale bar = 50 µm (D).

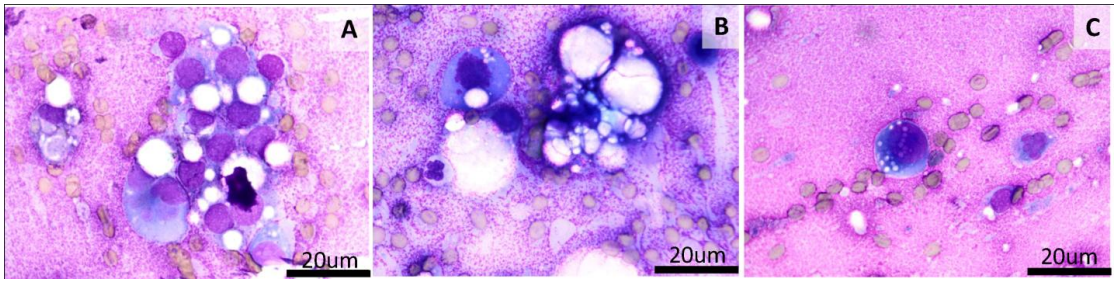

Fig. 10. Moderately pleomorphic lipoblasts with abundant cytoplasm and the presence of various number of lipid droplets; redish granular background material

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ARE THE CLIMATE CHANGES RESPONSIBLE FOR THE EMERGENCE OF NEW VECTORS AND VECTOR DISEASES

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Abstract

Based on data provided by the National Institute of Meteorology was implemented by our team a new mathematical ET30 model; the model is based on the construction of Lagrange polynomial interpolation function, using the FORTRAN programming language and applications OringiPro that allowed tracking of climate changes from 1961 up to 2013 and applying the temperatures extrapolation the predictions were made till 2030. Thus in Iasi was recorded an increase in temperature of 1.1 ° C, and at level of the entire country the increase was of 0.72 ° C, being expected the temperature to increase with 0.7-0.8 ° C by 2030.The estimates obtained with ET30 model, which show a warming of climate, keep the same tendency as the climate predictions made globally by the most prestigious research institutes in the world. The relative average humidity has recorded a decrease of 8.0%, being recorded an annual average relative humidity of 64% that is favorable to the mosquitoes development, an excessive humidity affecting their longevity.The optimal temperature of development, for the most of mosquitoes, specific to our country is 25-27 ° C, and the identification of a new species of mosquitoes, the Anopheles labranchiae, demonstrates the possibility to adapt to new vectors, existing the possibly to emerge diseases such as malaria. In recent years, the climate changes have played a major role in the emergence and spread of vector diseases like heartworm disease, babesiosis and borreliosis.

Keywords: *new mathematical model, new vectors*

Introduction

The occurring climatic changes lead to a global warming, favouring the risk of appearance and development of diseases considered tropical diseases up to the present. Now, there is a global process of emergence and re-emergence of infectious diseases with vector transmission, since they install on very wide territories as a result of the adaptation of some pathogenic agents, becoming an integral part of local ecosystems.

The optimal temperature for development is 25-27°C for most of the species of culicids specific to our country. The development may stop completely at some species or at most of them over 40°C, when high mortality appears. The life cycle of culicids is also influenced by the relative humidity of the environment. The climatic changes with the increase of the temperature even by0.5°C may lead to an increase of the mosquito population of 30-100% (Jonathan A. Patz, 2006). Among the vector diseases with an explosive evolution in Romania in the latest years are dirofilariasis and babesiasis. The development of *Dirofilaria* in the intermediate host (mosquitoes) is possible only when the temperature of the environment is over 14°C, the spreading limitation is understandable at higher latitudes (Dărăbuş*et al.*, 2006, Genchi*et al.*, 2007, Cosoroabă and Chiţimia, 2008).

In Romania, the prevalence of *D. Immitis* species is between 23.07 and 65%, according to the area under study. The evolution of canine dirofilariasis in the West of the country has developed lately. Ilie *et al*., Ciocan *et al*. reported in the past a prevalence between 4.25% and 8%. In 2012,the seroprevalence of *D. Repens* species was reported as 16%, and *D. Immitis* had prevalence of 6% (Ilie *et al.*, 2012). By molecular biology tests, the prevalence of *D. immitis* was 2.7%, and of *D. repens* was 15%.

A study realised in West Romania by Imre *et al.* in 2013 showed a prevalence of *Babesia canis*of 28.4% in rural area, and of 15.4% in urban area.

Materials and method

With the purpose to follow the influence of the environmental factors over the life cycle of vectors (mosquitoes and ticks), and of the development of pathogenic agents inside them, we performed an analysis of the temperatures, humidity, and precipitations recorded in Iași (the place where the collection of mosquitoes was performed), and in 5 areas in Romania. Consequently, we performed the analysis of the temperatures from 1961 to 2013,recorded in five weather stations in the country (Iași, Cluj-Napoca, Arad, Bucharest,and Constanța), and the analysis of the humidity and precipitations recorded in Iași between 1961 and 2013.

In order to estimate the evolution of the weather in the following years in Iaşi metropolitan area, we performed an analysis of the main meteorological parameters. This analysis is based on a mathematical model using as input parameters the thermal values of the air recorded between January 1961 and December 2013, and generating as output the most probable values of the temperature after two decades, *i.e.* around 2030.

In our climatological analysis, we used the values of the meteorological parameters recorded on the meteorological platform of Iaşi meteorological station, identified in the international system with the index STAID: 000951,defined by the following geographical coordinates: latitude – North (+) 47°10'00'', longitude – East (+) 27°38'00'', and altitude – 102 m.

In general, a climatological forecast needs a mathematical model. We chose a model different from the numerical grid methods, used by the national institutes of forecast at fixed point, because it is impossible to perform the forecast at fixed point for a very long period of time. The numerical grid models involve an evaluation of air temperature based on synoptic measurements recorded in the period preceding the forecast, which makes the models capable of a forecast with a very good probability,over 95%. The mathematical model which we propose is based on the construction of a nonlinear mathematical function allowing the estimation of the average thermal values in the following years, avoiding the forecast at fixed point, which is very difficult for a short period of time (of days), and practically impossible to perform for a long period of time, such as decades. We called ET30 the model we developed and implemented as Temperature ForecastModelfor 2030.

From a mathematical point of view, the experimental measurements are solutions of an unknown function *f*, defined as table function, represented by air thermal values at the height of 2 m above the ground surface ($T_{\text{January1961}}$, $T_{\text{February1962}}$, ..., $T_{\text{December2013}}$), recorded in the period of time between January 1961 and December 2013), called interpolation points or interpolation knots.

The interpolation function *F* must have the value of the unknown function *f* in the interpolation points T_i,it must be a continuous function on the period of time for which the measurements were made (between January 1961 and December 2013), and it must be a nonlinear function of Lagrange polynomial type.

In order to increase the precision of temperature estimation on ground surface in the following decades, we used ET30 model for three sets of thermal values recorded from January 1st, 1961, to December 31st, 2013: the first set of values is represented by the diurnal

arithmetic mean values of air temperature calculated as arithmetic mean of the values recorded in a day, the second set by monthly arithmetic mean temperature calculated as arithmetic mean of average diurnal values in a month, and the last set by the arithmetic mean temperature over five consecutive years for the same month. We proposed this method of estimation with the purpose to reduce daily fluctuations.

As we have presented above, ET30 model was developed and implemented with the purpose to estimate the value of air temperature after two decades, *i.e.* after the year 2030. For reliability, this model was tested for the years 1991 and 1992, building a model as it was presented above for the values recorded from January 1st, 1961,to December 31st, 1990, and another model to estimate the values from the years 2011, 2012 and 2013, using values recorded from January 1st, 1961, to December31st, 2010. The program sequences of the ET30 model were calculated using FORTRAN programming language, and the data processing was realised with the help of OringiPro application.

Results and discussions

We present in figure 1.1the temperature evolution in the latest five decades, which shows a slight increase starting with the year 1990. This increase is due to the emphasis of the anthropic effect, especially of CO_2 emission. The maximum, average, and minimum annual temperatures were calculated as arithmetic means of maximum, average, and minimum daily temperatures, respectively, recorded in a year. The maximum, average, and minimum annual temperatures over five years are calculated as their mean over five consecutive years.

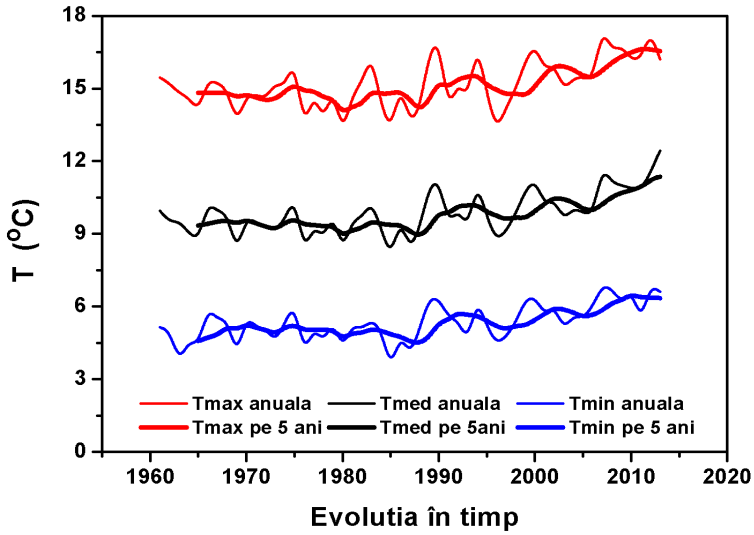

Figure 1.1. Evolution of maximum annual temperature, averageannual temperature, and minimum annual temperature from January 1st, 1961,to December 31st, 2013, calculated overfive consecutive years.

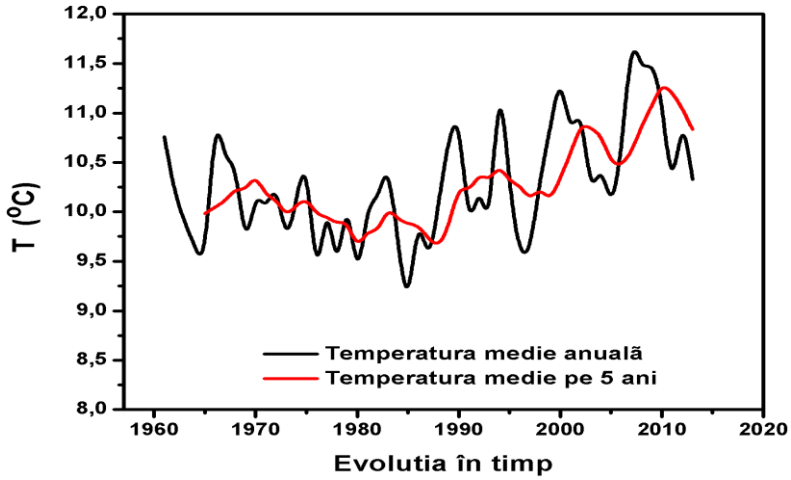

Figure 1.2. The average annual temperature, and the average temperature over five consecutive years, recorded between January 1st,1961, and December 31st, 2010, at 5 weather stations in the country

The temperatures calculated for the five weather stations in the country (Iaşi, Bucharest, Arad, Cluj-Napoca, Timişoara) also show an increase of the temperature, with a total of 0.72°C (Table 6.1). In Iaşi,the increase of the temperature by a season average of 1.1°C (Table 1.1) leads to the conclusion of a favourable climate for the development of culicids with the possibility of adaptation of new species.

Table 1.1. Increasing current temperatures by season, compared to the period when malaria was eradicated

Season	(2004-2013) (1961-1970) Iasi (°C)	(2004-2013) (1961-1970) Romania (°C)
Winter	1.2	1.4
Spring	1.3	0.8
Summer	1.7	0.9
Autumn	0.2	-0.2
TOTAL	1.1	0.725

\+ means increase of the temperature
\- means decrease of the temperature

The second column represents the increase of the temperature in 2013 (the average of 10 years,from 2004 to 2013), as compared to the average of 10 years for the period when malaria was eradicated (1961-1970).

The third column represents the increase of the temperature in 2013 as compared to the 60's, for the average of five weather stations in Romania (Iași, Cluj-Napoca, Arad, Bucharest, and Constanța weather stations).

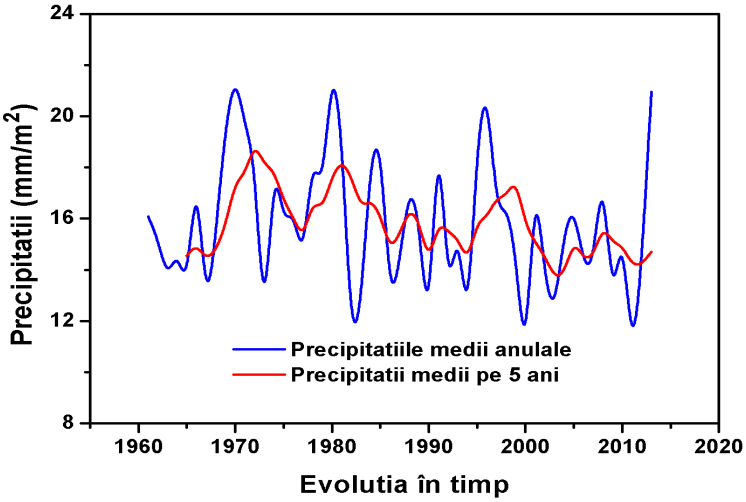

Figure 1.3. Average annual precipitations, and average precipitations over five consecutive years,recorded between January 1st, 1961, and December 31st, 2013

The precipitations recorded in Iași between 1961 and 2013 (fig. 1.3) do not show major changes, the variations being maintained within a narrow range.

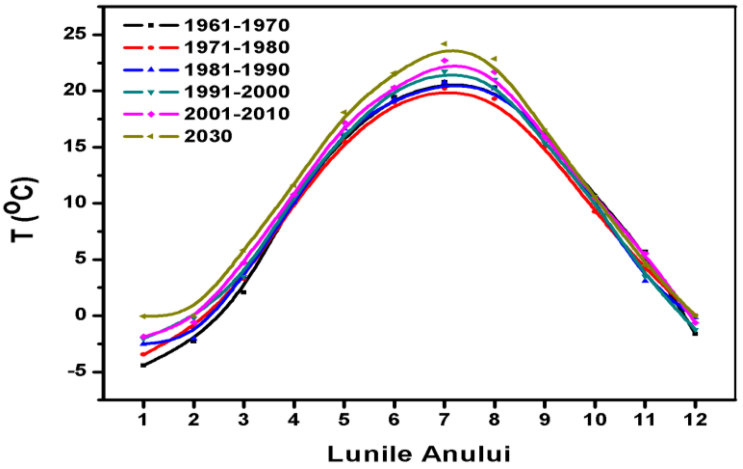

Figure 1.4 Average monthly temperatures calculated on decades, recorded at Iasi meteorological station between 1961 and 2013, and estimated for the twelve months of2030 with ET30 model.

Extrapolating the evolution of the temperatures in 2030 (fig.1.4), we can see a slight increase of the temperatures by an average of 24°C in 2030, which may assure a favourable climate for the development of culicids, the optimal temperatures for development being between 23 and 25°C.

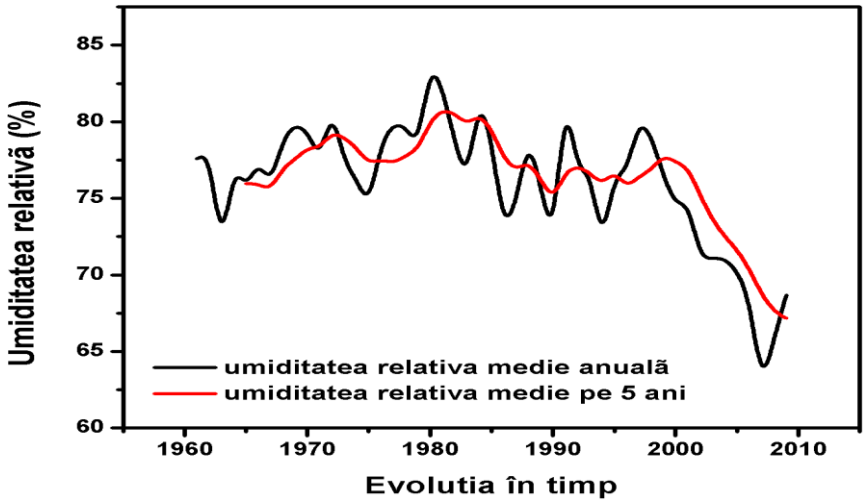

Fig. 1.5. Average annual relative humidity, and average relative humidity over five consecutive years,respectively,recorded between January 1st, 1961, and December 31st, 2010.

Table 1.2. Air relative humidity at a height of 2m above ground surface

Season	(2000-2009) (1961-1970) Iasi
Winter	-6.7 (%)
Spring	-10.6 (%)
Summer	-10.0 (%)
Autumn	-4.8 (%)
TOTAL	-8.0 (%)

- meansthe decrease of relative humidity, Iașiweather station

The average relative humidity registers a decrease by 8.0% as compared to the period when malaria was eradicated,recording an average annual relative humidity of 64% (fig.1.5), favourable to the development of culicids, an excessive humidity reducing their longevity.

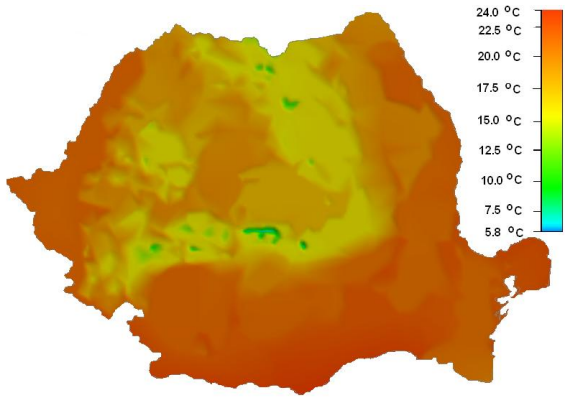

Figure 1.6. Map with arithmetic mean temperatures calculated for the month of July at weather stations of the network of National Administration of Meteorology in Romania for 2013

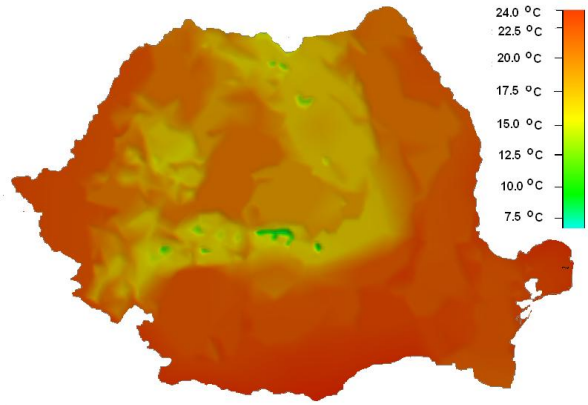

Figure 1.7 .Temperature map estimated in July of 2030, using ET30model

The representation of the temperature gradient inRomania for the month of July was realised with the use of ArcGis software.

The results obtained with ET30model, developed and implemented by us, show a different evolution in the four seasons of the year for air temperatures at ground surface in the months of the year 2030. Our analysis shows a slight increase over the value of 0.1°C in spring, a significant increase by 0.7-0.8°C in summer, especially in July – August. However, in the year 2030, the thermal characteristics of the air at ground level do not change compared with the average of the latest 30 years during autumn. In winter, the air will have a higher temperature in 2030, compared to the average of the latest 30 years, by 0.2-0.3°C, especially in the month of December. There is a slight warming in the month of July, 2030 (0.8°C) (Fig.1.7.).

The estimations obtained with ET30 model showing a warming of the climate in Iaşi metropolitan area keep the same tendency as the global weather forecast by the most famous research institutes in the world, like NIES (National Institute for Environmental Studies in Japan), CCCMA (Canadian Centre for Climate Modelling and Analysis), CSIRO (Commonwealth Scientific and Industrial Research Organisation in Australia), HCCPR (Hadley Centre for Climate Prediction and Research in United Kingdom), MPIM (Max-Planck-Institut für Meteorologie), and NCAR (The National Center for Atmospheric Research in USA). These institutes show a global warming by 0.8-1.7°C until 2030.

The hypothesis that a mini ice age would start globally (Easterbrook, D.J., 2001) after 2007 is infirmed by the values recorded after this year, which continue the warming tendency of the climate.

Conclusions

The implementation of a new mathematical model, the ET30 model,based on the construction of an interpolation function of Lagrange polynomial type, offers the possibility of a forecast with over 95% probability. The program sequences of the ET30 model were calculated using FORTRAN programming language, and the data processing was realised with the help of OringiPro application.

The average monthly temperatures calculated on decades, recorded at Iaşi meteorological station between 1961 and 2013, and estimated with the help of ET30 model for the twelve months of the year 2030, show an increase of average temperature up to 24°C. Comparing with the period of the 60's until 2013 (the average of 2004-2013), an increase by 1.1°Chas been recorded in Iaşi, and an increase by 0.72°Chas been recorded in the whole country, proving the risk of adaptation of new vector species to the climate of our country, as well as the possibility of transmission of new vector diseases.

The map which we realised with the implemented ET30 mathematical model concerning the current situation in Romania (analysis of the temperatures from the entire network of the National Administration of Meteorology in Romania) for the month of July, extrapolated over 30 years, shows a warming by 0.7-0.8°C, maintaining a favourable climate for the development of the culicids (on longer periods, at temperatures of 27-30°C, the life cycle of the culicidsis affected).

The values that we obtained with the ET30 mathematical model, showing a slight increase over 0.1°C in spring, a significant increase by 0.7-0.8°C in summer, especially in July – August, and by 0.2-0.3°C in the cold season, coincide with the global climate forecasts performed by the most famous research institutes in the world, which show an increase by 0.8-1.7°C until 2030, demonstrating therefore the validity of the mathematical model we implemented, ET30.

From a climatic point of view, the temperatures are favourable to the development of vectors and pathogenic agents inside them, leading to an increase of the prevalence of vector diseases, as well as to the possibility of emergence of new diseases on Romanian territor

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promote excellence in research development and innovation in priority areas- agronomic and veterinary medical knowledges based society"

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DETECTION OF BOVINE PAPILLOMAVIRUS -1/-2 DNA BY CLASSICAL PCR FROM SPONTANEOUS CUTANEOUS FIBROPAPILLOMAS IN CATTLE

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Abstract

Bovine papillomaviruses (BPV) are double-stranded DNA tumoral viruses able to induce epithelial lesions that occasionally are associated with uncontrolled cell division or malignant conversion. To the date, thirteen different BPV types were associated with skin and mucosal pathology in cattle and out of these, only two types (BPV-1 and -2) were incriminated to induce cutaneous fibropapillomas, characterized both epithelial and dermal proliferation. The aim of this study consisted in detection of BPV -1/-2 DNA in cattle cutaneous fibropapillomas. In order to detect the viral DNA, five samples collected from cattle suffering from cutaneous fibropapillomatosis were collected and tested by classical polymerase chain reaction using a consensus primer pair that is able to amplify the E5 open reading frame of both BPV -1 and -2. The PCR test confirmed the presence of the BPV -1/-2 DNA in the tumour samples and in the positive control, as well. The used primers are not able to discriminate these two types involved in the etiology of cutaneous fibropapillomas. Recent reports are describing a great number of co-infections with different BPV types in the same lesion, thus being almost impossible to link the specific morphology of the lesion with a specific BPV type. Since these two types are widely distributed among cattle population, the PCR test using these primers proves to be a useful tool for identification of BPV DNA in first instance, instead of using degenerated primers. This is the first time when cattle from Moldova affected by cutaneous fibropapillomatosis are proven BPV positive using BPV-1 /-2 specific primers.

Keywords: *DNA, BPV, Cattle*

Introduction

Bovine papillomaviruses (BPV) are DNA tumoral viruses able to induce epithelial lesions that occasionally are associated with uncontrolled cell division or malignant conversion (Nasir and Campo, 2008). To the date, thirteen different BPV types were associated with skin and mucosal pathology in cattle and further classified into three genera: Deltapapillomaviruses (BPV-1, -2, -13) inducing cutaneous and mucosal fibropapillomas (Nasir and Campo, 2008; Lunardi et al., 2012), Xipapillomaviruses (BPV-3, -4, -6, -9, -10, -11, -12) (Hatama et al., 2008; Hatama et al., 2011) responsible for the occurrence of true papillomas, strictly epitheliotropic and Epsilonpapillomaviruses (BPV-5, -8) inducing both true papillomas and fibropapillomas (Tomita et al., 2007a) and an as yet unassigned PV genus (BPV -7) (Ogawa et al., 2007). Out of these only two types (BPV-1 and -2) were incriminated to induce cutaneous fibropapillomas, characterized both epithelial and dermal proliferation (Bocaneti et al., 2014). Both BPV-1 and -2 have a genome of 7900 bp and are composed of early (E) and late (L) genes which can be divided into several open reading frames: viral replication (E1), regulation of transcription (E2), coding for cytoplasmic proteins (E4), transforming proteins (E5, E6 and E7) as the early genes and L1 and L2 coding for capsid proteins as late genes (Chambers et al., 2003; Nasir and Campo, 2008).However,

BPV-1 and BPV-2 are distinct genotypes, as defined by their 84% nucleotide homology of the L1 gene. Their L1 amino acid sequence identity reaches 92%, which is comparable to the similarity of HPV6/11 (92%) or HPV18/45 (87%) (Shafti-Keramat et al., 2009).

Cutaneous fibropapillomas are prevalent in almost every cattle herd causing economic losses when teats or skin are affected (Pangty et al., 2009).

The objective of this study was to determine the presence of BPV-1/-2 DNA in cattle cutaneous fibropapillomas by using PCR and BPV-1/-2 specific primers.

Material and method

Samples F1-F5 were collected from cows suffering from naturally occurring cutaneous fibropapillomas from Moldova, Romania. Briefly, the tissue samples were divided in two parts: one part was stored at -80^0 C for biochemical analysis and one part of each sample was fixed in 10% neutral buffered formalin and routinely embedded in paraffin. The samples were sectioned and stained with Haematoxylin & Eosin for histopathological assessment. The diagnosis was assessed following the guidelines proposed by Goldschmidt et al., 1998 and all the samples were diagnosed as cutaneous fibropapillomas.

Genomic DNA was extracted from the fibropapilloma samples F1-F5 using the DNeasy Blood and Tissue kit (Qiagen), according to the manufacturer's protocols.

PCR assay was performed using a Master Mix kit (Applied Biosystems) following the manufacturer's instructions. Amplification of the E5 open reading frame (ORF) was carried using a BPV-1 and -2 consensus primer pair, BPV-E5 F (TTGCTGCAATGCAACTGCTG corresponding to BPV nucleotides 3915 to 3934) and BPV-E5 R (TCATAGGCACTGGCACGTT corresponding to BPV nucleotides 4208 to 4225) amplifying a fragment of 311 bp from nucleotide 3915 to 4225.

The reactions were performed in a total volume of 25µL, containing 50ng of DNA, 3 mM MgCl2, 200 µM of each dNTP, 0.25 U AmpliTaq Gold Polymerase (Applied Biosystems), and 0.5 µM of each oligonucleotide primer. Reaction conditions were: denaturation for 5 min at 94^0 C, followed by 2 cycles of denaturation at 94^0 C for 1 min, annealing at 55^0 C and extension at 72^0 C for 1 min and followed by 35 cycles of denaturation at 94^0 C for 30 s, annealing at 52^0 C for 30 s and extension at 94^0 C for 30 s. Amplicons were separated by electrophoresis in 1% agarose gels with Tris acetate ethylene diamine tetraacetic acid (EDTA) buffer (TAE; 40 mM Tris, 1 mM Na_2EDTA, 20 mM acetic acid), stained with ethidium bromide and visualized under ultraviolet light. A blank sample consisting of reaction mixture without DNA and a positive sample consisting of BPV-2 cloned genome (Roperto et al. 2012) were used.

Results and discussions

The lesion morphologies detected included cauliflower kyperkeratotic appearance, multiple, with different body location (back, shoulder and head) (fig. 1A). Histopathologically, the tumours were characterized by epidermal hyperplasia, hyperkeratosis and koilocytosis and were all classified as cutaneous fibropapillomas (fig. 1B).

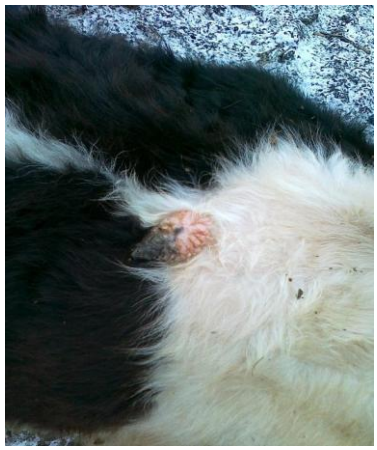

Fig. 1A Hyperkeratotic lesion, with back localization

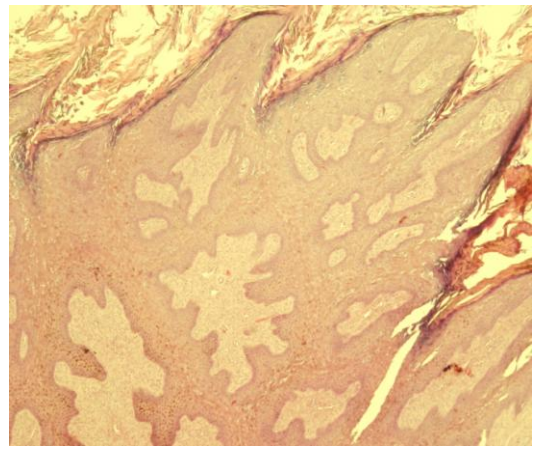

Fig. 1B Histological analysis of cattle cutaneous fibropapilloma; epidermal hyperplasia and hyperkeratosis. HE x 100.

The PCR assay confirmed the presence of the BPV -1/-2 DNA in the tested samples and yielded a specific product of 311 bp (fig. 2).

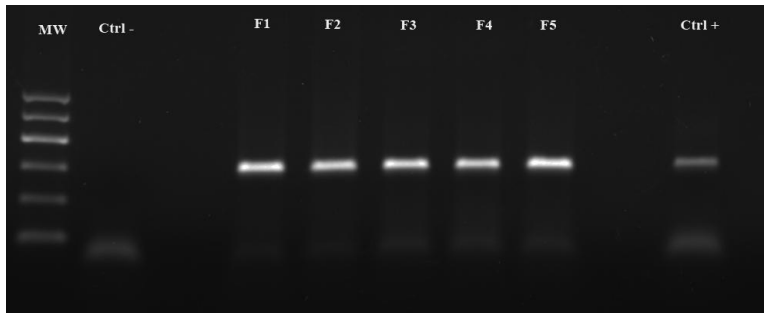

Fig. 2 Amplification of BPV-1/-2 DNA in cutaneous lesions. A representative electrophoresis gel of PCR products using E5 orf primers is shown. MW, 100 bp molecular weight marker; Ctrl-, no template control; Lanes F1–F5, samples; Ctrl+, positive control.

Similarly, Borzacchiello et al., (2008) used these primers in order to confirm the etiology of equin sarcoid, revealing the presence of BPV DNA. Since the BPV-1 and -2 types shows a high homology (Shafti-Keramat et al., 2009), it was possible to design primers that may recognize a region high conserved among these two types. However, to distinguish these two types it is necessary either the sequencing or the use of gene-specific primers for BPV-1 and BPV-2. The utility of these primers proves to be valuable since a rapid detection of the etiologic agent of fibropapilloma might be performed, avoiding the use of degenerated primers followed by the use of BPV specific primers.

Recent reports are describing a great number of co-infections with different BPV types in the same lesion, thus being almost impossible to link the specific morphology of the

lesion with a specific BPV type (Batista et al., 2013). Since these two types are widely distributed among cattle population, the PCR test using these primers proves to be a useful tool for identification of BPV DNA in first instance. This is the first time when cattle from Moldova affected by cutaneous fibropapillomatosis are proven BPV positive using BPV-1 /-2 specific primers.

Conclusions

This is the first time when cattle from Moldova affected by cutaneous fibropapillomatosis are proven BPV positive using BPV-1 /-2 specific primers. The presence of co-infections, mainly with BPV-1 and -2 types indicates a wide spread of these two types in cattle population.

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BRAINSTEM AUDITORY EVOKED RESPONSE IN DOGS WITH CENTRAL VESTIBULAR SYNDROME

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Abstract

Background *– Signs of vestibular disease include: nystagmus, hearing loss, vomiting, signs of brain stem dysfunction or of increased intracranial pressure. The* ***goal*** *of the curent study is to describe the brainstem auditory evoked response (BAER) acquired with surface electrodes in dogs with central vestibular syndrome (CVS). Other purpose of this study was to determine the prognosis value and the possibility of this test to differentiate central from peripheral origin of vestibular syndrome.* ***Material and methods*** *- BAER were recorded in ten dogs with CVS and in ten healthy dogs by monaural and binaural stimulation, with stimulus intensities of 90 dBSPL using surface electrodes and air-conducted clicks. To compare ears of patients with lateralised CVS with healthy dogs ears, the latencies and amplitudes waves I, II, III and V were analysed in SPSS 20 with Mann-Whitney test for 2 paired samples, at a significance threshold of P < 0.05. The differences between right and left sides were compared using Wilcoxon Signed Ranks Test.* ***Results*** *– BAER reflected morphological changes of waves I, II, III and V in 7/10 dogs with CVS and decreased amplitudes of all waves at all dogs with lateralised CVS. The amplitude values registered for all wave in ears of dogs with CVS were: 2.49±2.18 for wave I, 2.4±2.52 for II, 0.61±1.64 for III and 1.51±1.25 for V, being inferior to references values for monaural stimulation. P values obtained were = 0.014 for wave I amplitude, 0.031 for II and III and 0.032 for V. No statistical differences were observed for BAER latencies between patients with CVS and healthy dogs to mono- and binaural stimulation, which demonstrates that the latency is kept normal and the presence of biauricular interaction and CVS dogs did not perceive two sounds at the same time.* ***Conclusion*** *– In vestibular syndrome, brainstem auditory evoked response recorded with surface electrodes were characterised by decrease amplitudes without changes in latencies waves, which may show an impairment of strength, but not the speed of transmission of information between the nuclei of the auditory pathway.*

Keywords*: BAER, central vestibular syndrome, dog*

Introduction

All animals that have a vestibular system—from fish to mammals - can be afflicted, including cats and dogs.The vestibular system (VS) is the primary component of the nervous system responsible for maintaining equilibrium and balance. It is a sensory system that relays so called «special proprioception» (DeLahunta and Glass, 2009).

The VS is involved in the detection of the static position of the head as well as its acceleration, deceleration and rotational movements.The vestibular syndrome is not important in the initiation of movement, but is need to function efficiently to coordinate head position, eye movements and the extensor muscle tone – all of which depend on the position of the body in space and its movement (Jaggy and Platt, 2010).

The vestibular system consists of a peripheral (labyrinth with sensory receptors and the vestibular nerve) and a central part (vestibular nucleus in the brain stem) (Wilson, 2005).

The major causes of central vestibular disease are inflammatory/infectious diseases or neoplasia. Organophosphate intoxication, liver disease (with metabolic brainstem

degeneration), hypothyroidism (Jaggy, 1994) and thiamine deficiency can occasionally result in central vestibular disease (depending upon the species of animal), but these causes are far less than the inflammatory or neoplastic causes. In dogs, canine distemper virus, granulomatous meningoencephalitis, toxoplasmosis, neosporidiosis, aspergillosis, cryptococcosis, steroid-responsive meningoencephalitis, Lyme's disease, Rocky Mountain spotted fever and ehrlichiosis are the most common inflammatory and infectious diseases recognized. In cats, feline leukaemia(FeLV), feline infectious peritonitis (FIP), and cryptococcosis are the most common infectious diseases.

Any of the primary brain tumors can occur in dogs, while only meningiomas are common in cats. Cats who are not eating and stressed can easily develop thiamine deficiency and this should not be overlooked in treating sick cats with vestibular signs (Poncelet, 2004).

CVSis characterized byfalling, torticollis, head tilt, vestibular ataxia, and positional strabismus. Cranial nerve deficits, proprioceptive deficits and motor deficits indicate brainstem damage affecting the vestibular nuclei and sensor and motor pathways which course through the vestibular region of the brainstem. In addition, the nystagmus seen in central vestibular disease will often be vertical or positional in nature, supporting the location of the disease process within the brainstem or cerebellum. If there is whole body and head tremors, the lesion is likely to be within the flocculonodular lobe of the cerebellum (Thomas, 2000).

Brainstem auditory evoked potential study (BAER) - is probably the most used electrophysiological test in veterinary medicine. BAERs are generated by different tracts and nuclei that are part of the auditory pathways (cochlear receptor, acoustic nerve, cochlear ventral and dorsal nuclei, superior olivary nucleus, trapezoid body, lateral lemniscate, inferior colliculus, medial geniculate body, lobe projection area).

The goal of the current study is to describe the brainstem auditory evoked response (BAER) acquired with surface electrodes in dogs with central vestibular syndrome (CVS). Other purpose of this study was to determine the prognosis value and the possibility of this test to differentiate central from peripheral origin of vestibular syndrome.

Material and methods

This study was conducted inFaculty of Veterinary Medicine,Department of Clinical Sciences,Internal Medicine (Neurology).The group was composed of 20 dogs, out of whom 10 had vestibular signs suggestive of deficit central vestibular syndrome: falling, torticollis, head tilt, vestibular ataxia,positional strabismus,alteration of consciousness and proprioceptive sensitivity of the forelimbs (fig. 1) and 10 healthy dogs, belonging to the control group. Patients were ofmixed breeds, between 3 and 11 years old.

The diagnosis was made on the basis on anamnesis, clinical appearance and imagistic examinations (MRI, CT, radiology and ultrasonography). Each patient was tested by brainstem auditory evoked response. Radiographic evaluation (tympanicbulla and thoracic cavity) and ultrasonography examination (abdomen) were performed to exclude the systemic conditions with spread to the nervous system (liver and kidney diseases or metastatic neoplasia), in all patients.

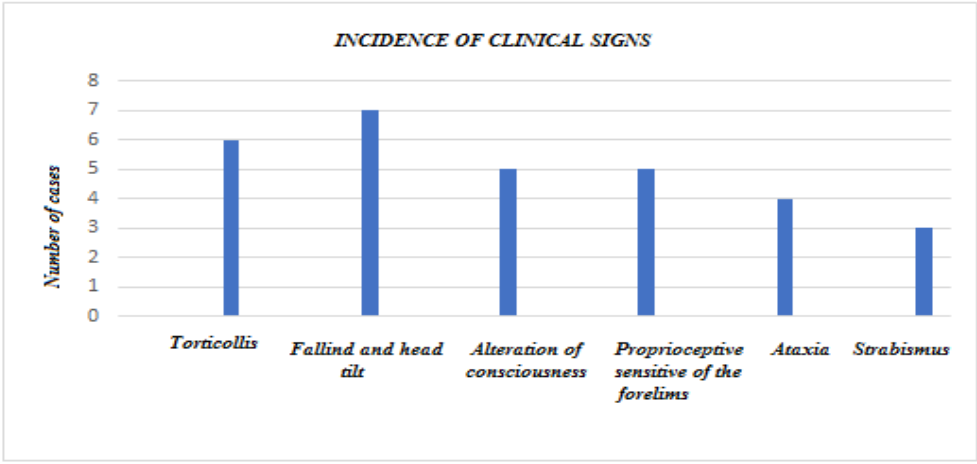


Figure 1. Incidence of clinical signs

The electrophysiological evaluation was carried out with the Neuropack S, MEB 9400K electrodiagnostic system (Nihon Kohden) in the auditory brainstem response program (ABR). Examination was made under generalanaesthesia with medetomidine hydrochloride (Domitor, Pfizer, Finland) in dose of 0.05 mg kg^{-1} inj. i.m. 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 paste (EEG Paste Elefix® NIHON KOHDEN).

Impedance was lower than 5Ω. Alternating click stimuli of 0.1 ms were applied through earphones inserted into the auditory canal. Monaural and binaural stimulation were performed at the intensity of the stimulus of 90 dB SPL (decibel sound pressure level). The non-tested ear was masked with white noise of an intensity 40 dB lower than that used on the tested ear. Each waveform was the average of 1000stimulations (Venker-van Haagen et al., 1989), using a High-cut filter of 100 Hz and a Low-cut filter of 3000 Hz (Kawasaki and Inada, 1992; Arnold, 2007). Artifactual data were automatically rejected; when rejected waveforms represented more than 5% of the average, the tests were repeated. The waves were manually labelled by the same examiner, each positive peak receiving a roman score from I through to V, and latencies of waves I, III, and V were measured, as well as the intervals I-III, III-V and I-V.

The statistical interpretation of the results was made with the software Statistical Package for the Social Sciences for Windows (SPSS) 16. Wilcoxon Signed Ranks Test for 2 paired samples was used to determine the presence/absence of statistical differences between the two ears(right ear and left ear) or between a ear(right ear or left ear) and binaural stimulation. The significance threshold was $P< 0.05$.To compare ears of patients with lateralised CVS with healthy dogs ears, the latencies and amplitudes waves I, II, III and V were analysed with Mann-Whitney test.

Results and discussions

Vestibular syndrome was clinically diagnosed based on falling torticollis, head tilt, alteration of consciousness, proprioceptive sensitivity of the forelimbs, vestibular ataxia and positional strabismus.

Torticollis was observed in 6 of 10 dogs and range from a few degrees to 45 degrees, being caused by the loss of the antigravity muscle tone of the neck on the same side as the lesion (Jaggy and Platt, 2010). This created difficulty to keep the patients' position.

Falling and the head tilt were seen in 7 cases and vestibular ataxia met 4 of them. After Kent and Platt (2009), they are caused by damage of the lateral vestibular nucleus ipsilateral to the lesion which transmits wrong information to the vestibulospinal tract the same defective part.

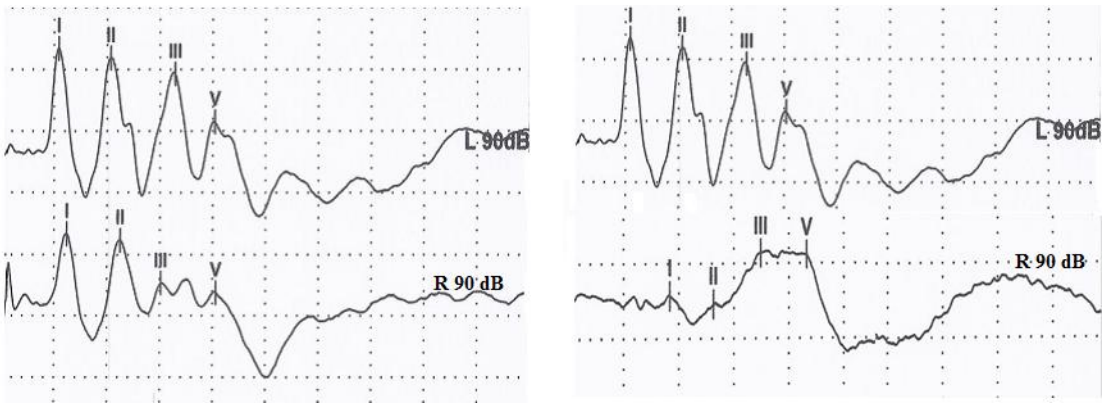
In 5 patients, central vestibular system damage was not difficult to diagnose after neurological examination, due to alteration of consciousness and proprioceptive sensitivity of the forelimbs. Cortical depression may be due to damage to ascending reticular system. Vestibular nuclei transmitted from vestibulo-thalamic tracts information to thalamus, so that depression may occur without affecting the ascending reticular system, but secondary to maltransmission of the information from affected vestibular nuclei to the thalamus (Kent and Platt, 2009).Three of themmanifested positional strabismus.

BAER reflected morphological changes of waves I, II, III and V in 8/10 dogs with CVS (presence of waves III-V plateau, and multiple discharges between waves III-V, absence of waves II-V) and decreased amplitudes of all waves at all dogs with lateralised CVS (table 1).

Table 1. Waves morphological changes in BAER testing and the final diagnosis in patients in the study

PATIENTS	*PRESENCE OF WAVE III-V PLATEAU*	*MULTIPLE DISCHARGES*	*ABSENCE OF WAVE II-V*	*DIAGNOSIS*
1	+			Unknown
2		+		Cranio-cerebral trauma
3		+		
4			+	AVC
5	+			Tumour
6				Carre's disease
7				Unknown
8	+			Hydrocephalus
9		+		Tumour
10	+			Hydrocephalus AVC

Morphological analysis of waves I, II, III and V in dogs with central vestibular syndrome has revealed presence of multiple discharges in 4 cases (figure 3), presence of plateau between waves III-V in 3 cases (figure 2) and absence of waves II, III and V in a dog (figure 3a). Most profound changes of the brainstem auditory evoked response route are determined by the conditions that lead to compression of the brainstem (hydrocephalus, tumour).

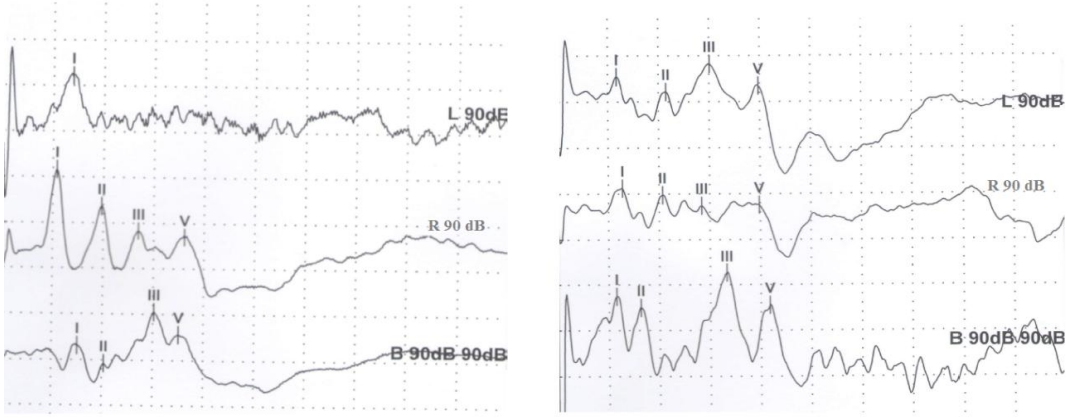


A. ***B.***

Figure 2. BAER trace done for an intensity of sound of 90 dB A. 3 years old crossbreed dog. The amplitudes of waves I-V are lower on right ear stimulation than on the left. B. 8 years old Caniche bitch. Presence of a plateau between waves III-V on right ear stimulation

In hydrocephalus, an explanation of these results would be that the excessive accumulation of CSF exert compression of the brainstem. Wave amplitudes were affected II-V, brainstem damage extending throughout its length, including both the cochlear nucleus and inferior coliculli.A similar hypothesis issues and Arnold et al. (1975) in children hydrocephalus, considering changes in BAER wave amplitudes are due to ventricular dilatation.

In veterinary medicine, disappearance of one or all waves have been reported in tumour pathology (Steiss et al., 1994; Fischer et al., 1994).



A. ***B.***

Figure 3. Vestibular syndrome.BAER trace done for an intensity of sound of 90 dB. A.5 years old Rotweiller.Presence of wave I, there is a significant decrease in amplitudine and dispersion of all centrally generated waveforms in this side - absence of waves II-V on left ear stimulation and presence of multiple discharges on right and binaural stimulation B. Golden Retriever, 11 years old, male. Presence of multiple discharges.

Mean values ± SD for latencies and amplitudes for an intensity of sound of 90 dB SPL, obtained for the diseased ear and the contralateral one in dogs with central vestibular syndrome are shown in table 2.

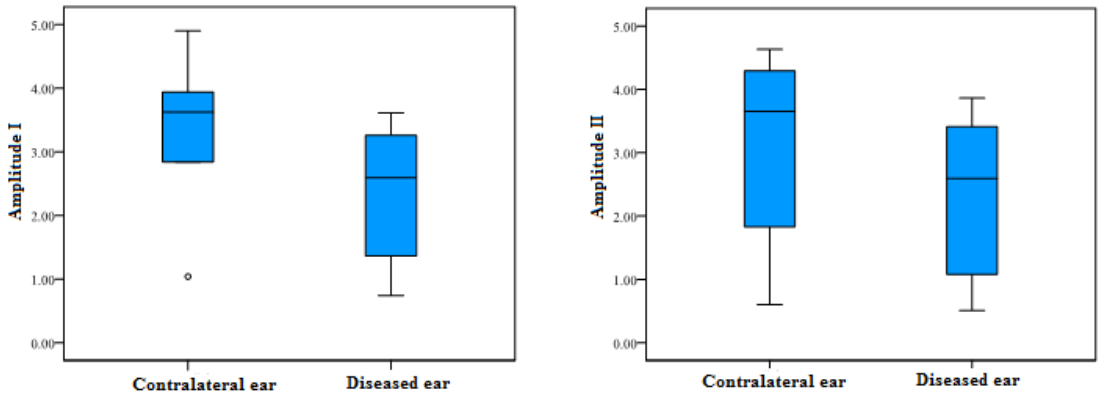
Table 2. Means values ± SD for latencies and amplitudes obtained for the diseased ear and the contralateral one in dogs with central vestibular syndrome

	Diseased ear	***Contralateral ear***
Latencies		
Wave I	1.09±0.015	1.11±0.105
Wave II	1.97±0.05	1.97±0.20
Wave III	2.93±0.083	3.01±0.27
Wave V	3.84±0.020	3.85±0.40
Amplitudes		
Wave I	2.49±2.18	3.78±2.19
Wave II	2.4±2.52	3.89±2.97
Wave III	0.61±1.64	1.96±1.92
Wave V	1.51±1.25	2.49±1.28

Comparing the latency I, II, III and V to between left and right ear of dogs suffering from CVS no statistically significant differences were obtained.No statistical differences were observed for BAER latencies between patients with CVS and healthy dogs to mono- and binaural stimulation, which demonstrates that the latency is kept normal and the presence of biauricular interaction.

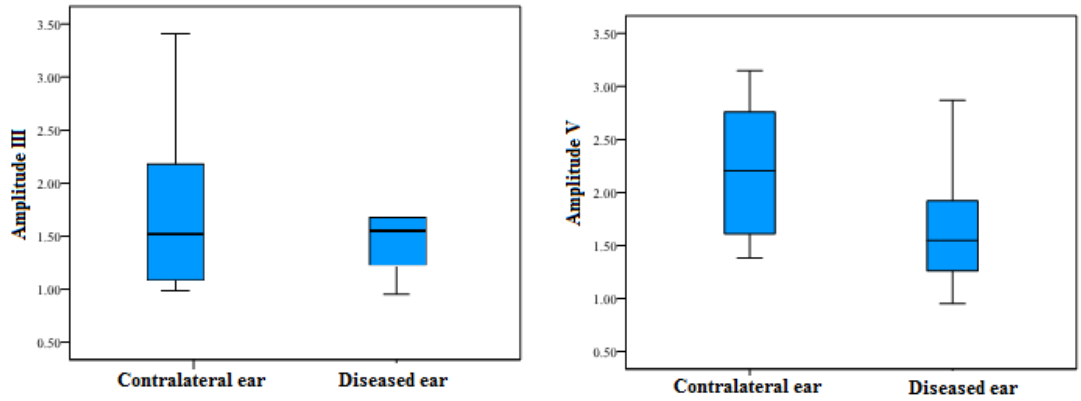
In dogs difficulties occurs when the BAER`s amplitudes are analysed. Anyway, there are some reports which describe the changes in amplitudes as sole modification in BAER pathological traces (Spremo and Stupar, 2008).However, comparing the amplitudes of these waves between diseased and contralateral ear were observed differences in all waves. P values obtained were = 0.014 for wave I amplitude, 0.031 for waves II and III and 0.032 for wave V. In all these situations amplitude diseased ears were smaller than contralateral ear (figure 3 and 4). Amplitudes decrease without changes in wave latencies may show an impairment of strength, not speed transmission of information between the nuclei of the auditory pathway (Armașu et al., 2011).

If the amplitudes of waves II, III and V, lower values obtained from stimulation of the ear affected versus contralateral are widely recognized in the literature (Steiss et al., 1994; Fischer, 1994;Wilson, 2005; Colson et al., 2012), impaired wave I was mentioned only in human pathology, in coma, demyelination of the brain stem (Arnold and Joseph, 1975), brain tumours (Legatt, 2005). I wave amplitude decrease is attributed ischemia or infarctions of the cochlea, compressions due to labyrinthine artery or basilar artery.



A. ***B.***

Figure 4. BAER test performed on dogs with vestibular syndrome
A. Values for the amplitudes of waves I obtained by stimulation of the healthy ear and the diseased one. B. Values for the amplitudes of waves II obtained by stimulation of the healthy ear and the diseased one.



A. ***B.***

Figure 5. BAER test performed on dogs with vestibular syndrome
A.Values for the amplitudes of waves III obtained by stimulation of the healthy ear and the diseased one.B.Values for the amplitudes of waves V obtained by stimulation of the healthy ear and the diseased one.

No statistical differences were observed for BAER latencies and amplitudes(P>0.05) between patients with CVS and healthy dogs to mono- and binaural stimulation, which demonstrates that the latency and amplitude is kept normal and the presence of biauricular interaction and CVS dogs did not perceive two sounds at the same time, contrary to the results of Steiss (1994), that in the central vestibular system disorders she found increased latency of all waves in the affected ear.

Conclusion

In vestibular syndrome, the morphological change of the BAER waves has revealed presence of waves III-V plateau in 4/10 dogs (40%), and multiple discharges between waves III-V in 3 cases (30%) and absence of waves II-V in a dog (10%).

Statistically significant differences obtaining between the amplitudes of the BAER trace of the diseased ear and those of the contralateral one, demonstrate impairedof nuclear response.

BAER recorded with surface electrodes were characterised by decreased amplitudes without changes in latencies waves, which may show an impairment of strength, but not the speed of transmission of information between the nuclei of the auditory pathway.

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VERTEBRAL HEART SCORES IN FOUR DOG BREEDS

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Abstract

The aim of this study was to asses the measurements of cardiac silhouete and to see if the Romanian mongrel breed is a species that developed from interracial breeds and they can have various VHS values. Radiographic examination is one of the most important diagnostic tools available for both physicians and veterinarians in the diagnosis of cardiac diseases. This diseases in small animals recorded an increasing frequency in the recent years expressing themselves through a wide range of signs. Diagnosis of heart disease is made from history, physical examination and imaging examinations. The mean for the 60 dogs was 9.8 v(SD ± 0.39). Doberman had the highest mean of VHS 10.2 ± 0.26 (9.7 to 10.6 v) than other races. Romanian mongrel breed had the lowest mean of VHS 9.5 ± .,43 (8.8 to 10.5 v). German Shepherd mean was 9.9 ± 0.45 (9 to 10.8 v) and Dachshund 9.7 ± 0.40 (9 to 10.2 v). In our study we find differences between breeds with shallow and wide, intermediate and deep and narrow. The Romanian mongrel breed have various VHS and they can be included in all the thorax sizes. This study can be use as a guide in veterinary practice.

Keywords: *computerized radiographic technique, dogs, VHS, thorax.*

Introduction

Cardiac diseases in small animals recorded increasing frequency in recent years. Diagnosis of heart disease is based on anamnesis signs, clinical examination and imaging.

The first who developed and used VHS (vertebral heart scale) method were Buchanan and Bucheler (1995) on a study of 100 clinically healthy dogs and a wide variety of breeds. The VHS they obtained was 9.7 (SD ± 0.5). Lamb et al. (2000, 2001) notes that when making VHS is necessarily to take into account race and crosses between them.

The objective of this study was to measure the cardiac silhouette by computerized radiographic technique to four dog breeds with different thorax conformations.

Materials and methods

The study evaluated 60 clinically healthy dogs, aged between 1 and 14 years, of which 28 males and 32 females, belonging to four breeds. The animals were divided into three categories, depending on the thorax conformation in agreement with literature (Burk and Feeney, 2003): deep and narrow thorax (Doberman) with intermediate thorax (German Shepherd) and shallow and wide thorax (Dachshund). Mongrels were analyzed separately in a special category, as they can present all three conformational categories.

VHS (vertebral heart scale) method was used, described by Buchanan and Bucheler (1995), which involves measuring the long axis (L - representing the distance from the carina to the cardiac apex) and short axis (l - representing the maximum diameter perpendicular on the axis of the heart term) in cm, and their sum was compared with the length of thoracic vertebrae (v), from cranial edge of thoracic vertebra 4 (T4) from right side incidence.

Average and standard deviation were calculated using Microsoft Office 2007 (Microsoft Excel) for each breed separately.

Results and discussion

In Table 1 are the VHS values for each race separately. The average for the 60 dogs was 9.8 v (SD ± 0.39). VHS of the Doberman (Fig. 1) had the highest mean value of 10.2 ± 0.26 (9.7 to 10.6 V) than other races. Romanian mongrels (Fig. 2) had the lowest average of VHS 9.5 ± 0.43 (8.8 to 10.5 v). The German Shepherd (Fig. 3) mean of VHS was 9.9 ± 0.45 (9 to 10.8 v) and Dachshund (Figure 4) of 9.7 ± 0.40 (9 to 10.2 v).

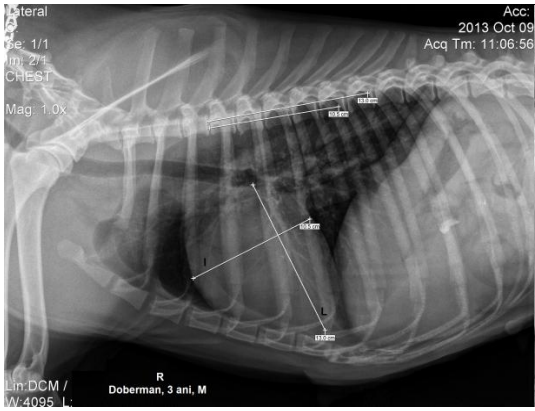

Figure 1 – Thorax of a 3 years old, male, Doberman from right side incidence.

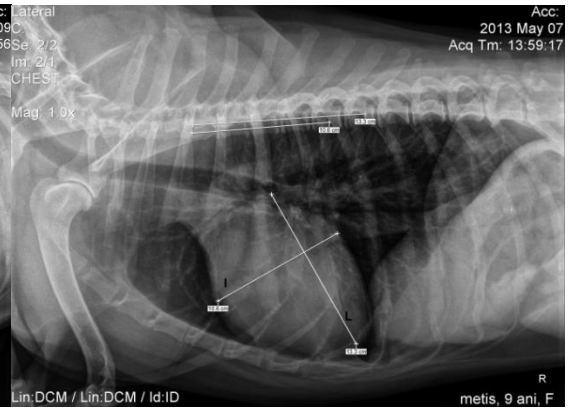

Figure 2 – Thorax of a 9 year old female, Mongrel, from right side incidence.

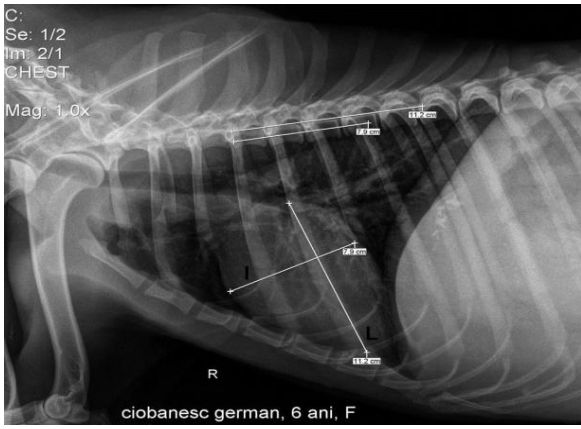

Figure 3 – Thorax of a 6 years old, female, German Shepherd, from right side incidence.

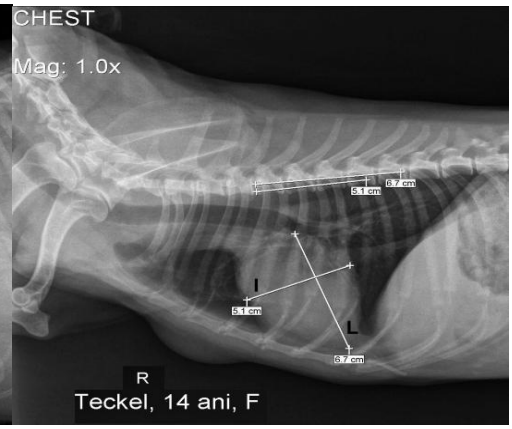

Figure 4 – Thorax of a 14 years, female, Dachshund, from right side incidence.

Table 1. Values of cardiac silhouette (mean, standard deviation, minimum and maximum) from right side incidence

Measurements	Dog breeds				Total (n=60)
	Dachshund (n=15)	German Shepherd (n=15)	Doberman (n=15)	Mongrels (n=15)	
L-long cardiac axis (v)[1]	5,2 ± 0,32 4,8-5,9	5,5 ± 0,39 4,5-6	5,7 ± 0,13 5,5-5,9	5,4 ± 0,37 4,8-6	5,4 ± 0,30 4,5-6
l-short cardiac axis (v)[1]	4,5 ± 0,23 4,1-4,8	4,4 ± 0,27 4-4,7	4,5 ± 0,24 4,1-4,8	4,1 ± 0,20 4-4,8	4,3 ± 0,24 4-4,8
VHS (v)[1]	9,7 ± 0,40 9-10,2	9,9 ± 0,45 9-10,8	10,2 ± 0,26 9,7-10,6	9,5 ± 0,43 8,8-10,5	9,8 ± 0,39 8,8-10,8

[1]Length measured in vertebrae

The results obtained in this study showed that VHS values are relatively similar to those reported in previous studies (Bucheler and Buchanan, 1995; Lamb et al., 2000).

Doberman has the highest average value of VHS in this study and were slightly higher compared to the results of Ghadir et al. (2008).

Mongrels in our country are not a registered breed, therefore there is no reference range of they're VHS. In our study mongrels recorded an average value of VHS to other races less than 9.5 V (SD ± 0.43), the difference being given by the result of uncontrolled crosses between individuals of different constitution and conformational types. Because of this the VHS value of these individuals ranges between 8.8 to 10.5 v. No significant difference has been seen between males and females, similar observations were found by Kumar et al. (2011) from the Indian mongrels.

Average value of the German Shepherd VHS was relatively higher than in previous studies (Buchanan and Bucheler, 1995; Lamb et al., 2001; Ghadir et al., 2010), the average value of VHS was 9.7 (SD ± 0.5), 9.7 (SD ± 0.8) and respectively 9.8 (SD ± 0.59). The VHS differences may be caused by the different number of dogs in the study or different ages of individuals evaluated (Gaschen, 1999; Sleeper and Buchanan, 2001).

Values recorded at Dachshund breed are similar to those obtained by Jepsen-Grant et al. (2013), which indicate that the Dachshund is a breed with a well-defined conformation with a wide and small thorax.

Conclusions

Radiological method remains an essential means to assess cardiac silhouette, data interpretation can be done in conjunction with anamnesis data and clinical examination.

Measurement of cardiac silhouette with VHS method remains one of the easiest ways, but at the same time it is important to take into account the age, race, constitution and conformation and the state of each individuals evaluated.

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RESEARCH ON THE HISTOLOGICAL STRUCTURE OF THE DEEP PECTORAL MUSCLE OF MEAT-TYPE COMMERCIAL HYBRID COBB-500, SLAUGHTERED AT DIFFERENT AGES

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Abstract

From 30 specimens, males and females, belonging to avian meat-type hybrid COBB-500, which were sacrificed at ages of 35, 42 and 49 days, histologic samples were collected from the deep/profound pectoral muscle (PP). These samples were processed by paraffin sectioning techniques to give the 60 histological blades, which were studied using a binocular-type photonic MC3 microscope. In the microscopic field, using cyto and histometric techniques, we measured the thickness of the muscle cells and primary muscle fascicles (PMF). Based on these measurements we calculated: the average thickness, the perimeter and the cross section surface of PMF; the number of myocytes and muscle and connective tissue proportion of PMF. The following results were obtained. In males, the average thickness of PMF was 266.5-355.75-278 μ, depending on the age, increasing from 35 to 42 days with 33.49%. Similarly it varies the number of myocytes in the PMF (47.8-63.63-39.3) and also the cross section surface of PMF (53.250-91.650-56.655 μ^2). The proportion of muscle tissue increased from 45.72% to 35 days, up to 61.01% at 49 days. In females, the average thickness of PMF was of 317.75-345.25-298μ, again depending on the age and the development is similar to that found in males. Between males and females are significant differences (of 2.95-7.19-19.23%), depending on age, for the average thickness and perimeter of PMF. The number of myocytes in PMF is higher in females than in males, with 52.72% at 35 days and 16.03% at 49 days. The proportion of muscle tissue decreased from 40.09% to 35 days at 38.69% at 42 days and rose to 48.31% at 49 days. In shape and appearance, PMF are cylindroid in both sexes and at all slaughter ages studied.

Key words: *COBB-500, Profound pectoral, PMF, myocyte, muscular tissue*

Introduction

Known and appreciated by consumers and manufacturing field, poultry meat and especially chicken meat, is produced in the industrial system using a series of high-performance hybrids such as: ROSS-308, COBB-500, SHAVER -STARBRO, etc.

Although the bio-economic performance of these hybrids are well known [13], the quality of their meat products are known less. Poultry meat quality is defined and assessed by several parameters such as: chemical composition, physical and technological characteristics, somatic muscle histological structure, etc.

For the meat-type avian hybrid COBB-500 there are already made studies regarding some muscle in carcass composition (breast, thighs, calves), in terms of their chemical composition and especially on histological structure [7]; [8]; [9]; [10]. But the avian body is composed of many myologic entities of various sizes and shapes and it is necessary that the research began to be continued and extended in depth, approaching (in study) more and more histological, biochemical, physical, organoleptic and other parameters.

In the present paper we studied the deep/profound pectoral muscle (PP) [2]; [3] of COBB-500 avian hybrid, in both sexes and three different slaughter ages, regarding the myocytes and primary muscle fascicles (PMF).

Material and method

The biological material was represented by 15 young males and 15 young females from meat type COBB-500 hybrid hen, which were sacrificed at ages of 35, 42 and 49 days. The average weight of the birds at the time of slaughter has been: of 1406-1614 grams at 35 days; of 2168-2296 grams at 42 days and of 2350 to 2378 grams at the age of 49 days, being higher in males than in females. From the carcases obtained at slaughter we extracted histologic samples from deep pectoral muscle (*Pectoralis profundus*) (PP) [11] [2]; [3], which were processed by paraffin sectioning technique [1]; [14], yielding, after following all operating steps, 60 histologic slides.They had on them cross sections through the middle of the studied muscle (PP), colored with HE (hematoxylin-eosin) and they were then studied using a MC3 binocular photon microscope, calibrated in advance. The calibration was performed at three ocular and objective associations namely OC10 x OB10; OC10 x OB20; OC10 x OB6 and the micrometer values calculated with this occasion were as following: 9.011 μ; 4,441 μ and 15,000 μ.

In the microscopic field we photographed and measured (with an ocular micrometer) the muscle fibers and primary muscle fascicles (PMF), and, using a micrometer grid we determined the density of myocytes. Measurements aimed at small and large diameter of myocytes and PMF.

The calculations we determined were: the mean diameter; perimeter and cross-sectional area of the PMF; profile and format index for the PMF; the proportion of the two tissue types (muscular-M and connective-C) and myocyte density of PMF. We used the following mathematical formulas:

(1) For average diameter ($D\bar{x}$) of PMF: $D\bar{x} = \frac{LD+SD}{2}$, where: LD=large diameter (μ); SD=small diameter (μ)

(2) For PMF perimeter: $P=\frac{LD+SD}{2} x\, \pi$, where: π =3,141592654

(3) For the format index (Fi): $Fi=\frac{LD}{SD}$ (Fi=x/1)

(4) For the profile index (Pi): $Pi=\frac{SD\ x\ 100}{LD}$ (Pi %)

(5) For the cross sectional area of PMF: C.s.a.=$\frac{DM+Dm}{4}\ x\ \pi$ (μ^2)

(6) For the proportion of muscular tissue (MT%): $MT=\frac{nmf\ x\ 100\ x\ C.s.a.fm.}{C.s.a.FMP.}$, where: nmf=number of muscular fibers; $C.s.a.fm.$=cross sectional area of muscular fibers (μ^2); $C.s.a.FMP$=cross sectional area of PMF (μ^2)

(7) For the proportion of connective tissue (CT%): $P_{CT}=100-P_{MT}$

(8) For the density of muscular fibers (D_{mf}): $D_{mf}=\frac{n\ x\ 10^6}{C.s.a.FMP}$, unde n=number of myocytes from primary muscular fascicles (PMF).

All data obtained from micrometer measurements and calculations have been tabulated and then analyzed and interpreted statistically, calculating: mean and standard error of the mean, standard deviation, variance and coefficient of variation. In order to test the statistical significance of the differences between the sexes and of the three studied ages we calculated Fischer (F) and Tukey (W) values for all parameters included in the study. For all

statistical calculations we used Anova Single Factor (ASF) algorithm, included in the software package of Microsoft Excel (MsEx) [5].

Results and their discussion

For the commercial COBB-500 meat type hybrids the pectoral muscles are the most valuable: the four muscles (two shallow and two deep) are often mentioned by poultry breeders, livestock industry and food technology.

From the quantitative point of view (their size and weight) these pectoral muscles are very well characterized, but we wanted to obtain data on their quality, as it is assessed here by a series of histological parameters regarding the muscle fibers and fascicles. Thus, the deep pectoral muscle (PP), the myocytes have an average thickness of 22.925 μ for females and one of 25.355 μ for males and an average of the two sexes of 24.14 μ. The difference between the sexes is 9.58% at the expense of females. These numbers are found in birds slaughtered at the age of 35 days (table 1).

Table 1. The myocytes thickness and cross-sectional surface (area) from Gastrocnemius lateralis muscle of avian hybrid COBB-500, regarding the slaughtering age and sex

Specification	MU	The slaughtering ages and sex of chickens											
		35 days				42 days				49 days			
		Male	Female	Sexes mean	±%F/M *	Male	Female	Sexes mean	±%F/M *	Male	Female	Sexes mean	±%F/M *
The average diameter of myocytes	μ	25.355	22.925	24.140	-9.58	30.743	26.056	28.400	-15.24	33.288	29.680	31.484	-10.84
Cross sectional area of myocytes	μ^2	507.524	415.951	461.737	-18.04	695.392	720.833	646.226	-28.96	687.914	691.642	784.841	-19.83

* Percentage comparison between female and male

At the age of 42 days, the male chickens myocytes from the studied muscle (PP) had an average thickness of 30.743μ, for females the same parameter had values of 26.056μ and the mean of the two sexes was of 28.40μ (table 1). The difference between sexes was of 15.24%, in the detriment of females.

For the chickens sacrificed at the age of 49 days, the PP myocytes had an average thickness of 33.288μ, for males; of 29.68μ, for females and of 31.484μ, as mean of sexes, the difference between them was of 10,84%, in the detriment of females (table 1).

By analizing these numbers we observed the fact that the myocytes thickness from the studied muscle grows at the same time as the age, phenomen that appears at both sexes being reduced at females with 9,58-10,84-15,24% (table 1).

The muscular fibers form the primary mucle fascicles whitch form the secundary muscle fascicles and these are present at all somatic muscle. In our case (study) the PMF had an average thickness of 266.5±9.031μ, for males and one of 317±9.185μ, for females, the mean of the two sexes was of 292.125μ (tables 2, 3, 5). The difference between sexes is of 19.23%, for females (table 4).These values are for the age of 35 days.

Table 2. Statistical estimators regarding some structural parameters of PMF from PP muscle for COBB-500 **males**, regarding the slaughtering ages

Specification			MU	n	Statistical indicators			Variation limits	
MF*	Age	Parameters			$\bar{x}\pm s_{\bar{x}}$	s	V(%)	Min.	Max.
PMF**	35 DAYS	Large diameter	µ	30	329±14.825	81.2022	24.68	210.0	600.0
		Small diameter	µ	30	204±7.699	42.1696	20.67	135.0	270.0
		Average diameter	µ	30	266.5±9.031	49.4652	18.56	180.0	405.0
		PMF** perimeter	µ	30	837.234±28.372	155.3997	18.56	565.487	1272.345
		Format index (If)	x/1	30	1.666±0.085/1	0.4683	28.10	1.000/1	2.856/1
		Profile index (Ip)	%	30	64.635±3.223	17.6535	27.31	35.00	100.00
		Number of MF	n	30	47.80±3.04	16.6804	34.90	21	91
		Cross-sectional area	µ²	30	53249.996±3439.93	18841.245	35.38	24740.04	98960.17
		Muscular tissue proportion	%	30	45.72±0.64	3.5163	7.69	40.6141	54.9426
		Connective tissue proportion	%	30	54.28±0.64	3.5163	6.48	45.0574	59.3859
PMF**	42 DAYS	Large diameter	µ	30	462.5±18.18	99.5832	21.53	255.0	660.0
		Small diameter	µ	30	249.0±9.718	53.2301	21.38	150.0	405.0
		Average diameter	µ	30	355.75±11.517	63.0789	17.73	240.0	532.5
		PMF** perimeter	µ	30	1117.622±36.18	198.1681	17.73	753.982	1672.898
		Format index (If)	x/1	30	1.919±0.094/1	0.5146	26.81	1.133/1	3.0909/1
		Profile index (Ip)	%	30	55.956±2.84	15.5548	27.80	32.353	88.235
		Number of MF	n	30	63.633±4.197	22.9909	36,14	31	145
		Cross-sectional area	µ²	30	91650.075±6139.14	33625.429	36.69	44178.65	209936.929
		Muscular tissue proportion	%	30	52.548±0.183	0.9998	1.90	49.665	54.882
		Connective tissue proportion	%	30	47.452±0.182	0.9998	2.11	45.118	50.335
PMF**	49 DAYS	Large diameter	µ	30	354.5±16.009	87.6843	24.73	225.0	600.0
		Small diameter	µ	30	201.5±8.127	44.5117	22.09	120.0	300.0
		Average diameter	µ	30	278±9.628	52.7371	18.97	202.5	390.0
		PMF** perimeter	µ	30	873.363±30.249	165.6786	18.97	636.1725	1225.2211
		Format index (If)	x/1	30	1.826±0.104/1	0.57236	31.34	1.000/1	4.000/1
		Profile index (Ip)	%	30	59.297±3.075	16.8251	28.37	25.0	100.0
		Number of MF	n	30	39.3±2.388	13.08105	33.285	17	66
		Cross-sectional area	µ²	30	56654.696±3655.5	20022.068	35.34	26860.617	105145.172
		Muscular tissue proportion	%	30	61.01±1.22	6.66185	10.92	50.327	87.573
		Connective tissue proportion	%	30	38.987±1.216	6.66185	17.09	12.427	49.673

*MF=muscular fascicle; **PMF=primary muscular fascicle.

Table 3. Statistical estimators regarding some structural parameters of PMF from PP muscle for COBB-500 **females**, regarding the slaughtering ages

Specification			MU	n	Statistical indicators			Variation limits	
MF*	Age	Parameters			$\bar{x}\pm s_{\bar{x}}$	s	V(%)	Min.	Max.
PMF**	35 DAYS	Large diameter	µ	30	388.5±14.867	81.4328	20.96	225	540
		Small diameter	µ	30	247.0±9.595	54.5451	22.08	150	315
		Average diameter	µ	30	317.75±9.185	50.31088	15.83	195	397.5
		PMF** perimeter	µ	30	998.241±28.857	158.0563	15.83	612.611	1248.783
		Format index (If)	x/1	30	1.648±0.091/1	0.50148	30.43	1.000/1	3.000/1
		Profile index (Ip)	%	30	66.05±3.48	19.03713	28.82	33.33	100.00
		Number of MF	n	30	73.0±4.0	21.9624	30.08	26.0	108.0
		Cross-sectional area	µ²	30	75563.157±3996.64	21890.5132	28.97	28274.334	111330.189
		Muscular tissue proportion	%	30	40.09±0.40	2.170999	5.42	36.248	45.899
		Connective tissue proportion	%	30	59.91±0.40	2.17100	3.623	54.100	63.752
PMF**	42 DAYS	Large diameter	µ	30	440.5±25.796	141.28932	32.075	210.0	795.0
		Small diameter	µ	30	250±10.052	55.05483	22.02	120.0	330.0
		Average diameter	µ	30	345.25±15.845	86.78489	25.14	165.0	517.5
		PMF** perimeter	µ	30	1084.635±49.777	272.64276	25.14	518.363	1625.774
		Format index (If)	x/1	30	1.791±0.100/1	0.549877	30.70	1.1053/1	3.3636/1
		Profile index (Ip)	%	30	60.133±2.84	15.55675	25.87	29.73	90.476
		Number of MF	n	30	61.2±3.856	21.120998	34.51	19	89
		Cross-sectional area	µ²	30	89199.733±6848.02	37508.1292	42.05	19792.034	149853.969
		Muscular tissue proportion	%	30	38.69±1.12	6.108925	15.79	28.285	52.322
		Connective tissue proportion	%	30	61.31±1.11	6.108925	9.96	47.678	71.715
PMF**	49 DAYS	Large diameter	µ	30	369±12.897	7064237	19.14	180.0	525.0
		Small diameter	µ	30	227±9.749	53.3951	23.52	135.0	300.0
		Average diameter	µ	30	298±8.903	48.76227	16.36	180.0	390.0
		PMF** perimeter	µ	30	936.195±27.969	153.19118	16.36	565.487	1225.221
		Format index (If)	x/1	30	1.709±0.102/1	0.55605	32.54	1.000/1	3.8889/1
		Profile index (Ip)	%	30	63.20±2.99	16.39956	25.95	25.714	100.00
		Number of MF	n	30	45.60±2.61	14.2940	31.35	20	70
		Cross-sectional area	µ²	30	66421.123±3974.59	21769.716	32.78	25446.90	108149.327

		Muscular tissue proportion	%	30	48.31±0.75	4.108997	8.51	42.566	56.735
		Connective tissue proportion	%	30	51.69±0.75	4.108997	7.95	43.265	57.434

*MF=muscular fascicle; **PMF=primary muscular fascicle.

Table 4. Some structural parameters of PMF from profound pectoral muscle of the avian hybrid COBB-500, regarding the age of slaughter and sex

Specification	MU	Slaughter age and sex								
		35 days			42 days			49 days		
		Male	Female	±%F/M*** *	Male	Female	±%F/M** **	Male	Female	±%F/M****
Large diameter	µ	329.0	388.5	**+18.09**	462.5	440.5	**-4.76**	354.5	369.0	**+4.09**
Small diameter	µ	204.0	247.0	**+21.08**	249.0	250.0	**+0.40**	201.5	227.0	**+12.66**
Average diameter	µ	266.5	317.75	**+19.23**	355.75	345.25	**-2.95**	278.0	298.0	**+7.19**
PMF perimeter	µ	837.234	998.241	**+19.23**	1117.622	1084.635	**-2.95**	873.363	936.195	**+7.19**
Format index	x/1	1.666/1	1.648/1	**-1.08**	1.919/1	1.791/1	**-6.67**	1.826/1	1.709/1	**-6.41**
Profile index	%	64.635	66.05	**+1.415pp**	55.956	60.133	**+4.18pp**	59.297	63.20	**+3.903pp**
Number of myocytes from PMF	n	47.80	73.0	**+52.72**	63.633	61.20	**-3.82**	39.3	45.6	**+16.03**
Cross-section surface of PMF	μ^2	53249.996	75563.16	**+41.90**	91650.075	89199.733	**-2.67**	56654.696	66421.123	**+17.24**
Muscular tissue proportion	%	45.72	40.09	**-5.63pp****	52.55	38.69	**-13.86**	61.01	48.31	**-12.7pp**
Connective tissue proportion	%	54.28	59.91	**+5.63pp**	47.45	61.31	**+13.86**	38.99	51.69	**+12.7pp**

* Percentage comparison between female and male; **pp=procentual points.

For the birds sacrificed at the age of 42 days the average thickness of PMF was of 355.75±11.571µ, for males; 345.25±15.845µ, for females and of 350.50µ, as a mean of the sexes (v=17.73 to 25.14%) (tables 2, 3). At this age (42 days) the differences between sexes is small (2.95%), not in favor of females (table 4).

At the age of 49 days the PMF from studied muscle (PP) had an average thickness of 278±9.628µ, at males; of 298±8.903µ, at females and of 288µ, as a mean of sexes. In this case the difference between females and males is of 7.19%, for females (table 2, 3, 4).

The age of slaughter had influenced the thickness of PMF thus it was 16.65% lower at 35 days (average of both sexes) and less than 17.83% in 49 days compared to 42 days at which had a mean value of 350.5 µ (table 5).

Similar data have evolved for the perimeter of PMF (tables 2, 3, 4, 5).

Regarding the primary muscular fascicles aspect their shape is cilindrical, at the age of 35 days (Fi=1.648/1-1.666/1) (Fi=64.635-66.05%) and oval at the ages of 42 and 49 days (Pi=1.919/1-1.791/1)(Pi=1.826/1-1.709/1) (Pi=55.96-60.13%) (Pi=59.30-63.20%) (table2, 3, 4, 5). For this character the differences between sexes are of 10.67%, at the age of 35 days and of 4.72%, for the age of 49 days, compared to the standard age (technological age) of slaughter of 42 days (tables 4, 5).

Regarding the number of myocytes from PMF, the data we obtained confirms that what is known from other studies [7]; [8]; [9]; [10] there is a inverse relationship between

their thickness and their number. Thus, for female sex, myocytes are thinner and their number in a PMF, is greater, in particular at the age of 35 days, when the its body is in full growth.

Table 5. Comparations between the three slaughtering ages of males and females of avian hybrid COBB-500, regarding the age of slaughter and sex

Specification		MU	Age of slaughtering and sex of chickens											
			35 days (V1)				42 days (V2)*				49 days (V3)			
			Male	Female	Mean of sexes		Male	Female	Mean of sexes		Male	Female	Mean of sexes	
			±% V1/V2	±% V1/V2	Abs. val.	±% V1/V2	±% V1/V2	±% V1/V2	Abs. val.	±% V1/ V2	±% V1/V2	±% V1/V2	Abs. val.	±% V1/V2
Pectoralis profundus	Large diameter	µ	**-28.86**	**-11.80**	358.75	**-20.54**	100	100	451.50	100	**-23.35**	**-16.23**	361.75	**-19.88**
	Small diameter	µ	**-18.07**	**-1.20**	225.50	**-9.62**	100	100	249.50	100	**-19.08**	**-9.20**	214.25	**-14.13**
	Mean diameter	µ	**-25.09**	**-7.97**	292.125	**-16.65**	100	100	350.50	100	**-21.86**	**-13.69**	288.0	**-17.83**
	PMF perimeter**	µ	**-25.09**	**-7.97**	917.7375	**-16.65**	100	100	1101.1285	100	**-21.85**	**-13.69**	904.779	**-17.83**
	Format index	x/1	**-13.18**	**-7.98**	1.657/1	**-10.67**	100	100	1.855/1	100	**-4.85**	**-4.58**	1.7675 /1	**-4.72**
	Profile index	%	**+8.679 pp*** **	**+5.917 pp**	65.3425	**+7.30 pp**	100	100	58.0445	100	**+3.341 pp**	**+5.10 pp**	61.2485	**+3.204 pp**
	Myocytes number from PMF	n	**-24.88**	**+19.28**	60.40	**-3.23**	100	100	62.4165	100	**-38.24**	**-25.49**	42.45	**-31.99**
	Cross-section surface/area of PMF	μ^2	**-41.90**	**-15.29**	64406.578	**-28.77**	100	100	90424.904	100	**-38.18**	**-25.54**	61537.909	**-31.95**
	Muscular tissue proportion	%	**-6.83 pp**	**+1.40 pp**	42.905	**-2.715 pp**	100	100	45.62	100	**+8.46 pp**	**+9.62 pp**	54.66	**+9.04 pp**
	Connective tissue proportion	%	**+6.83 pp**	**-1040 pp**	57.095	**+2.715 pp**	100	100	54.38	100	**-8.46 pp**	**-9.62 pp**	45.34	**-9.08 pp*** **

*the technological age of slaughter for this avian hybrid; ** PMF=primary muscular fascicles; ***pp=procentual points.

So, if we analise the data from tables 2 and 3 we observe that for this character we found: 47.8±3.04 f.m./PMF, for males and 73±4 f.m./PMF, for females, the mean of sexes has a value of 60.4 f.m./PMF (table 5).These numbers are available for the age of 35 days.

At the age of 42 days the number of myocytes was: 63.633±4.197 f.m./PMF, for males; of 61.2±3.856 f.m./PMF, for females and of 62.416 f.m./PMF, as a mean of the sexes (tables 2, 3, 5).

At the age of 49 days, the number of myocytes was of: 39.3±2.388 f.m./PMF, for males; 45.60±2.61 f.m./PMF, for females and of 42.45 f.m./PMF, as a mean of both sexes. For this character the difference between sexes was very big, at the age of 35 days (52.72%); smaller at the age of 49 days (16.03%) and very small at the age of 42 days (3.82%) (table 4).

Between the 3 slaughter ages that we studied the differences that we found were: very small for the age of 35 days (3.23%) (as a mean of sexes) and bigger for the age of 49 days (31.99%) if we compaire them with the values obtained with the age of 42 days (table 5).

The primary muscular fascicles (PMF) have an oval cross-section and the cross sections have a area size of approximately 53.250-75.563μ^2, at the age of 35 days; of 80.200-

91.650μ^2, at 42 days and of 56.655-66.421μ^2, at 49 days (tables 2, 3, 4), regarding the sex of chickens.

Table 6. Statistical significance of the differences found between males and females regarding all structural parameters studied from profound pectoral muscle of meat type hybrid COBB-500

Slaughter age	Studied parameters	Differences between mean of sexes	Tukey values (w=0.01)	Statistical significance	La 1; 58 GL, for:			
					P	p ≤ 0.05	p ≤ 0.01	p ≤ 0.001
					Fα	**4.008**	**7.103**	**12.034**
35 days	Large diameter (μ)	**M-F*=59.5**	**55.8317**	**	F	**8.0307605**		
	Small diameter (μ)	**M-F=43.0**	**33.4723**	**	F	**11.66942329**		
	Average diameter (μ)	**M-F=51.25**	**34.2538**	***	F	**15.829036**		
	FMP perimeter (μ)	**M-F=161.007**	**107.6115**	***	F	**15.829029**		
	Format index (x/1)	**M-F=0.018**	**0.33312**	**n.s.**	F	**0.02549**		
	Profile index (%)	**M-F=1.415**	**12.6046**	**n.s.**	F	**0.089178**		
	Myocytes number	**M-F=25.20**	**13.3895**	***	F	**25.047140**		
	Cross-sectional area	**M-F=22313.164**	**14022.1061**	***	F	**17.9052344**		
	MT proportion (%)	**M-F=5.63**	**2.006296**	***	F	**55.7847882**		
	CT proportion (%)	**M-F=5.63**	**2.006296**	***	F	**55.784782**		
42 days	Large diameter (μ)	**M-F*=22.0**	**83.9205**	**n.s.**	F	**0.4859522**		
	Small diameter (μ)	**M-F=1.0**	**37.17889**	**n.s.**	F	**0.00511554**		
	Average diameter (μ)	**M-F=10.50**	**52.08705**	**n.s.**	F	**0.2873448**		
	FMP perimeter (μ)	**M-F=32.987**	**163.6363**	**n.s.**	F	**0.2873446**		
	Format index (x/1)	**M-F=0.128**	**0.3656153**	**n.s.**	F	**0.865404**		
	Profile index (%)	**M-F=4.177**	**10.6804**	**n.s.**	F	**1.081285**		
	Myocytes number	**M-F=2.433**	**15.15699**	**n.s.**	F	**0.182247**		
	Cross-sectional area	**M-F=2450.342**	**24456.091**	**n.s.**	F	**0.07098453**		
	MT proportion (%)	**M-F=13.86**	**3.005289**	***	F	**150.28656**		
	CT proportion (%)	**M-F=13.86**	**3.005289**	***	F	**150.286635**		
49 days	Large diameter (μ)	**M-F*=14.5**	**54.66658**	**n.s.**	F	**0.4974809**		
	Small diameter (μ)	**M-F=25.50**	**33.74891**	**n.s.**	F	**4.0368745**		
	Average diameter (μ)	**M-F=20.0**	**34.87087**	**n.s.**	F	**2.3260477**		
	FMP perimeter (μ)	**M-F=62.823**	**109.55008**	**n.s.**	F	**2.3260475**		
	Format index (x/1)	**M-F=0.117**	**0.387415**	**n.s.**	F	**0.650144**		
	Profile index (%)	**M-F=3.903**	**11.40676**	**n.s.**	F	**0.827688**		
	Myocytes number	**M-F=6.30**	**9.406902**	**n.s.**	F	**3.171555**		
	Cross-sectional area	**M-F=9766.427**	**14359.418**	**n.s.**	F	**3.27101072**		
	MT proportion (%)	**M-F=12.70**	**3.800007**	***	F	**78.997495**		
	CT proportion (%)	**M-F=12.70**	**3.8000021**	***	F	**78.9980723**		

*M - F = differences between males and females.

Between the three ages of slaughter there are notable differences in this character, the highest values were found at the age of 42 days and the lowest at the age of 49 days (table 5).

In terms of the proportion of muscle tissue (MT) and connective tissue (CT) of muscle PP, data obtained by us are: for MT at 35 days, the value was of 45.72 ± 0.64% in males; of 40.09 ± 0.4% in females and 42.905%, the average of both sexes (tables 2, 3, 5). Atthe age of 42 days MT has been: 52.548 ± 0.183% in males; 38.69 ± 1.12% in females and 45.62% as an average of both sexes.

At 49 days, MT had a value of 61.01 ± 1.22% in males; of 48.31 ± 0.75% for females and 54.66%, as an average of sexes (tables 2, 3, 4).

The differences between the sexes are lower at 39 days (5.63 procetuale points) and very high (13.86 pp, respectively 12.70 pp) at the ages of 42 and 49 days (table 4). Between the three slaughter ages, differences in the proportion of muscle tissue and connective tissue are significant ie 2,715 pp at 35 days and 9.04 pp to 49 days compared to 42 days of age (table 5). In addition, the data in table 5 and figure 7 shows the fact that there is an increase in the proportion of muscle tissue from 35 days (42.905% as an average of sexes), to 42 days (45.62%) and to 49 days (54.66%).

Table 7. Statistical significance of the differences found between the three slaughter ages regarding some structural parameters of PMF from pectoral profound muscle of meat-type hybrid COBB-500

Sex	PMF studied parameters	Differences between the compaired means*	Tukey values (w=0.01)	Statistical significance	At 2; 357 GL, for:			
					P	p ≤ 0.05	p ≤ 0.01	p ≤ 0.001
					Fα	**3.1140**	**4.8945**	**7.5575**
MALE	Large diameter (μ)	V_1-V_2=133.5	**69.59116**	***	F	**18.6803142**		
		V_1-V_3=25.5		**n.s.**				
		V_2-V_3=108.0		***				
	Small diameter (μ)	V_1-V_2=45.0	**36.324214**	***	F	**11.11971921**		
		V_1-V_3=2.5		**n.s.**				
		V_2-V_3=147.5		***				
	Average diameter (μ)	V_1-V_2=89.25	**42.925195**	***	F	**23.04160082**		
		V_1-V_3=11.5		**n.s.**				
		V_2-V_3=77.75		***				
	FMP perimeter (μ)	V_1-V_2=280.388	**134.8535**	***	F	**23.04141605**		
		V_1-V_3=36.129		**n.s.**				
		V_2-V_3=244.259		***				
	Format index (x/1)	V_1-V_2=0.253	**0.40304**	**n.s.**	F	**1.81937463**		
		V_1-V_3=0.160		**n.s.**				
		V_2-V_3=0.093		**n.s.**				
	Profile index (%)	V_1-V_2=8.679	**12.93997**	**n.s.**	F	**2.061254**		
		V_1-V_3=5.338		**n.s.**				
		V_2-V_3=3.341		**n.s.**				
	Myocytes number of PMF	V_1-V_2=15.833	**13.989712**	***	F	**14.03554437**		
		V_1-V_3=8.50		**n.s.**				
		V_2-V_3=24.333		***				
	Cross-section surface of PMF	V_1-V_2=38400.079	**19430.668**	***	F	**21.5539594**		
		V_1-V_3=3404.7		**n.s.**				
		V_2-V_3=34995.38		***				
	Muscular tissue proportion	V_1-V_2=6.83	**3.399447**	***	F	**91.4645635**		
		V_1-V_3=15.29		***				
		V_2-V_3=8.46		***				
	Connective tissue proportion	V_1-V_2=6.83	**3.399447**	***	F	**91.464558**		
		V_1-V_3=15.29		***				
		V_2-V_3=8.46		***				
FEMALE	Large diameter (μ)	V_1-V_2=52.0	**79.50415**	**n.s.**	F	**3.89267664**		
		V_1V_3=19.5		**n.s.**				
		V_2-V_3=71.5		**n.s.**				
	Small diameter (μ)	V_1-V_2=3.0	**42.10206**	**n.s.**	F	**1.58853072**		
		V_1-V_3=20.0		**n.s.**				
		V_2-V_3=23.0		**n.s.**				
	Average diameter (μ)	V_1-V_2=27.5	**49.89703**	**n.s.**	F	**4.074011**		
		V_1-V_3=19.75		**n.s.**				
		V_2-V_3=47.25		**n.s.**				
	FMP perimeter (μ)	V_1-V_2=86.394	**156.7559**	**n.s.**	F	**4.074023**		
		V_1-V_3=62.046		**n.s.**				
		V_2-V_3=148.44		**n.s.**				
	Format index (x/1)	V_1-V_2=0.143	**0.415594**	**n.s.**	F	**0.539438**		
		V_1-V_3=0.061		**n.s.**				
		V_2-V_3=0.082		**n.s.**				
	Profile	V_1-V_2=5.917	**13.22066**	**n.s.**	F	**0.9026233**		

	index (%)	V_1-V_3=2.850		n.s.		
		V_2-V_3=3.067		n.s.		
	Myocytes number of PMF	V_1-V_2=11.80	15.05646	n.s.	F	15.0079635
		V_1V_3=27.40		***		
		V_2-V_3=15.60		***		
	Cross-section surface of PMF	V_1-V_2=13636.57	21732.406	n.s.	F	5.011058027
		V_1V_3=9142.037		n.s.		
		V_2-V_3=22778.61		**		
	Muscular tissue proportion	V_1-V_2=1.40	3.43377	n.s.	F	41.27251
		V_1V_3=8.22		***		
		V_2-V_3=9.62		***		
	Connective tissue proportion	V_1-V_2=1.40	3.43376	n.s.	F	41.272912
		V_1V_3=8.22		***		
		V_2-V_3=9.62		***		

*Slaughter ages for chickens: V_1=1.4035 days; V_2=42 days; V_3=49 days.

The proportions of connective tissue from PP muscle have evolved opposite from the muscle tissue (tables 2, 3, 4, 5), decreasing with the age of slaughter.

In all the studied parameters, differences between the two sexes and between the 3 ages of slaughter were tested in terms of their statistical significance, the results being presented in tables 6 and 7. Thus, at the age of 35 days, only the differences between the sexes on the index size and index profile are statistically insignificant, all other differences are distinct and highly statistically significant (table 6). At the age of 42 days, only the differences in the proportion of the two tissue types (MT and CT) are statistic significant, while all other differences were not statistically significant. The same situation is for the age of 49 days (tablel 6). Regarding the differences between the three slaughter ages, the situation is different and varied, finding both statistically significant differences (12 = 40% for males and 23 = 76.67% for females) and significantly distinct differences (1 = 3.33% in females) and statistically significant differences (18=60%, for males and 6=20%, for females) (table 7). From the presented and discussed data, it appears that the muscle fibers of PMF in the PP muscle, there is an increasing in their thickness with age in both males and females. In the PMF the situation is different in the sense that their thickness is increased until the age of 42 days, as as well as the number of myocytes, but the proportion of muscle tissue is growing throughout all the studied period (35-42-49 days).

Conclusions

1. The average thickness of the PP muscle myocytes increases with age, both in males and females, as well as the average value of the sexes (24.14-28.40-31.484 μ).
2. Average thickness of PMF from PP muscle has values as follows: 266.5 to 317.75 μ, at 35 days; from 345.25 to 355.75 μ at 42 days and 278 to 298 μ, at 49 days, gender differences are 2.95%, 7.19% and respectively 19.23%.
3. The perimeter and cross-section surface of PMF evolves similar to the average thickness of them, both about sex and age of slaughter.
4. PMF appearance of PP muscle is cylindroid at the age of 35 days (Fi= 1.666 to 1.648 / 1), there are no big differences between sexes (only 1.08%) and it has oval shape at the ages of 42 and 49 days (Fi = 1.855 / 1 - 1768/1), with differences of 6.41 to 6.67% between the sexes.
5. The number of myocytes in PMF is higher in females than in males at the ages of 35 and 49 days and decreased slightly to 42 days, with an average value of sexes of:

60.4 f.m./PMF, at 35 days; of 62.42 f.m./PMF, at 42 days and of 42.45 f.m./PMF, at 49 days.

6. The proportion of muscle tissue (MT) from PMF of the PP muscle increases with the slaughter age of male chicks from 45.72% to 52.55% to 61.01% while for females there id no developments of this type (40.09% -38.69% -48.31%).
7. The differences between males and females, for the ratio of MT are of 5.63 percentage points for the age of 35 days; of 13.86 pp at 42 days and 12.7 percentage points for the age of 49 days, to the detriment of the females chickens.
8. At the age of 35 days, the majority of the differences between males and females (80%) were found to be distinct and highly statistically significant, while at the ages of 42 and 49 days, the majority of differences (between males and females) are not statistically significant.
9. The differences between the three investigated slaughter age, fo males were found to be statistically significant at the rate of 40%, the rest being highly statistically significant. For the females, the differences are not statistically significant for the three ages studied, in proportion of 76.67%, the rest being distinct and highly statistically significant (23.33%).

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RESEARCH REGARDING THE FINENESS AND DENSITY OF MYOCYTESAS WELL AS THE PROPORTION OF MAIN TISSUE CATEGORIES FROM THE LATERAL GASTROCNEMIUS MUSCLE OF AVIAN HYBRID COBB-500, BY SEX AND AGE OF SLAUGHTER

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Abstract

We collected histological samples from 30 individuals, males and females (in equal numbers), from the COBB-500 avian hybrid at slaughter. The samples were taken from the lateral gastrocnemius muscle (GL) and were processed by paraffin sectioning techniques obtaining 60 histological samples. The ages of slaughtering for chickens were: 35, 42 and 49 days . The blades were studied using a MC3 type microscope to discover: the large and small diameters of myocytes and primary muscle fascicles (FMP). Then we calculated: the average diameter; the cross-sectional area and the area of myocytes and FMP; myocyte density and the proportion of the two main types of tissue. All data were statistically processed and interpreted. The following results were obtained: the average thickness of muscle cells increased, in males, with 28.908 μ, from 35 days to 37.519 μ to 49 days; for females it increased from 26.197 to 32.593 μ μ at the same time. The myocytes perimeter evolved similar both for males and females. The Myocyte density decreased with age in both sexes and the proportion of muscle tissue had values from 53.047 to 61.441 % in males and from 47.753-49.978-50.109 % for females. The vast majority of the differences between the sexes and between the ages of slaughter were shown to be distinct and highly statistically significant.

Key words: *hybrid, COBB-500, gastrocnemius, myocytes, density*

Introduction

On our planet the population growth is developing outstanding, which requires increasingly important food resources.

Agriculture and especially livestock sciences (Zootechnics) are economic sectors that provide food for human population, placing at our disposal the protein and energy required for a normal, healthy and hight quality life.

Among many species, races, varieties and hybrids of animals (and plants), mammals, birds, fish, it stands particularly those raised in poultry domains.

Meat type avian hybrids are very effective in terms of rapid growth and their feed conversion capacity, but their meat is poor in terms of its quality. The quality of the poultry meat is defined by a multitude of physical, chemical, organoleptic and histological parameters. We are interested particularly of the histological structure of the most important somatic muscles (of the chest and legs area) from the composition of poultry carcasses.

We addressed in these studies a number of histological parameters for the chest muscles of avian hybrid COBB-500, and in this paper we deal with one of the calf muscles (*Gastrocnemius lateralis*) of the same avian hybrid, in both sexes and the three slaughter ages (35, 42, 49 days).

Material and method

To organize and conduct our research we required the use of many materials and working methods.

The biological materials were the 30 chicken (15 males and 15 females) of meat type commercial hybrid, COBB-500, which were sacrificed at different ages, namely 35, 42 and 49 days (table 1). At these ages the chickens had different body weights, the males were heavier than the females.

Thus, at the age of 35 days, the weight of male sex chicks was 1406 grams (with 12.89% less) (table 1). The sexes average for this indicator was 1510 grams for the age of 35 days.At the age of 42 days males weight was of 2296 grams, and of females was 2168 g (with 5.57% less) and the gender average weight was 2232 grams. At the age of 49 days, males had a weight of 2378 grams and females one of 2350 grams (1.18% less), the gender average weight is 2364 grams.

From these chickens we collected some histological samples from the lateral gastrocnemius muscle (Gastrocnemius lateralis) (GL) [2]; [4]; [12], using a specific working technique and proper tools.

Table 1. The plan of our research

Biological and non-biological materials used in research							The followed parameters in the gastrocnemius muscle
Avian hybrid	The muscle	Age of slaughter (days)	After slaughter weight (grams)	Sex	Number of studied chickens	Number of histological slides	
„COBB-500"	Gastrocnemianul lateral (Gastrocnemianus lateralis) (GL)	35	1614	M*	5	10	- average thickness, perimeter and the cross sectional area of myocytes in the studied muscle; -densitatea acestor miocitemyocytes density - proportion of the two main tissue types (MT and CT) of the studied muscle
			1406	F**	5	10	
		42	2296	M	5	10	
			2168	F	5	10	
		49	2378	M	5	10	
			2350	F	5	10	
		TOTAL	-	-	30	60	

The amples were processed using a paraffin sectioning technique, by going through all specific stages of work (fixing in 10% formalin and then Bouin solution, dehydrated, clarified, parafining, sectioning, staining, etc.) [1]; [15]. Finally, we obtained 60 slides having on them the studied muscle (GL) cross-sections which have been coloured with hematoxylin and eosin dyes (HE) [1]; [15].

These blades have been studied using a MC3-type binocular photon microscope which has been calibrated previosly for three ocular (OC) and objective (OB) associations. Thus, for the OC10xOB10 association we determined a micrometer value (MV) of 9.011 μ; for OC10xOB20 association we determined a different amount of micrometers, namely 4.441μ , and for the OC10xOB6 association, VM was 15.000 μ.

Inside of the microscopic field we identified, photographed, counted and measured the myocytes that make the primary muscle fascicles. To measure them we used a small micrometer scale and with it we determined the following: the large and small diameters of 120 myocytes an of 30 primary muscle fascicles (PMF) for each sex and slaughter age (120x2x3=720) (30x2x3=180).

The specific calculations were determined: the mean diameter, perimeter, area and density of myocytes cross-section; the proportion of muscle and connective tissue from GL.

For these calculations we used a scientific calculator Casio-fx-82ES-type and a HP-530 laptop. Other nonbiological materials that we used: semiautomatic microtome; diverse instruments (forceps, scalpels, scissors, knives, dissecting needles, micrometers, micrometer scales); dyes; paraffin; various reagents, etc. For determination of the parameters mentioned above we used some mathematical relationships which are listed below.

To determine the average myocytes and PMF thickness ($D\bar{x}$):

(1) $D\bar{x} = \frac{LD+SD}{2}$, where LD=large diameter (μ); SD=small diameter (μ).

To determine de myocytes perimeter (Pmi):

(2) $Pmi=\frac{LD+SD}{2} x\ \pi$, where π=3,141592654= the coeficient used to calculate the length and the area of the circle.

To determine the cross-section surface of myocytes: (3) $S.c.s.m.=\frac{LD+SD}{4}\ x\ \pi$.

To determine the myocytes density (Dmi):

(4) $Dmi=\frac{n\ x\ 10^6}{S.c.s.PMF}$, where: n=number of myocytes from a PMF; S.c.s.FMP.=cross-section surface of PMF (μ^2).

To determine the proportion of muscular and connective tissue (MT and CT):

(5) $MT=\frac{n\ x\ 100\ x\ S.c.s.m.}{S.c.s.m.FMP.}$ and (6) CT=100-MT.

We used tables for all our data obtained from micrometer measurements and determinations then we statistically processed and interpreted them. We calculated the general statistical indicators such as: the average; standard error of the mean; standard deviation; variance; geometric mean; the average deviation and the coefficient of variation. We also determined the analysis of variance, namely the Fischer ($\tilde{F}$) and Tukey (W) values. For statistical calculations we used INSTAT-3 algorithm and Anova SingleFactor algorithm, from Microsoft Excel software package [6].

Results and their discussion

Regarding the birds, their leg muscles (thighs and calves), are certainly a valuable part of the carcass, along with the pectoral muscles. These somatic muscles, although quantitatively, they do not ranks first, however, in terms of quality, are superior to those of the breast, and this because they contain both white and red fibers fiber and the amount of fat that they contain is much higher.

From a histological point of view, the muscle that we studied – the gastrocnemian muscle (GL) has a series of interesting parameters whose values are different depending on the sex and age of slaughter of these chickens.

Thus, at the age of 35 days, the average thickness of the myocytes had values of: 28.908 ± 0.388 μ, for males and of 26.197 ± 0.423 μ for females (v = 14.72 to 17.67%).

At the same age, the cross-section surface of the muscular fibers had a statistical mean of 653.957 ± 17.781 μ^2, for males and one of 546.079 ± 16.297 μ^2, for females (v =

29.78 to 32.69%)(table 2). The myocytes perimeter was of 90.819 ± 1.22 μ, for males and of 82.3 ± 1.328 μ, for females (14.72 - 17.67 %).

Table 2. Statistical indicators for the thickness and profile of myocytes* from lateral Gastrocnemius muscle of meat-type hybrid, COBB-500, regarding the age and sex

Specification			UM	N	Statistical indicators			Limits of variations	
Age of slaughter	Sex	Studied parameters			x̄±sx̄	s	V(%)	minimum	maximum
35 days	Mascul	Average diameter	μ	120	28.908±0.388	4.2555	14.72	20.151	42.189
		Myocytes perimeter	μ	120	90.819±1.22	13.3691	14.72	63.3062	132.5421
		Cross-section surface	μ^2	120	653.957±17.781	194.7798	29.78	316.285	1363.119
		Myocytes density	fm/mm^2	30	840.856±24.305	133.126	15.83	667.743	1122.786
		MT** proportion	%	30	54.989±1.589	8.7057	15.832	43.668	73.425
		CT** proportion	%	30	45.011±1.589	8.7057	19.341	26.575	56.332
	Femel	Average diameter	μ	120	26.197±0.423	4.6292	17.67	11.1025	33.3075
		Myocytes perimeter	μ	120	82.300±1.328	14.5431	17.67	34.879	104.639
		Cross-section surface	μ^2	120	546.079±16.297	178.5275	32.69	92.9399	867.4393
		Myocytes density	fm/mm^2	30	874.48±4.143	22.6924	2.595	835.965	925.992
		MT** proportion	%	30	47.753±0.226	1.2391	2.595	45.650	50.566
		CT** proportion	%	30	52.247±0.226	1.2391	2.372	49.434	54.350
42 days	Mascul	Average diameter	μ	120	32.883±0.388	4.2539	12.94	22.205	43.9659
		Myocytes perimeter	μ	120	103.305±1.22	13.364	12.936	69.759	138.1228
		Cross-section surface	μ^2	120	848.921±20.466	224.196	26.41	371.760	1495.868
		Myocytes density	fm/mm^2	30	624.876±4.863	26.6377	4.263	577.674	688.492
		MT** proportion	%	30	53.047±0.413	2.2614	4.263	49.040	58.448
		CT** proportion	%	30	46.953±0.413	2.2614	4.816	41.552	50.960
	Femel	Average diameter	μ	120	30.052±0.282	3.0865	10.27	21.2675	37.1934
		Myocytes perimeter	μ	120	94.41±0.885	9.6965	10.27	66.8138	116.8464
		Cross-section surface	μ^2	120	700.503±13.081	143.2915	20.4555	342.6935	1074.6192
		Myocytes density	fm/mm^2	30	715.326±16.717	91.5631	12.80	554.090	881.898
		MT** proportion	%	30	50.109±1.171	6.4139	12.80	38.815	61.777
		CT** proportion	%	30	49.891±1.171	6.4139	12.856	38.223	61.185
49 days	Mascul	Average diameter	μ	120	37.519±0.378	4.1456	11.0493	24.703	46.6305
		Myocytes perimeter	μ	120	117.869±1.189	13.0237	11.05	77.607	146.4939
		Cross-section surface	μ^2	120	1092.199±22.545	246.9717	22.612	465.6672	1703.8986
		Myocytes density	fm/mm^2	30	562.543±10.904	59.7266	10.617	435.296	654.742
		MT** proportion	%	30	61.441±1.191	6.5237	10.618	47.539	71.511
		CT** proportion	%	30	38.559±1.191	6.5237	16.919	28.489	52.461
	Femel	Average diameter	μ	120	32.593±0.360	3.944	12.10	23.3153	41.0793
		Myocytes perimeter	μ	120	102.395±1.131	12.3904	12.10	73.247	129.0542
		Cross-section surface	μ^2	120	823.528±18.085	198.1156	24.057	418.2297	1277.9239
		Myocytes density	fm/mm^2	30	606.874±9.216	50.4751	8.317	507.670	707.360
		MT** proportion	%	30	49.978±0.759	4.1567	8.317	41.808	58.253
		CT** proportion	%	30	50.022±0.759	4.1567	8.310	41.747	58.192

* myocytes =striated muscle fibers; **MT=muscular tissue; ***CT=connective tissue.

The density of myocytes from the studied mucle (GL) had mean values of 840.865 ± 24.305 f.m./mm^2, for males and one of 874.48 ± 4.143 f.m./mm^2, for females (table 2). In terms of the proportion of pure muscle tissue, this had a value of 54.989 ± 1.589%, for males and of 47.753 ± 0.226%, for females (table 2).

At the age of 42 days, the age that is considered to be the technological age of slaughtering for this COBB-500 meat-type hybrid, the values of the studied parameters were as following: the average thickness of myocytes was of 32.883 ± 0.388 μ, for the male sex and of 30.052 ± 0.282 μ, for female sex (v=10.27 to 12.94 %) (table 2).

The perimeter of the studied muscular fibers had an average of 103.305±1.22μ, for males and of 94.41±0.885μ, for females (v=10.27-12.94%). The myocytes cross-section surface had a statistical mean of 848.921±20.466μ^2, for males and one of 700.503±13.081μ^2,

for females (20.455-26.41%). For the myocytes density we found some average values like 624.876±4.863 f.m./mm^2, for males and like 715.326±16.717 f.m./mm^2, for females (v=4.263-12.80%) (table 2).

At the age of 42 days, in the studied muscle (GL) the pure muscle tissue proportion was of 53.047±0.413%, for males and of 50.109±1.171%, for females (v=4.263-12.80%) (table 2).

At the age of 49 days the muscular fibers average thickness was of 37.519±0.378µ, for males and of 32.593±0.36µ, for females (v=11.05-12.10%). The perimeter of these muscular fibers had a average value of 117.869±1.189µ, for males and one of 102.395±1.131µ, for females (v=11.05-12.10%) (table 2).

Myocytes cross-section surface of this muscle had, at the age of 49 days, a value of 1092.199±22.545µ^2, for males and one of 823.528±18.085µ^2, for females (v=22.612-24.057%), as for the proportion of muscular tissue, this had a statistic mean of 61.441±1.191%, for males and one of 49.978±0.759%, for females. For the density of these muscular fibers we found values of 562.543±10.904 f.m./mm^2, for males and of 606.874±9.216 f.m./mm^2, for females (v=8.317-10.617%) (table 2).

If we compare the two sexes for all the parameters that we studied in this case we can conclude that there are differences between the two, for all three slaughter ages.

Thus, at the age of 35 days, the average thickness and perimeter of myocytes, for female sex it is smaller with 9.38% than the male sex. Regarding the cross-section surface of these myocytes, the difference between sexes has a value of 16.50%, being smaller in the case of females (table 3).

Table 3. The myocytes thickness and profile from Gastrocnemius lateralis muscle from the avian hybrid COBB-500, regarding the age and sex

Studied muscle*	Studied parameters of myocytes and PMF**	MU	Slaughter age and sex								
			35 days			42 days			49 days		
			Male	Female	±%F/M****	Male	Female	±%F/M****	Male	Female	±%F/M****
Gastrocnemius lateralis	Average thickness of myocytes	µ	28.908	26.197	**-9.378**	32.883	30.052	**-8.609**	37.519	32.593	**-13.129**
	Myocytes perimeter	µ	90.819	82.300	**-9.38**	103.305	94.41	**-8.610**	117.869	102.395	**-13.128**
	Myocytes cross-section surface	µ	653.957	546.079	**-16.496**	848.921	700.503	**-17.483**	1092.199	823.528	**-24.599**
	Myocytes density	fm/mm^2	840.865	874.48	**+4.00**	624.876	715.326	**+14.475**	562.543	606.874	**+7.88**
	MT proportion***	%	54.989	47.753	**-7.236pp**	53.047	50.109	**-2.938pp**	61.441	49.978	**-11.463pp**
	CT proportion***	%	45.011	52.247	**+7.236pp**	46.953	49.891	**+ 2.938pp**	38.559	50.022	**+11.463pp**

*SM=studied muscle; **PMF= primary muscle fascicle; ***MT=muscular tissue, CT=connective tissue; **** Percentage comparison between female and male; pp=procentual points.

Because the myocytes thickness is inversely proportional with their density, for this index, the differences between sexes is very reduced: 4 %, for females (table 3). Regarding the proportion of muscular tissue, the value of this index is very small for females compared with males, the difference between the two has a value of 7.236% (table 3).

At the age of 42 days, the differences between sexes are present and thei are evolving in a similar way with those of 35 days. Thus, for the myocytes average thickness and perimeter the differeces were of 8.61%. For the cross-section surface the difference has a value of 17.483%; for the muscular fibers density, the values obtained by females exceed males with 17.483% and for the proportion of muscular tissue, the difference was of 2.94% (tabelul 3).

At the age of 49 days, the differences between sexe was intensified for the thickness, the perimeter and the cross-section surface of the myocytes, with a valuae of 13.13% respectively 24.60%, females being inferior to males (table 3).

For the myocytes density, the difference between females and males was of +7.88% in this case (table 3). Regarding the muscular tissue proportion, the two sexes have very different values: 11.463 procentual points (pp), in favor of females (table 3).

We also studied the statistical significance of our data and the majoriry (83.33%) of the differences found between males and females were distinct and very statistical significant ($\hat{F}$>Fα) (D>$W_{0,01}$) (tabelul 5). The differences that were not significant (between the two sexes) were those regarding the density of myocytes, at the age of 35 days and those regarding the proportion of muscular and connective tissue, for the age of 42 days (table 5).

Table 4. Evolution of some morphological parameters of muscular fibers and fascicles from gastrocnemius lateralis muscle of COBB-500 avian hybrid, depending on sex and slaughter ages

SM*	Studied parameters of myocytes	Slaughter age and sex			
		35 days(V1)			
		Males	Females	Sexes average	
		±% V1/V2**	±% V1/V2**	Absolute values	±% (pp) V1/V2
Gastrocnemius lateralis (GL)	Average thickness of myocytes (μ)	**-12.088**	**-12.828**	**27.5525**	**-12.441**
	Myocytes perimeter (μ)	**-12.086**	**-12.827**	**86.5595**	**-12.440**
	Cross-section surface of myocytes (μ^2)	**-22.966**	**-22.045**	**600.018**	**-22.550**
	Myocytes density (f.m./mm^2)	**+34.565**	**+22.249**	**857.6725**	**+27.992**
	Muscular tissue proportion (%)	**+1.942pp**	**-2.356pp**	**51.371**	**-0.207pp**
	Connective tissue proportion (%)	**-1.942pp*****	**+2.356pp*****	**48.629**	**+0.207pp*****
		42 days (V2)			
	Average thickness of myocytes (μ)	100	100	**31.4675**	100
	Myocytes perimeter (μ)	100	100	**98.8575**	100
	Cross-section surface of myocytes (μ^2)	100	100	**774.712**	100
	Myocytes density (f.m./mm^2)	100	100	**670.101**	100
	Muscular tissue proportion (%)	100	100	**51.578**	100
	Connective tissue proportion (%)	100	100	**48.422**	100
		49 days (V3)			
	Average thickness of myocytes (μ)	**+14.098**	**+8.455**	**35.056**	**+11.404**
	Myocytes perimeter (μ)	**+14.098**	**+8.458**	**110.132**	**+11.405**
	Cross-section surface of myocytes (μ^2)	**+28.657**	**+17.562**	**957.8635**	**+23.641**
	Myocytes density (f.m./mm^2)	**-9.975**	**-15.161**	**584.7085**	**-12.743**
	Muscular tissue proportion (%)	**+8.394pp**	**-0.131pp**	**55.7095**	**+4.131**
	Connective tissue proportion (%)	**-8.394pp*****	**+0.131pp*****	**44.2905**	**-4.131**

*SM=studied muscle; ** percentage comparisons between the slaughter ages of chickens (V1=35 days; V2=42 days; V3=49 days); ***pp=procentual points.

If we compare the three slaughtering ages of the chickens for all six studied parameters we observe that there are also some important differences. Thus, regarding the values obtained for the age of 42 days, we can observe the fact that the myocytes parameters, for the age of 35 days are smaller, for males and also for females, as an average value. The differences found are of 12.088-12.441-12.82%, for the cross-section surface and of 22.25-27.99-34.565%, for myocytes density (table 4). Regarding the proportion of muscular tissue, the situation is pretty unusual with a difference between males and females. The mean value of the muscular tissue proportion for the age of 35 days is 51.371% and this value is very close of the one of the age of 42 days: 51.578% (a mean of sexes). There is a small difference of 0.207 procentual points (table 4).

The values of all parameters studied, for the age of 49 days can be characterised by a big significance, both in males and females, compaired with the values obtained for the age of 42 days. Thus, for myocytes thickness and their perimeter, the difference is of 14.098%, for males; de 8.455%, for females and of 11.404%, as mean of sexes (table 4). For the cross-section surface, this difference is of 28.657%, for males; de 17.562%, for females and of 23.641%, as mean of sexes (table4). Regarding the myocytes density, the difference found has a value of 9.975%, for males; de 15.161%, for females and of 12.743%, as the mean of sexes (table 4).

Table 5. Statistical significance of the differences found between males and females regarding all structural parameters studied from gastrocnemian lateral muscle of meat type hybrid COBB-500

Slaughter age	Studied parameters	Differences between sexes	Tukey values (w=0,01)	Statistical signification	At 1; 238 GL, for:			
					P	p ≤ 0,05	p ≤ 0,01	p ≤ 0,001
					Fα	**3.84**	**6.64**	**10.83**
35 days	Average thickness of myocytes (μ)	**M-F*=2.711**	**1.4774**	***	F	**22.315347**		
	Myocytes perimeter (μ)	**M-F=8.519**	**4.6415**	***	F	**22.316203**		
	Cross-section surface of myocytes (μ^2)	**M-F=107.878**	**62.08095**	***	F	**20.0042566**		
	Myocytes density (f.m./mm^2)	**M-F=33.615**	**65.6579**	**n.s.**	F	At 1; 58 GL, for:		
						4.008	**7.103**	**12.034**
						1.858758		
	Muscular tissue proportion (%)	**M-F=7.236**	**4.27527**	***	F	**20.310884**		
	Connective tissue proportion (%)	**M-F=7.236**	**4.27527**	***	F	**20.310899**		
42 days	Average thickness of myocytes (μ)	**M-F=2.831**	**1.2349**	***	F	**34.827131**		
	Myocytes perimeter (μ)	**M-F=8.895**	**3.8795**	***	F	**34.826804**		
	Cross-section surface of myocytes (μ^2)	**M-F=148.418**	**62.5174**	***	F	**37.3631724**		
	Myocytes density (f.m./mm^2)	**M-F=90.45**	**46.3624**	***	F	At 1; 58 GL, for:		
						4.008	**7.103**	**12.034**
						26.991070		
	Muscular tissue proportion (%)	**M-F=2.938**	**3.3065**	**n.s.**	F	**5.599554**		
	Connective tissue proportion (%)	**M-F=2.938**	**3.3065**	**n.s.**	F	**5.599554**		
49 days	Average thickness of myocytes (μ)	**M-F=4.926**	**1.3444**	***	F	**88.91402**		
	Myocytes perimeter (μ)	**M-F=15.474**	**4.2237**	***	F	**88.913755**		

	Cross-section surface of myocytes (μ^2)	**M-F=268.671**	**74.39209**	***	$\hat{F}$	**86.4098176**		
	Myocytes density (f.m./mm^2)	**M-F=44.331**	**38.0192**	**	$\hat{F}$	At 1; 58 GL, for:		
						4.008	**7.103**	**12.034**
						9.641694		
	Muscular tissue proportion (%)	**M-F=11.463**	**3.76087**	***	$\hat{F}$	**65.8793532**		
	Connective tissue proportion (%)	**M-F=11.463**	**3.76084**	***	$\hat{F}$	**65.879400**		

*M- F = differences between males and females.

For the muscular tissue proportion, for males, the difference between the age of 49 days and the one of 42 days is of 8.394 procentual points, but for females the difference is very small. For the sexes average, the difference (for this specific character) is of 4.131 procentual points (table 4).

Regarding the statistical significance, the difference between those 3 slaughter ages, for all 6 studied parameters, the results have been highly significant (table 6) ($\hat{F}$<Fα) (D<$W_{0,01}$). For females there are six significant differences (particularly for connective and muscular tissue proportion), and for males there are only three differences all nonsignificant (for the same character) (table 6).

Table 6. Statistical significance of the differences found between the 3 slaughter ages, regarding some myocytes parameters of GL muscle of COBB-500 hybrid meat type chickens, by gender

Sex	Studied parameters	Differences between the 3 ages of slaughter*	Tukey Values (w=0,01)	Statistical significance	At 2; 357 GL, for:			
					P	p ≤ 0.05	p ≤ 0.01	p ≤ 0.001
					Fα	**2.99**	**4.60**	**6.91**
MALE	Average thickness of myocytes (μ)	V_1-V_2=3.975	**1.5866**	***	$\hat{F}$	**125.2210876**		
		V_1-V_3=8.611		***				
		V_2-V_3=4.636		***				
	Myocytes perimeter (μ)	V_1-V_2=12.486	**4.9846**	***	$\hat{F}$	**125.213016**		
		V_1-V_3=27.050		***				
		V_2-V_3=14.564		***				
	Cross-section surface of myocytes (μ^2)	V_1-V_2=194.964	**83.8740**	***	$\hat{F}$	**116.322486**		
		V_1-V_3=438.242		***				
		V_2-V_3=243.278		***				
	Myocytes density (f.m./mm^2)	V_1-V_2=215.989	**66.3528**	***	$\hat{F}$	At 2; 87 GL, for:		
		V_1-V_3=278.322		***		**3.114**	**4.8945**	**7.5575**
		V_2-V_3=62.333		**n.s.**		**87.27515035**		
	Muscular tissue proportion (%)	V_1-V_2=1.942	**4.9707**	**n.s.**	$\hat{F}$	**14.075423**		
		V_1-V_3=6.452		***				
		V_2-V_3=8.394		***				
	Connective tissue proportion (%)	V_1-V_2=1.942	**4.9707**	**n.s.**	$\hat{F}$	**14.0754355**		
		V_1-V_3=6.452		***				
		V_2-V_3=8.394		***				
FEMALE	Average thickness of myocytes (μ)	V_1-V_2=3.855	**1.4809**	***	$\hat{F}$	At 2; 357 GL, for:		
		V_1-V_3=6.396		***		**2.99**	**4.600**	**6.910**
		V_2-V_3=2.541		***		**80.2816997**		
	Myocytes perimeter (μ)	V_1-V_2=12.110	**4.6459**	***	$\hat{F}$	**80.5067182**		
		V_1-V_3=20.094		***				
		V_2-V_3=7.985		***				
	Cross-section surface of	V_1-V_2=154.424	**65.7389**	***	$\hat{F}$	**75.9110443**		
		V_1-V_3=277.449		***				

	myocytes (μ^2)	V_2-V_3=123.025		***				
	Myocytes density (f.m./mm^2)	V_1-V_2=159.154	47.8619	***	F	At 2; 357 GL, for:		
		V_1-V_3=267.606		***		3.114	4.8945	7.5575
		V_2-V_3=108.452		***		142.643137		
	Muscular tissue proportion (%)	V_1-V_2=2.356	3.4638	n.s.	F	2.630297		
		V_1-V_3=2.225		n.s.				
		V_2-V_3=0.131		n.s.				
	Connective tissue proportion (%)	V_1-V_2=2.356	3.4638	n.s.	F	2.630286		
		V_1-V_3=2.225		n.s.				
		V_2-V_3=0.131		n.s.				

*The slaughter age of chickens: V_1=35 days; V_2=42 days; V_3=49 days.

Conclusions

1) The myocytes thickness from Gastrocnemianus lateralis muscle has an average value (for the two sexes) of 27.553μ, at the age of 35 days; of 31.467μ, at the age of 42 days and of 35.056μ, at the age of 49 days.
2) Myocytes perimeter has values of 82.30-90.891μ, at the age of 35 days; of 94.41-103.305μ, at the age of 42 days and of 102.395-117.859μ, at the age of 49 days, being small in females than in males with 9.38%, 8.61% and also with 13.13%.
3) The cross-section surface of GL muscle myocytes has values that grow at the same as the age and thei are bigger for males that for females.
4) The myocytes density from the studied muscle has values of 840.865-874.48 f.m./mm^2, at the age of 35 days; of 624.876-715.326 f.m./mm^2, at the age of 42 days and of 562.543-606.874 f.m./mm^2, at the age of 49 de zile, being bigger for females than males with 4%, 7.88% and 14.47%.
5) The GL muscle myocytes density evolves inversely with their thickness.
6) The GL muscle tissue proportion has values of 54.99-47.75%, at the age of 35 de zile; of 53.05-50.11% at the age of 42 days and of 61.44-49.98%, at the age of 49 days, being smaller for females than for males with 7.24, 2.94 and 11.46 procentual points.

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THE MORPHOLOGY AND CYTOCHEMISTRY OF THE UTERUS IN LOHMANN BROWN LAYING CHICKENS

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Abstract

The aim of the present study is to reveal the anatomical, histological, cytochemical and ultrastructural aspects of the uterus in Lohmann brown laying hens. Also we aim to show differences in the morphology of this oviduct segment during the egg mass passing through. The samples were taken from 30 Lohmann Brown chickens aged between 19 and 50 weeks and from three areas in the uterus: anterior, medium and posterior. The samples were fixed in 4% buffered formalin, embedded in paraffin, sectioned at the size of 5 µm and stained H-E, PAS, van Gieson, Masson, von Kossa, Novelli and Gomori. The uterus is structured from a mucous tunic with an epithelium and lamina propria; a submucous tunic with connective tissue and blood vessels; a muscular tunic with two layers of smooth muscle tissue and a serous tunic on the outside that is a fasciculated mesothelium. The epithelium consists of secreting goblet cells and prismatic cells with cilia. These two types of cells alternate in the epithelium but before the egg passage the goblet cells seem to dominate because they are filled with secretion and after the egg passage the prismatic cells apparently dominate because once the goblet cells release their secretion they become very thin and are overlaid by the other cells. These secreting cells from the epithelium have an important role in the mineralisation of the eggshell, the von Kossa stain highlighting the mineralisation sites in the epithelium. The mucosa presents leaf-shaped primary folds with secondary and tertiary folding that enlarge the secreting surface of the epithelium detrimental to the development of the tubular glands in the lamina propria. The muscle tunic is very well developed, having the main role of keeping the egg in this segment and rotating it for about 19 hours in order for the elaborated material to be deposited on the egg mass. As this role is realised in particular using the inner circular group of smooth muscle tissue, this layer is much more developed.

Key words*: anatomy, chicken, histology, SEM*

Introduction

Although there is some data in the scientific literature regarding the morphology and cytochemistry of the uterus in chickens, the present study highlights new aspects on the morphology, cytochemistry and ultrastructure of the uterus in one of the best egg-laying crossbreeds of chicken - Lohmann Brown.

Uterus is the penultimate segment of the oviduct, continuing the isthmus and being continued by the vagina (Hodges, 1974). Differently from the rest of the oviduct that has a tubular shape, the uterus presents a sack-like appearance given by its' very well developed muscular tunic (Todireanu, 2014). In the uterus, the eggshell is elaborated in about 19 hours (Jamieson, 2007). The structure of this segment is similar to the general structure of the oviduct: mucous, submucous, muscular and serous tunics (Kawashima *et al.*, 1999; Cotea, 2013). In order to expand the secreting surface, the mucous tunic of the uterus expands into primary, secondary and tertiary folds (Cotea, 2013; Todireanu 2014).

This research is aimed to bring new data on the subject and also to compare it to the results already published.

Materials and methods

For the present study we used 30 Lohmann Brown chickens, aged between 19 and 50 weeks. This particular crossbreed has been chosen because it is one of the most popular among chicken farmers and also for the very good egg production given for a longer egg-laying period. The samples were harvested from three areas of the uterus (anterior, medium and posterior) and fixed in 4% buffered formalin. After fixation, samples were trimmed and embedded in paraffin, sectioned at 5 µm and stained H-E, PAS, Van Gieson, Masson, von Kossa, Novelli and Gomori.

Results and discussions

The uterus, which continues the isthmus, is presented as a dilated area, with a sack-like shape that has the role of secreting the eggshell (fig. 1). Between the isthmus, vagina and uterus there are intermediate areas that present a different but similar morphology than that of these segments (fig. 1). The uterus has a length of 12-13 cm and a diameter of 40 mm. The diameter of the other segments in the oviduct is given by the height of the mucosa folds, but in this case it is given by the well developed muscle tunic that maintains the sack shape although the mucosa folds are lower and leaf-shaped.

The shell is elaborated at this level, process that requires about 19 hours. The egg mass spends more than three times more time in this segment than in all the other ones together and expands the walls (fig. 2). The reason why this time is required is that while in the other segments the egg just passes through in a spiral direction and gathers the secretion, in the uterus the egg stops and rotates to add the shell components.

In fig. 3 and fig. 4, the secretion of the eggshell in the uterus is highlighted. The egg mass just after leaving the isthmus and entering the uterus presents both shell membranes completely elaborated (fig. 3). After 19 hours, the eggshell is also fully secreted (fig. 4) and the egg is ready to be moved to the next segment, the vagina, where the cuticle is added, and then to be layed.

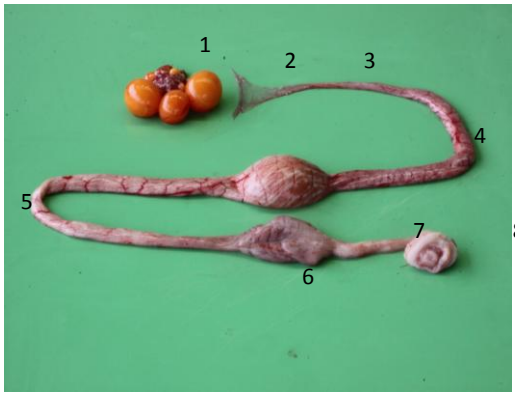

Fig. 1. Chicken oviduct: 1-ovary, 2-infundibulum funnel, 3-neck of the infundibulum, 4-magnum, (with egg mass),5-isthmus, 6-uterus, 7-vagina, 8-cloaca

Fig. 2. Chicken oviduct with egg mass in uterus

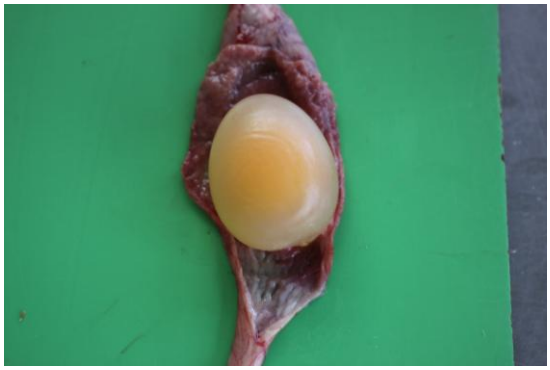

Fig. 3. Chicken isthmus-uterus junction with egg mass in the uterus presenting the shell membranes fully formed

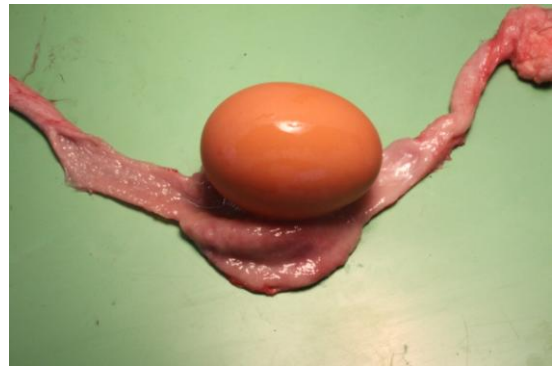

Fig. 4. Chicken uterus with eggshell completely secreted

The structure of the uterus consists of a mucous tunic with surface epithelium that covers the lamina propria with fewer tubular glands than in magnum or isthmus. These glands open at the surface of the epithelium through short and twisted ducts that are structured from glandular cells (fig. 5).

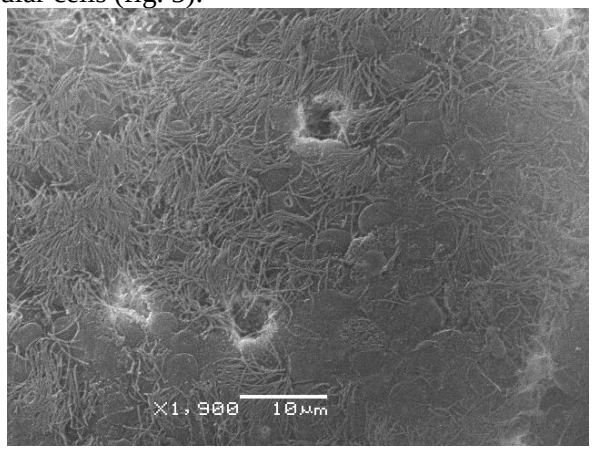

Fig. 5. Uterus. Lohmann Brown chicken. The epithelium with ciliated cells, secretory cells and the openings of the tubular glands, SEM, x1900

The primary mucosa folds are lower and thinner than those of the magnum and isthmus, because of the fewer tubular glands in the lamina propria. To enlarge the secretion surface of the epithelium the primary fold presents secondary and even tertiary folding (fig. 6). The epithelium presents both secreting goblet cells and prismatic cells with cilia at the apical pole. These cells alternate and are in an approximate 1:1 ratio. Given that there are fewer tubular gland cells in the lamina propria and more secreting cells in the epithelium, we highlight the important role that the epithelium has in the elaboration of the eggshell (fig. 7).

The prismatic cells in the epithelium are high and thin, with the nucleus positioned in the middle of the cell and presenting cilia at the apical pole. The goblet cells are thicker, with

secretion granules at the apical pole, and the nucleus pushed in the lower third of the cells. This alternation between the cells gives the epithelium a pseudostratified appearance (fig. 7). After the secretion granules are released from the goblet cells, these become very thin, seem higher, and are shadowed by the prismatic cells. These cells rest on the very thin basal lamina.

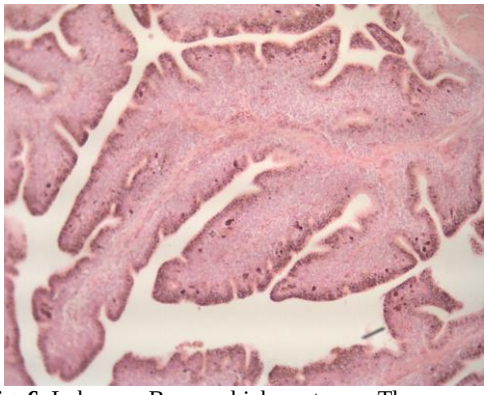

Fig. 6. Lohmann Brown chicken uterus . The mucosa presents leaf shaped primary folds with secondary folding, the calcium being highlighted in the epithelium, von Kossa stain, x100

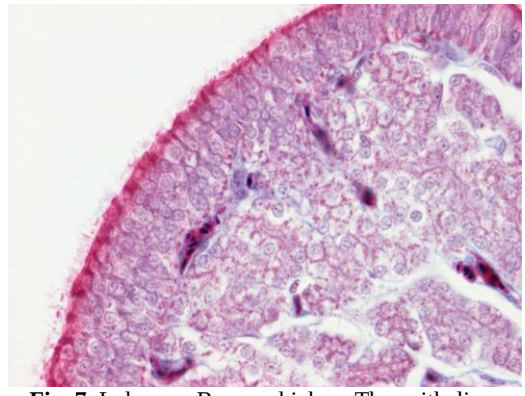

Fig. 7. Lohmann Brown chicken. The epithelium with secretion granules at the apical pole and secreting cells filled with secretion in the lamina propria. Masson stain, x800

In the lamina propria, the tubular glands are structured from 5 to 7 glandular cells, which also present secretion granules at the apical pole and the nucleus pushed lower, in the basal third of the cell (fig. 8). Before the egg passage through this segment, the secreting cells both of the epithelium and of the lamina propria are filled with granules at the apical pole, afterwards the cells are emptied or present lesser granules (fig. 7, fig. 8).

The submucous tunic is structured from connective tissue with blood vessels that irrigate the tubular glands and are have smaller branches that assure the blood irrigation to the epithelium (fig. 9).

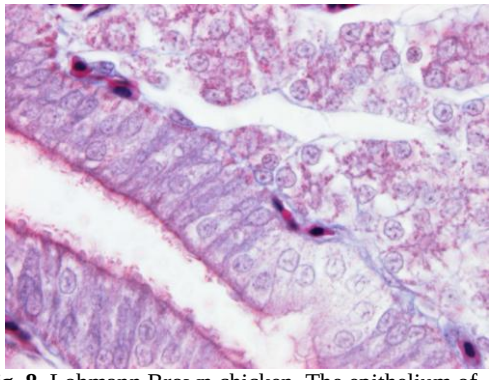

Fig. 8. Lohmann Brown chicken. The epithelium of the uterus after the egg passage with goblet cells emptied of secretion. Masson stain, x800

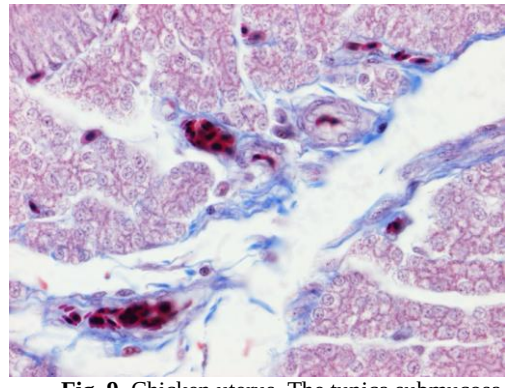

Fig. 9. Chicken uterus. The tunica submucosa structured from connective tissue with large blood vessels, surrounded by tubular glands with capillaries, Masson stain, x400

The muscular tunic is very well developed, being the largest between all the oviducts' segments. This tunic is structured from two distinct layers of smooth muscle cells with different orientation. The most developed is the circular layer, that is just beneath the submucosa. This layer is required to be this developed in order for the egg mass to be maintained and rotated for a long period of time, 19 hours, while the secretion material structures the shell on the surface. The outer longitudinal layer of the muscular tunic is also very well developed, but still less than the circular stratum. Between the two layers of smooth muscle cells there are large blood vessels surrounded by connective tissue. These blood vessels irrigate through smaller ramifications the mucosa folds (fig.10).

The uterus is covered on the outside by a very thin serous tunic which represents a fasciculated mesothelium consisting of a connective tissue associated with an epithelium with a single line of flattened cells (fig. 10).

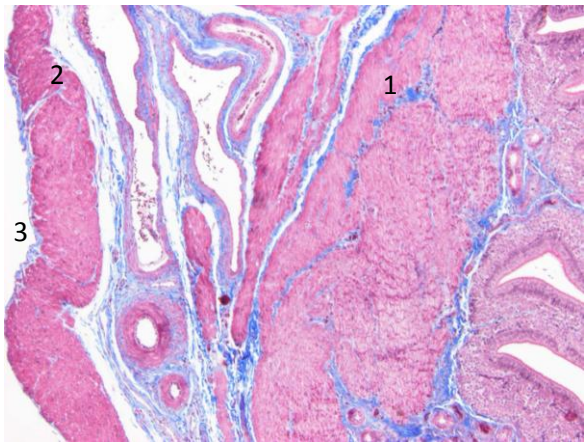

Fig. 10. Chicken uterus. The muscular tunic with the two rows of smooth muscle cells - inner circular layer (1) and outer longitudinal layer (2). Covering the longitudinal row there is a fasciculated mesothelium- the serous tunic (3) Masson stain, x200

Using scanning electron microscopy (SEM) details regarding the epithelium and the secreted material were highlighted (fig. 11, fig. 12, fig. 13).

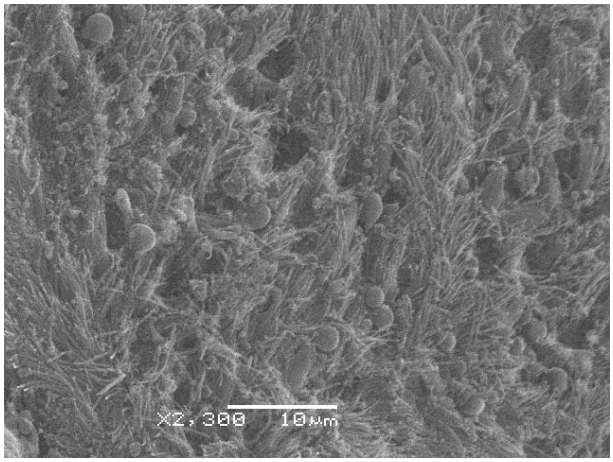

Fig. 11. Lohmann Brown chicken uterus. The epithelium with numerous ciliated cells and presenting secretion granules in the lumen, SEM, x2300

There are numerous cells with cilia lining the mucosa folds and secreting cells mostly at the base and edges of these folds. The secreted material takes the shape of granules and filaments that combine into an amorphous mass that constitutes the eggshell (fig. 11, fig. 12, fig. 13).

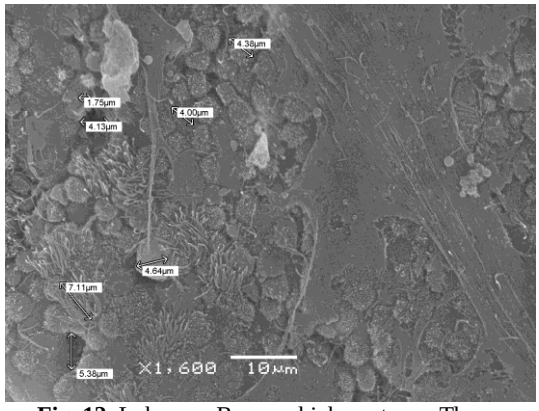

Fig. 12. Lohmann Brown chicken uterus. The epithelium prismatic cells with cilia and secreting cells presenting a secretion mass in the lumen, SEM, x1600

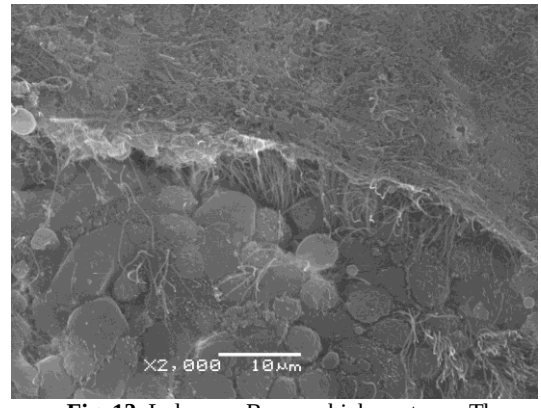

Fig. 13. Lohmann Brown chicken uterus. The epithelium with numerous secreting cells, ciliated cells and the amorphous secretion mass in the lumen, above the epithelium, SEM, x2000

On the transmission electron microscope we highlighted the alternation between the prismatic cells with cilia at the apical pole and the secreting cells in which there are numerous well-defined secretion vesicles compacted and ready for exocytosis (fig. 14). The secreting cells present a nucleus with nucleolus and active heterochromatin that is accessible by the RNA polymerase. Heterochromatin presents as small blocks and the X facultative heterochromatin is present on the inner side of the nuclear membrane, which confirms the female sex. In some areas of the uterus the mucous tunic is invaginated to increase the secreting surface. In this diverticulum we highlighted the cilia of the prismatic cells and the well developed organelles of the secreting cells, represented by numerous mitochondria with frequent folds and the endoplasmic reticulum tanks in which the secretion is gathered (fig. 15).

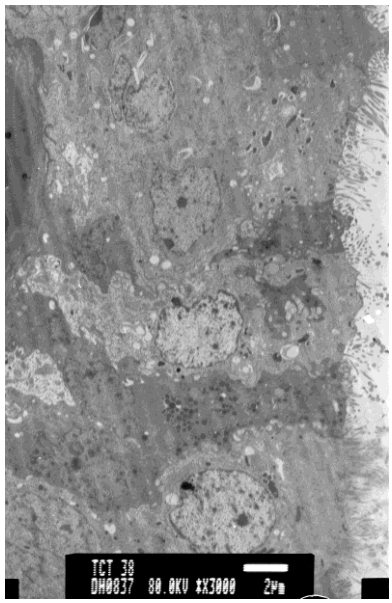

Fig. 14. Lohmann Brown chicken uterus. The alternation of the ciliated and secretory cells, TEM, x3000

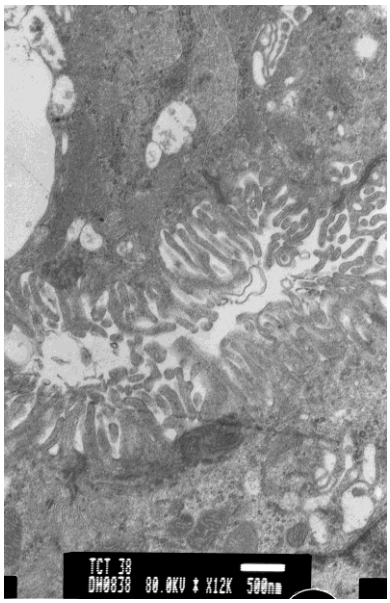

Fig. 15. Lohmann Brown chicken uterus The secretory cells with numerous mitochondria, TEM, x12000

Conclusions

Uterus presents some differences to the general histological structure of the oviduct. The mucous tunic presents folds that are primary, secondary and tertiary, with the role of expanding the secreting surface of the epithelium. Secreting cells present numerous granules at the apical pole before the egg passage, and afterwards they are empty or present less granules. These granules combine into an amorphous mass in the lumen that structure the eggshell. The muscular tunic is highly developed, based especially on the inner circular layer of smooth muscle cells.

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EVALUATION OF THE ACROSOME STATUS OF FROZEN-THAWED BULL SPERMATOZOA USING LIGHT MICROSCOPY METHODS

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Abstract

Background: *The acrosome is a highly specialized region of the spermatozoon, essential for the fertilization process.Defects or dysfunction of this structure have been associated with fertility problems in both man and domestic species including bulls. Since artificial insemination with frozen-thawed spermatozoa is the most commonly used reproductive biotechnology in cattle breeding industry, a simple and fast procedure for routine evaluation of the acrosomal status of frozen-thawed bovine sperm is needed. Current methods of evaluating the acrosome of bull spermatozoa are time consuming and some of them require specialized equipment such as flow cytometry or fluorescence microscopy, which is cost-prohibitive to the average practitioner.* ***Aim of the study:*** *Two methods for evaluation of an acrosome reaction of capacitated bovine spermatozoa, using light microscopy have been compared in this study. Calcium ionophore was used as an inducing agent of acrosomal exocytose. We tested two methods, Spermac Stain and dual staining, which are suitable for light microscopy.* ***Material and Methods****: Capacitacion of the bovine spermatozoa was achieved by incubating the sperm suspension at 39 °C and 5%CO2 for 3 hours in TALP medium. Calcium ionophore A23 187 (Sigma, St. Luis MO) was added and the sperm suspension was incubated for 1 hour in the same conditions in order to induce the acrosome reaction. Wet smears were prepared, fixed and stained according to the protocol for both methods, Spermac and dual staining.* ***Conclusions:*** *Our results suggested that Spermac Stain and dual stain are simple, fast and never the less cheap assays for detection of the acrosomal status of bovine sperm.*

Key words*: bull sperm, acrosome, Spermac stain*

Introduction

In both humans and mammals, capacitation followed by the acrosome reaction of spermatozoa are essential steps for the fertilization process and formation of a zygote. The acrosome reaction is characterized by the vesiculation of the outer acrosomal membrane and subsequently, the release of the acrosomal content. The acrosomal integrity status may be used as a marker for fertilization ability of spermatozoa. Therefore, reliable assays for the evaluation of the acrosome reaction of spermatozoa without special or expensive equipment are needed in both research laboratories and semen production centers.

Material and Methods

Capacitacion and induction of the acrosome reaction of bovine sperm

Frozen-thawed sperm were isolated using a swim-up preparation described previously by Shamsuddin et al. with minor modifications. Briefly, 400 µL were placed under 1 mL TALP, and incubated at 39°C, 5% CO_2 and maximum humidity. After 1 h, 1 mL of the upper fraction were collected and placed in 3 mL of TALP, centrifuged (200 × g, 10 min). Afterwards, the pellet was then resuspended with 3 mL TALP and centrifugated for 10 min at 200 × *g*. Finally, the pellet was resuspended in 150 µl TALP and the sperm concentration was adjusted to 1×10^6spz/mL.To achieve capacitacion, sperm suspension was incubated at 39 °C and 5%CO_2 for 3 hours in TALP medium (Chioham et al., 2004). After capacitacion, 5mM

calcium ionophore A23 187 (Sigma, Europe MO) as the inducing agent of the acrosome reaction was added and the sperm suspension was incubated for 1 hour in the same conditions as for capacitation. For the control samples we used sperm suspension in a medium only (without calcium ionophore).

Dual staining protocol

Sperm membrane and acrossome integrity were assessed using the vital stain Congo Red/Giemsa (Sigma-Aldrich Europe, MO, USA), as described by Kovacs and Foote (1992). An aliquot of semen (20µL) was added to the stain solution (20µL Congo Red0, 18% in 0, 9% NaCl) and incubated at 37ºC for 20 minutes. Slides were washed in distilled water, air dried at a room temperature and fixed with methanol for 5 minutes. The final step consisted in theGiemsa (10%) staining for 20 hours. For all samples, 100 cells were examined in each slide at 1000 X magnification.

The binding site for Giemsa stain during the acrosome reaction is on the outer acrosomal membrane due to the fusion and exclusion of the plasma membrane. The inner acrosomal membrane comes to be exhibited and there is no binding site for acrosome-reacted spermatozoa thus, sperm with detached acrosomes are stained white-grey/white and intact acrosomes are not stained.The sperm was classified as: live with intact acrosome(dark blue/purple acrosomal region and white-grey/white posterior region), live reacted acrosome (unstained, white-grey/white whole sperm head), dead with intact acrosome(blue/dark blue acrosomal and postacrosomal region), and dead with reacted acrosome (white-grey/white acrosomal region and dark blue postacrosomal region) Fig.1.

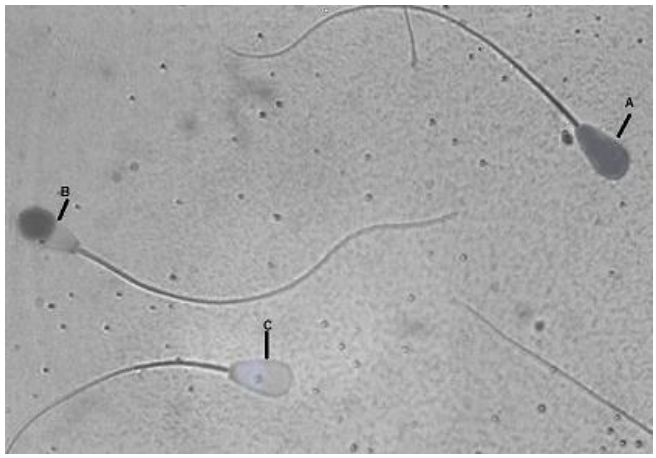

Figure 3 Acrosome status assessed by dual stain (Red Congo/Giemsa). A- dead sperm cell with intact acrosome (blue/dark blue acrosomal and postacrosomal region). B- live sperm cell with intact acrosome (dark blue/purple acrosomal region and white-grey

Spermac Stain protocol

Semen smears from each sample were prepared for determination of acrosomal status using the dichromatic Spermac® stain. After staining, each sample was examined under oil immersion and 1000 × magnification using a bright field microscope. From each smear a

total of 100 spermatozoa were evaluated for acrosome integrity. The sperm cells were assessed as having normal or altered acrosomes (Oettlé EE, 1989;Paulenz H et al., 1995). The acrosomal status was expressed as percentage of sperm cells with normal acrosomal morphology.The normal (acrosomal intact) sperm present a green acrosomal and pink postacrosomal regions with a well-defined thick green band forming a semi-circle at the tip of the sperm head. The acrosome-reacted/abnormal acrosome are those cells that present a pink acrosomal region, or it is green but the semi-circle band is either broken-up, discontinuous, vesiculated, missing or fuzzy, without a distinct dark-green band at the tip of sperm head.

Statistical analysis

Data analysis was performed using the statistical software package Statistical Analysis Systems (Ver. 9, SAS Institute Inc, Cary, NC, USA) using analysis of variance (PROC MIXED) for each parameter. The statistical model included the fixed effect of treatment and the random effect of bull. Least-squares mean for treatment and control was compared using Student's t-test, and p-values < 0.05 were regarded as significant.

Results

The percentage of sperm cells with intact and reacted acrosome was determined using the two methods. The percentage of sperm cells with intact acrosome (live and dead) for the dual staining was calculated in order to compare the results with the dichromatic Spermac stain. A percentage of 66 sperm cells with intact acrosome was obtain using the dual staining technique for both dairy and beef bulls, whereas using the Spermac stain, a percentage of 64 sperm cells with intact acrosome for dairy, respectively 61% for the beef bulls was observed. However, no significant statistical differences were determined between the two methods (Table1; Table 2). A visible increasing number of sperm cells with reacted acrosome was observed in the samples treated with calcium ionophoreA23 187 (Sigma, Europe MO) but no significant statistical difference was observed between breeds or stainings (Table 1; Table 2).

Table1. The acrosome status of frozen-thaw bull sperm from dairy and beef bulls in Response to Calcium Ionophore Influx as assessed by dual staining technique (mean±SD)

	Dairy bulls		**Beef bulls**	
Parameter	**Control**	**Treated**	**Control**	**Treated**
Dead, reacted acrosome	25	29	26	31
Dead, intact acrosome	36	26	33	23
Live, reacted acrosome	9	35	8	37
Live, intact acrosome	30	13	33	8

Table2. The acrosome status of frozen-thaw bull sperm from dairy and beef bulls in Response to Calcium Ionophore Influx as assessed by dual staining technique (mean±SD)

Parameter	Dairy bulls		Beef bulls	
	Control	Treated	Control	Treated
Intact acrosome	64	33	61	31
Reacted/damaged acrosome	36	67	39	69

Discussions

Once sperm are ejaculated, they must undergo structural and metabolic modifications, called capacitation, that take place inside the female reproductive tract (Pereira et al., 2000). Capacitation confers sperm the ability to gain hyperactive motility in order to interact with the oocyte zonapellucida, undergo the acrosome reaction and finally initiate the fusion of the plasma membrane with the oocyte (Rajesh &Preeti, 2004; Yanagimachi, 1994). During the acrosome reaction, the outer acrosomal membrane suffers a fusion process with the plasma membrane, and theacrosomic enzymes are released (Pereira et al., 2000). Therefore, sperm must retain an intact acrosome, so that acrosome reaction can occur at the proper time in order to perform fertilization. However, false acrosome reaction may occur during the freezing and thawing process (Bedford, 1990; Hou et al., 2002).

In our experiments we detected the acrosome status of frozen-thaw bovine spermatozoa from dairy and beef bullusing two evaluation methods double staining and dichromatic Spermac stain. We concluded thatboth staining procedures are cheap and do not required expensive equipment such as flow cytometer or fluorescence microscop in order to evaluate the acrosome status of the frozen-thaw bull sperm. The slides can be examined under a light microscopy, and the preparations are stable for a long time compared to other expensive procedures such as fluorescence microscopy. The samples can be analyzed successfully regardless if the semen is diluted in extender with or without egg yolk. However, dual staining appears to be more suitable method for the evaluation of the acrosome reaction of bovine sperm and possesses some advantages compared with the Spermac stain. The dual stain permits facilitates the simultaneous evaluation of both viability andacrosomal status in one smear, thus true and false acrosome reaction (dead sperm with detached acrosome) can be determined using this technique.

As artificial insemination with frozen-thawed spermatozoa is a routinely used technique in cattle breeding, these methods could be useful for the evaluation of sperm acrosome status in the practice.

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CORRELATION AMONG MOTILITY, MORPHOLOGY, VIABILITY AND DAMAGED CHROMATIN OF CRYOPRESERVED BULL SPERMATOZOA

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Abstract

Background: *Chromatin integrity is currently considered a determinant part of sperm quality thus its evaluation is more over introduced as mandatory parameter in the routine evaluation of sperm quality. Toluidine blue (TB) is a nuclear dye used to evaluate sperm chromatin integrity by detecting the absence or rupture of disulfide bonds. The methods represents a simple and nevertheless, economical alternative to determine the chromatin integrity status and its implications in the reproductive potential of bulls.* ***Aim of the study:*** *The aim of this study was to determine the percentage of sperm with damaged chromatin measured by toluidine blue stain and its relationship with motility, viability and normal morphology in cryopreserved bull semen.* ***Material and Methods****: Immediately after thawing, wet smears were prepared and stained with toluidine blue to establish the percent of sperms with damaged chromatin (sperms with damaged chromatin were stained dark blue or violet while sperms with normal chromatin were light blue). Eosin-nigrosin stain was used to assess both viability and morphology. Motility was subjectively measured using light microscopy. Effect of bull, and the interaction between variables were also assessed using Statistical Analysis System for Windows, software 9 (SAS Inst. Inc.; Cary, NC. USA). Analysis of variance was conducted with the General Lineal Model (GLM procedure) and results are shown as means ± SD. A Spearman's correlation test was used to establish correlation among the measured parameters.* ***Results and Conclusions:*** *The mean percentage of sperm cells with damaged chromatin was 4.65 ± 1.89 while the percentage of live sperms was 54.25 ± 10.44. The percentage of sperm with normal morphology ranked between 72% and 93%, with a mean of 83.33± 8.89. Motility was positively correlated with viability (r=0.796, p=0.0002) and normal morphology (r=0.725, p=0.0002). Viability was positively correlated (r=0.852, p=0.0002) with normal morphology and negatively with the damaged chromatin percent (r= -0.453, p=0.008). Normal morphology and motility were also negatively correlated with the percentage of damaged chromatin (r=-0.867, p=0.0421) and r=-0.568, p=0.028).* ***Conclusions:*** *the cryopreserved semen analyzed presented a relatively low level of chromatin damage, and this trait was negatively correlated with sperm motility, morphology and viability. Further studies, on a larger scale are needed for a better observation of the breed effect on chromatin integrity and possible relationship with other semen parameters.*

Key words: *bull spermatozoa, cryopreservation, sperm chromatin, toluidine blue stain*

Introduction

The biological value of sperm has an essential role in the reproductive efficiency in herds and *in vitro* embryo production systems, with direct impact on both fertilization and embryo development (Hansen, 2002). Along with classical parameters that are evaluated in the routine spermiogram, chromatin integrity is currently considered an essential parameter in the estimation of sperm quality (Gillan *et al.*, 2008; Januskauskas *et al*., 2001),

Studies carried out before on bulls, revealed a negative correlation between motility, viability and damaged chromatin (Kasimanickam *et al.*, 2006). Sperm chromatin integrity has been shown to affect the reproductive potential (Jane Morrell, 2008).

DNA integrity is affected by several factors, including cryopreservation, but the precise underlying mechanism is still much debated. Studies conducted by Thomson *et al.*, 2009 revealed that sperm DNA injuries induced by cryopreservation are primarily

mediated by reactive oxygen species rather than apoptosis. Semen cryopreservation induces sperm DNA fragmentation, which is comparatively higher in bulls with poor semen quality (Mukhopadhyay *et al.,* 2011).

The toluidine Blue (TB) is a nuclear dye that has been used to evaluate sperm chromatin integrity by detecting the absence or rupture of disulfide bonds in the sperm DNA. This stain has been used to evaluate sperm chromatin integrity in several species, such as bulls, horses, rabbits, buffaloes and humans (Beletti *et al*., 2005; Zúccari *et al*., 2008), high correlations between this method and other more sophisticated method such as SCSA and TUNEL have being observed (Erenpreiss *et al*., 2004).

Material and methods

Motility and viability in frozen-thawed bull semen

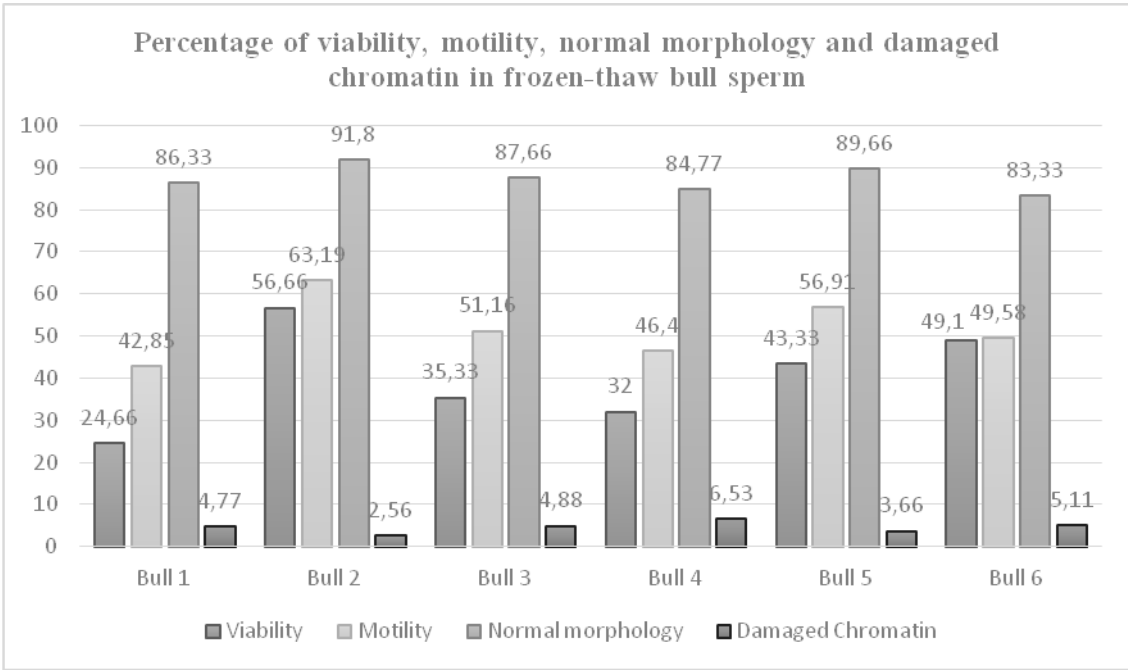
Straws with frozen semen from 6 bulls were used in this study. The straws were thaw in a water bath at 37 °C for 30 seconds and immediately after, motility was estimated under light microscope at x 400 magnification in bright field illumination. Sperm viability was measured with eosin-nigrosin stain (Tamuli and Watson, 1994) by mixing 15 µL of semen and 10 µL of stain for 30 seconds. An aliquot of sperm was smeared onto a pre-warmed glass slide and air dried. The percentage of viable sperm, represented by the unstained sperm cells was determined by smear observation under light microscope at x 1000 magnification.

Chromatin integrity in frozen-thawed bull semen

Toluidine blue stain was used to assess the sperm chromatin integrity (Agarwal and Said, 2004). An aliquot of semen was spread onto a glass-slide, air dried and fixed in an solution composed of ethanol:acetic acid (3:1 v/v) for 30 minutes. The fixed smears were hydrolysed for 5 minutes in 0.1 N HCl, washed in distilled water, air dried and stained with a 0.05% toluidine blue (pH 4) in McIlvain buffer for 10 minutes. Smears were observed under a light microscope at x 1000 magnification and classified as it follows: Light blue or green sperm were considered as having normal chromatin, whereas dark blue or violet sperm were considered as having damaged chromatin.

Results

The results per bull for all the routine parameters analyzed and for the chromatin integrity evaluation using TB stain are are shown in Figure 1. The mean percentage of sperm cells with damaged chromatin was 4.658 ± 1.89 while the percentage of live sperms was 51.68 ± 9.44. The percentage of sperm with normal morphology ranked between 83.3% and 91.8%, with a mean of 87.24±4.89. The mean motility was 40.18±10.33 and Spearman's correlation test revealed a positive correlation with the percentage of live sperm cells ($r=0.828$; $p=0.04156$). The percentage of sperm cells with damaged DNA was negatively correlated with morphology ($r=-0.885$; $p=0.018$) and viability ($r=-0.657$; $p=0.156$) and motility ($r=-0.714$; $p=0.0424$).



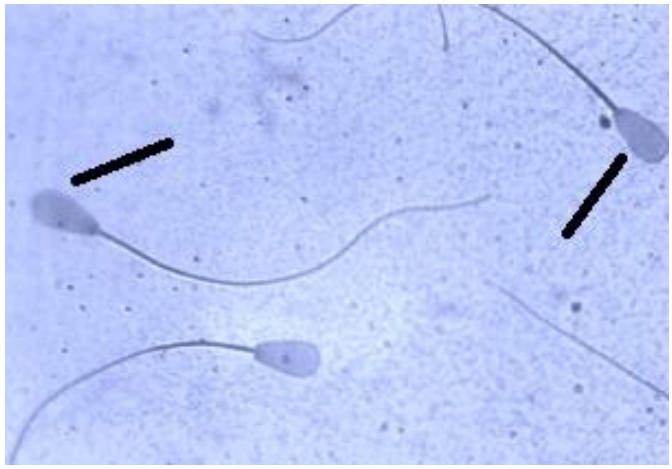


Figure 4 Frozen-thaw bull spermatozoa stained with Toludine blue. Arrows indicate sperm cells stained dark violet, which are presumed to have damaged chromatin.

Discussions and Conclusions

The present study aimed to identify any possible relationships between the percentage of damaged chromatin from frozen-thaw bull sperm, assessed using the toluidine blue stain and parameters which are routinely analyzed such as viability, motility and normal morphology. According to some studies (Slowínska *et al*., 2008), the cryopreservation process might be one of the main causes that determines the damage of the sperm chromatin.

The bull effect significantly affected the level of damaged chromatin, this being in agreement with other studies conducted by Gillan et al. (2008). Fertility variations in bulls might be explained by the evaluation of sperm chromatin integrity alone or in combination with other parameters (Gillan *et al.*, 2008) which could lead to the selection of superior bulls since higher levels of abnormal chromatin were observed in poorly fertile bulls.

In previous studies conducted by Januskauskas *et al.*, 2003, a negative correlation between sperm chromatin integrity and other routine semen parameters was observed. Since negative association between sperm chromatin damage and fertility were observed in several studies conducted Fatehi *et al*., 2006; García-Macías *et al*., 2008, the chromatin integrity should be studied as an independent complementary parameter for the better assessment of bull sperm quality.

In humans, the assessment of chromatin integrity using the TB stain was compared with other more sophisticated methods such as SCSA, TUNEL or Acridine Oranj (Erenpreiss *et al*., 2004), high correlation coefficients being observed. However, similar studies on frozen-thaw bull sperm are lacking, only a moderate correlation being observed with Acridine-oranj on a study regarding the cryopreserved semen from buffaloes (*Bubalus bubalis*). On the other hand, TB stain represents a practical methodology to study sperm morphometric features and to identify nuclear regions susceptible to chromatin alterations (Beletti *et al*., 2005) and nonetheless is a fast, simple and accesible method to evaluate the integrity of the sperm DNA compared to SCSA and TUNEL.

In conclusion, the cryopreserved semen from the bull taken into study presented low level of sperm with damaged chromatin, as assessed by TB stain. The percentage of damaged chromatin was negatively correlated with viability, motility and morphology and this parameter could be used supplementary to estimate the biological value of frozen-thaw bull sperm.

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PREVALENCE OF GASTROINTESTINAL PARASITOSES IN HARE (LEPUS EUROPAEUS) IN WEST ROMANIA

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Abstract

During the period September 2013 - January 2014, 26 hare cadavers (LepusEuropaeus), from three hunting places from Timis County were necropsied, 14 were males and 12 females. The gastrointestinal mass from each cadaver was collected to determine the digestive parasites. Macroscopic examination was performed: each segment of the digestive tube was sectioned, the mucosa and the gastrointestinal content were examined carefully, and for the gastrointestinal mass successive washes method was used. The gastric content were microscopically examined, the intestinal content and each segment of digestive tube (previously washed) were examined with the aid of a magnifying glass. From 26 samples examined, 24 were positive, the prevalence was 92.30%. The parasites identified were Eimeria spp. found in 20 samples, with the prevalence of 76.92%, Cysticercuspisiformis - intwo samples (7.69%), Tricostrongylus spp.- in six samples (23.07%) and Trichocephalusleporis - in nine samples (34.61%).

Key words*: LepusEuropaeus, digestive system, necropsy.*

Introduction

In the begining the hare (LepusEuropaeus) was considered belonging to the order Rodentia and after the researches/studies of Gildy in 1912, the hare was correctly classified in the order Lagomorpha, Family Leporidae (7). The living area for hares spreads through entire Europe, from fields until the alpines area.

Description: body lengths varies from 60 cm to 70 cm, weights between 4-4.5 kg. Its colour matches the environment, ensuringa good stealth. The fur colour is grizzled yellow-brown on the back; with rufous sides; white on the underside and black on the tail and ear tips. Hares are herbivorous and feed on grasses, herbs, twigs, leaves, buds, bark and field crops (8).Sexual dimorphism is absent (7).

Afrenie et al. in 2008 in a study made at Timisoara's Zoo Garden, have found in faeces samples from european hares (*Oryctolaguscuniculus*) the parasites: *Eimeria spp.* (1).

After examining 24 gastrointestinal mass samples from hares (*Lepus europaeus*) belonging to a hunting place from Finland, the parasites *Trichostrongylus spp.*, *Dicrocoeliumdendriticum*, *Eimeria spp.*, in 88% were identified (3).

Epidemiological aspects, increased health standards for the hunting products, the lack of references on the parasitoses in hares, are the motivation for studying the prevalence of gastrointestinal parasites infestation in hares (*Lepus europaeus*) from three hunting places from Timis County.

Materials and methods

From September 2013 till January 2014, 26 hare (Lupus europaeus) cadavers were necropsied, out of three hunting estates from Timiş County. Out of 26 cadavers, 14 were males and 12 females. The hares age was not identified, yet, they were adults. All hares were brought from Timiş County, eight samples from Cheveresu Mare (Woods), ten from Folvodia Frontiera, and eight samples from Banloc. All cadavers have been examined at Parasitology and Parasitical diseases Clinique at Veterinary Medicine from Timisoara.

For identification of digestive parasites, the gastrointestinal mass has been collected from each cadaver and stored (refrigerated) at 5º C. Macroscopic examination has been chosen: each section of the digestive tract has been open, by means of longitudinal cuts, the mucous membrane and its content was carefully examined and the gastrointestinal mass by successive washes method (Fig. 1, 2) (2).

Fig. 1. Gastrointestinal mass prepared for examination.

Fig.2 Examination of the gastrointestinal mass- Successive wash method

The adult parasites have been collected from each section of the digestive tract (stomach, small intestine, large intestine). These were washed with physiological serum 0.9%, to eliminate the impurities, and then, preserved in ethanol 70% (2).
Were examined under the microscope the gastric and under the magnifying glass the intestinal content as well as each segment of the digestive tract, following a pre-washing procedure. The faeces have been examined using the flotation (Willis) method (2). Identification of the *Eimeria* has been made in accordance with the identification keys described by Pellerdy (1974) (4).

Results and discussions

Macroscopic exam:

Following the analysis of the gastrointestinal mass probes, was identified the presence of *Trichostrongylus spp.* adults, after the content of the small intestine has been emptied**.** In the large intestine was identified the presence of *Trichocephalus leporis.*

On liver, was identified*Cysticercus pisiformis,* larva form of *Taenia pisiformis* (Fig.4, 5, 6).

Fig. 4.*Trichostrongylus spp.*adult

Fig.5. *Trichocephalus leporis* adult

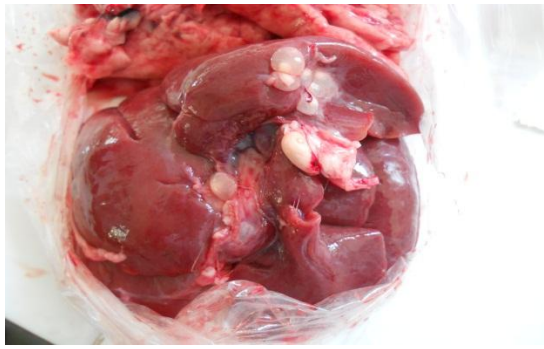

Fig.6. Liver with *Cysticercus pisiformis*

Microscopic exam:

Following coproparasitological exams using flotation method has been identified parasitism with *Eimeria spp.* and*Trichostrongylus spp.* (Fig.7, 8).

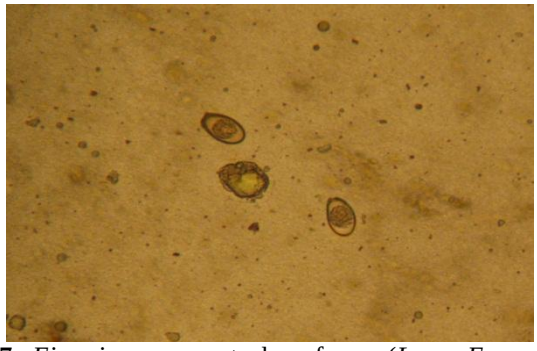

Fig. 7. *Eimeria spp*.oocysts, hare faeces (*Lepus Europaeus*)

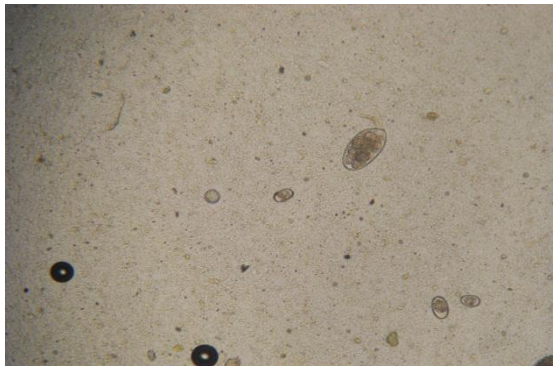

Fig. 8. *Tricostrongylus spp*.eggs, *Eimeria spp*. oocysts

Out of 26 probes, 24 were positives with a prevalence of 92,30%.

Of all the examined probes 21 were found with multiparasitism (80,76%), and five with monoparasitism (19,23%). Monoparasitism has been marked by trichostrongyles (2/26 –

7,69%) and *Eimeria* (3/26 –11,53%). Multiparasitism was encountered in 21 (80,76%) animals, beeing marked by a combination of protozoars, cestodes and nematodes.

With regard to the prevalence of identified parasites it has been acknowledged that parasitism with *Eimeria spp.* has been found in 21 samples (76,92%), with *Cysticercus pisiformis* two samples (7,69%), *Trichostrongylus spp*., in six samples (23,07%), and *Trichocephalus leporis* in nine samples (34,61%).

From 26 examined samples, 14 were male (53.84%) and 12 female (46.15%).

From the examined male samples, nine samples (64.28%) were parasitized with *Eimeria spp.*, three samples (21.42%) with *Tricostrongylus spp.*, no sample (0%) was parasitized with *Cysticercus pisiformis*, six samples (42.85%) with *Trichocephalus leporis* and two samples(14.28%) were negative.

From the examined female sampled, 11 samples (91.66%) were parasitized with *Eimeria spp.,* three samples (25%) with *Trichostronglus spp.,* two samples (16.66%) with *Cysticercus pisiformis* and three samples (25%) with *Trichocephalus leporis.*

Young hares live together with female hares even after weaning, thisposing an increased risk of contamination with *Eimeria spp.* to young hares from parasitized females.

There is no difference in parasitism ratio between females and males for parasitism with *Trichostrongylus spp., Cysticercus pisiformis, Trichocephalus leporis.*

In this study the parasitism prevalence was: in Cheveresu Mare 100% (8/8 analyzed), in Folvodia Frontiera 80% (8/10 analyzed) Banloc 100% (8/8 analysed).

In Cheveresu Mare out of eight examined samples all (100%) were positive for *Eimeria spp.*, three (37.5%) were parasitized with *Trichostrongylus spp.*, two samples (25%) with *Trichocephalus leporis.*

In Folvodia Frontiera out of ten examined samples, two samples (20%) were negative, for samples (40%) were parasitized with *Eimeria spp.*, two samples (20%) with *Trichocephalus leporis,* two samples (20%) with *Cysticercus pisiformis.*

In Banloc, eight out of eight (100%) examined samples have been parasitized with *Eimeria spp.*, three samples (37,5%) with *Trichostrongylus spp.*, andtwo (25%) with *Trichocephalus leporis.*

In the study conducted by us, a 76.92% prevalence for *Eimeria spp.* and a similar prevalence-88% for *Trichostrongylus spp.,Dicrocoelium dendriticum*, *Eimeria spp.*,have been identified in a study in Finland (3).

After examining 24 samples of gastrointestinal mass in hares *(Lepus europaeus)* originated from a hunting estate in Finland, the diagnostic of parasitismwith *Trichostrongylus spp., Dicrocoelium dendriticum*, *Eimeria spp.*, totals a percentage of 88%, and in our study a 92,30%, % (3) prevalence of gastrointestinal parasitosis.

The parasitism with *Eimeria spp.* in hares (Lepus europaeus) was identified only at the species raised in the wild, and not at hares raised in cages. The species identified were: *E. leporis, E. semisculpta, E. robertsoni, E. townsenai, E. hungarica* and *E. europeea* (4).

Afrenie et al. in 2008 in a study made at Timisoara's Zoo Garden, have found in faeces samples from european hares (*Oryctolaguscuniculus*) the parasites: *Eimeria spp.* (1).

McCulloch et al. in 2004 have identified in one hare cadaver lesions and nodules in jejunum caused by*Eimeria leporis* (3).

Conclusions

Gastrointestinal parasitoses prevalence in hare (*Lepus europaeus*) in Timis County was 92.30%, the most frequent parasitism being *Eimeria spp.* in 76.92%.

Depending on the hunting places the gastrointestinal parasitoses prevalence in hare (*Lepus europaeus*) was different: in Cheveresu Mare and Banloc 100% and Folvodia Frontiera 80%.

From all examined samples were identified with *Eimeria spp.* 11.53%, with *Trichostronylus spp.* 7.69%monoparasitism and 80.76% with poliparasitism.

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CYSTIC ENDOMETRIAL HYPERPLASIA (CEH)-PYOMETRA COMPLEX IN BITCH

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Abstract

Pyometra, namely the accumulation of pus into the lumen of the uterine cavity, is a common and important condition among intact bitches, to which the currant veterinarian must give serious attention. Cystic endometrial hyperplasia(CEH)-pyometra complex in bitch: should these two entities be differentiated? It is known that both problems evolve as a result of an abnormal response of the uterus to the repeated stimulating action of progestagens during the luteal phase of the oestral cycle. Although some authors believe that CEH leads to the appearance of pyometra, these two entities can developed independently of each other. Thus, other authors propose the differentiation between CEH and pyometra because of their clinical and histopathological aspects. Diagnosis of pyometra in bitch can be difficult to distinguish from that of CEH, combined with the accumulation of sero-mucous fluid (mucometra or hydrometra). However, this diagnosis is of particular importance, given the systemic impact that pyometra can have. Pyometra (especially open-cervix pyometra) represents a medical emergency and may lead to animal exitus, while CEH it is not a life threatening for the animal, its main negative effect being infertility. This paper shows information about: incidence, pathogenesis, latest diagnostic trends, and pharmacologic advances in canine pyometra therapy.

Keywords: *bitch, pyometra, cystic endometrial hyperplasia (CEH), treatment.*

Introduction

Although it is one of the most common uterine ailments among bitches, the exact mechanisms triggering cystic endometrial hyperplasia (CEH) are still unclear at this time (*Borresen B, 1977*). It is known however that CEH evolves as a consequence of abnormal uterine response to prolonged and repeated action of progestagen hormones, during the luteal phase of the sexual cycle (*Dow C, 1958, 1959; Sandholm M et al., 1975*). Some authors consider that the CEH is the element triggering the pyometra (*Dow C, 1958; Sandholm M, 1975; Arora N, 2006*), seeing the two diseases as a whole: CEH-pyometra complex, while other authors (*DeBosschere H, 2001*,) have pointed out, after histological examination of the uterus, using a computerized image analysis system, that the two pathological entities should be considered separately, due to clinical and histological discrepancies. *DeBosschere*'s computer analysis revealed that pyometra does not necessarily succeed cystic endometrial hyperplasia, but both can evolve de nuovo.

Pyometra, respectively chronic purulent endometritis, is a common condition for unspayed bitches, especially the nulliparous older than 4 years, evolving usually 4 weeks to 4 months post-estrus (*Smith F, 2006*). The condition may have a cumulative evolution, so that in the early stages of disease the signs can be mild to absent, it is therefore important that the diagnosis to be made at the first signs and the treatment to be administered properly to avoid disastrous consequences (*Hagman RM et al., 1974*).

Bitches with pyometra may experience vaginal discharge, in case of open cervix-pyometra, or not, if the pyometra evolves with closed cervix. The closed cervix pyometra is a

medical emergency, and can lead to sepsis, toxiemia and even death if the animal is not treated properly and promptly (*Arora N, 2006*).

From the clinical point of view, pyometra is difficult to distinguish from CEH evolving with sero-mucous fluid in the uterine lumen (hydrometera, mucometra or hematometra, depending on water content) (*Pratzer SD, 2008*). Usually, due to bacterial infection and the proper immune response, signs of pyometra are much more obvious than those of CEH's whose main symptom is infertility (sometimes abdominal distension when CEH evolves with mucometra). It is still of great importance to differentiate the two pathological entities given the risk of pyometra of developing sudden endotoxic shock that can be fatal, while bitches with CEH (uninfected uterus) does not represent a medical emergency (*DeBosschere H et al, 2001*).

Incidence

Normally, pyometra affects mature bitches, going through repeated cycles of heat (*Sandholm M et al., 1975*). Ages reported in various studies related to pyometra, varies between 4 months and 16 years, with a mean age of 7.25 years. It seems that the highest incidence of pyometra is found in unsterilized female dogs, aged 4 years (*Hardy RM, 1974*), and over 75% of female dogs with pyometra are nulliparous (*Chen YMM, 2001*).

Progestagen hormone therapy (to suppress oestrus) or estrogen (to induce estrus or abortion) are factors in the occurrence of pyometra, also there is a predisposition for developing this condition by young bitches with vagina or vaginal vestibule abnormalities, such as septums and strictures (*Chu PY, 2001*). Some breeds that are known to be prone to pyometra are: Rottweiler, Saint Bernard, Chow Chow, Golden Retriever, Miniature Schnauzer, Irish Terrier, Airedale Terrier, Cavalier King Charles Spaniel, Rough Collie, and Bernese Mountain dog, while Drevers, German Shepherd dogs, Daschunds, and Swedish hounds are breeds with low risk of developing pyometra, according to *Corada Y* (*2006*) while older studies showed that there was no racial predisposition (*Dhaliwal GK et al., 1998*).

Pyometra may occur at 12 weeks after cessation of heat, with a mean of 5.7 weeks, though it can develop at any time during the sexual cycle or during pregnancy (*Hardy RM, 1974*) . Other studies have shown that in 3/4 of the cases, pyometra was observed in 8 weeks after the end of oestrus (*Hauptmann JG, 1994*) No study has highlighted a link between pyometra and false pregnancy.

Pathogenesis

Most bitches develop pyometra during diestrus , although some cases of pyometra have been reported in anestrus (*Noakes DE et al., 2001*). Studies conducted by *Lesboyries* and *Bertheleon* in *1936,* who achieved clinical cure in bitches with pyometra in 5-7 days after the ovariotomia without hysterectomy, and those coordinated by and *Janssens* and *Janssens* in *1946* which demonstrated that pyometra never appeared in ovarioectomized bitches, have highlighted the role of ovarian steroids in the pathogenesis of pyometra (*Schlafer DH, 2008*).

The role of progesterone in the etiopathology of pyometra is explained by the mediation in the appearance of cystic endometrial hyperplasia, stimulating the secretion of the endometrial glands that favors bacterial development, also closing the cervix which

prevents drainage of the uterine exudate, and finally, suppressing the immune response (*Verstegen J, 2008*).

It is not known the exact mechanism that triggers pyometra, although the link between diestrus and pyometra is well established (*Boressen B, 1975*). Numerous studies have been directed towards establishing the exact cause of pyometra. Older studies have concluded that excessive exposure to exogenous progesterone increases susceptibility to pyometra for ovariotomized bitches (*Dow C, 1959*), while later reports were unable to highlight the predisposing role of progesterone, nor the one of irregular sexual cycles or pseudopregnancy (*Schlafer DH, 2008*).

In the literature, it is suggested that degenerative lesions of the uterus during the CEH, provide ideal conditions for installing uterine infections. Already-compromised uterus is susceptible to infection with bacteria that migrates from the vagina (*DeBosschere H et al*., *2001; 2002*). Other authors, such as *Nomura et al*. (*1994; 1995*) and *Koguchi* (*1995*) considered wrong the assumption that pyometra evolves strictly after endometrial proliferation from CEH, but rather CEH itself is the result of bacterial stimulation at the end of estrus or early diestrus that causes excessive endometrial hypertrophy and hyperplasia, the same as that encountered in the implantation (trophoblast or decidual reaction). This theory contradicted by *DeBosschere* is supported by clinical observations in young animals where the CEH has never occurred.

The bacteria most commonly isolated from the uterus affected by pyometra are *E. coli*, *Staphylococcus aureus*, *Streptococcus* spp. *Pseudomonas* spp. and *Proteus* spp (*Smith F, 2006*). These are the most frequently isolated bacteria from the vagina of normal healthy bitches (*Hardie EM, 1995*). Gram-negative bacteria release cell wall compounds into circulation, endotoxins that are biologically active, initiating the release of numerous inflammatory mediators (cytokine cascades) , and are therefore responsible for systemic symptoms in bitches with pyometra (*Mir F, 2012*).

Diagnosis

While CEH's only clinical manifestation is the failure to conceive (or abdominal distension rarely when CEH evolves with mucometra / hydrometra), clinical signs of pyometra in bitch differ depending on the type of pyometra, respectively after cervix ability to drain purulent fluid (*Pretzer SD, 2008*).

In bitches with open-cervix pyometra, the most common clinical sign is the presence of a malodorous , sero-bloody to muco-purulent vaginal discharge. Open cervix pyometra usually produces less systemic ill than the close-cervix one, so the only clinical sign is represented by the vaginal discharge, although the clinical picture can sometimes be completed by lethargy, loss of appetite / anorexia, depression, polyuria, polydipsia, vomiting and diarrhea (*Smith F, 2006*). On the other hand, in bitches with close-cervix pyometra, clinical signs are more pronounced, with marked signs of depression, lethargy, polyuria, polydipsia, vomiting, diarrhea and oftenly, abdominal distension. Bitches affected are often dehydrated, septicemic, in toxic shock. While in the open-cervix pyometra hipertermia is often encountered, in the close cervix pyometra, bitches can even have hypothermia, also in contrast, no vaginal discharge is noticed (*Hardy RM et al*., *1974*; *Dow C, 1957*)

Pyometra can also be diagnosed by cytological examination of the vaginal discharge, useful test in differentiating open-cervix pyometra of CEH evolving with mucometra. In pyometra, we see a large number of degenerative neutrophils, also intra- and extracellular bacteria are present. In mucometra, cytology reveales a lower number of neutrophils, with or without degenerative changes, red blood cells, endometrial cells (with foamy cytoplasm),also, cell debris. Similarly, if CEH's evolving with hydrometra,a small number of red and white blood cells, moderate number of endometrial cells, mucus and less cell debris are present. In hematometra, red blood cells are predominant (*Pretzer SD, 2008*).

When performing cell count, a left shift is also a common finding. It is often seen a normochromic, normocytic anemia (*Hardy RM, 1974*; *Dow C, 1957*), with packed cell volumes ranging from 21% to 48% (*Pretzer SD, 2008*).

Serum chemistry reveales hypergammaglobulinemia, azotemia and hypoalbuminemia, metabolic acidosis can also be seen (*Arora N, 2006*). Urinary analysis is not a enlightening criterion, because of dehydration that often installs, affecting specific gravity. In less than 20% of cases, specific gravity decreases (*Hardy RM et al., 1974*). Usually, pyometra causes decreasing of the ability of renal tubules to concentrate urine (due to the effect of bacterial endotoxins), which explains the decrease in specific gravity and the subsequent clinical signs of polyuria and polydipsia, also proteinuria can be present (*Hardy RM et al., 1974*). In mucometra both serum cheminstry, white blood count, and urinalysis are normal (*Pretzer SD , 2008*).

Diagnosis of pyometra is best put by using ultrasound and radiology (*Bigliardi E et al., 2004*). Ultrasonography findings typically include an enlarged uterus, convoluted uterine horns that contain anechoic to hypoechoic fluid. Luminal contents is usually homogeneous but may contain ecodense patterns (*Noakes DE et al., 2001*). In CEH, a thickened endometrium with cystic structures can be seen (*Nerstegen J et al., 2008*). If the luminal content is ecodens, hematometra and mucometra can be suspected, and hydrometra is usually suspected in the presence of anechoic fluid in combination with the absence of clinical signs associated with pyometra (*Bigliardi E, 2004*).

Abdominal radiographic examination can be used to identify a tubular organ, similar in appearance to that of a sausage (sausage-like fluid filled tubular organ), located between the descending colon and bladder, although some authors have pointed out that it is difficult to distinguish a uterus in early pregnancy of pyometra, uterine torsion or mucometra , all having similar soft tissue radiographic characteristics (*Gropetti D, 2009*).

A recent study conducted by a Swedish team (*Hagman R. et al., 2005*) highlighted the role of the analysis of prostaglandin F2α metabolite in the differentiation of pyometra versus CEH / mucometra. The authors noted that the analysis of PGFmetabolite alone is the only parameter with the highest sensitivity (98.3%) and specificity (80.0%) for the differentiation of pyometra versus CEH / mucometra in bitches who noted the presence of uterine fluid. Their discovery is particularly valuable for the clinician, sometimes being difficult to distinguish the two diseases when clinical signs are mild. A sensitive, rapid and inexpensive PG-metabolite test for the prediction of pyometra would enable clinicians to decide whether it is possible to postpone surgery (in CEH/mucometra) until optimal resources are available or whether the bitch needs to be monitored as an emergency and ovariohysterectomised as soon as the clinical condition allows (pyometra).

A newer method of identification and differentiation of various types of uterine pathology was recently tested by a team from the University of Milan (*Gropetti D et al., 2010*), who first used endometrial cytology and computerized morphometric analysis of epithelial nuclei. Samples for endometrial cytology were collected in vivo by uterine flushing with transcervical uterine cannulation. The computer-assisted nuclear morphometry proved to be not only useful for determining the stage of the reproductive cycle, but also a valid support to diagnose and distinguish uterine disorders.

Advances in treatment and assessement of future reproductive success

The treatment of pyometra tipically involves intravenous rehydration and antibiotics, immediately followed by ovariohisterectomy, once the bitch is stabilized. It remains the standard treatment for any bitch that does not present a great reproductive value, or if the owner does not want to breed the bitch. The main advantage of ovariohysterectomy is that any risk of relapse is excluded. However, often the attending physician is unable to perform the surgery, since animals are brought too late, in serious condition, and procedures such as anesthesia and surgery are life threatening (*Verstegen J. et al., 2008*).

In the last 10-15 years, other treatment strategies have been developed. Since ancient times, medical treatment involves the simple use of local and systemic antibiotics. However, this strategy only worsens or delays the worsening of the disease, with the need for the additional treatment later (*Verstegen J et al., 2008*). Older therapies involving repeated administration of prostaglandin F2alfa (PGF), which determines luteolisis, reducing the serological concentration of progesterone, thus producing relaxation of the cervix and decrease of uterine secretions. However, it is known that high doses of PGF used produce some serious side effects (salivation, vomiting, straining, diarrhea, pyrexia, some occasional respiratory distress, including shock and death) and is also associated with the risk of uterine rupture, especially in cases of close-cervix pyometra (*Johnston SD et al., 2001*).

Over the time, it was also proposed estrogen administration to relax and open the cervix, but this was associated with a steep increase absorption of toxins and increase toxiemia which dramatically worsens the condition of the animal, which led to the abandonment of this therapy (*Williams BJ et al., 1999*).

During the last 10 years, new approaches have been proposed and numerous successful results of medical treatment for canine pyometra have been reported. In pyometra, drug therapy should firstly follow the removing of the progesterone effects, either directly through luteolisis, or indirectly by progesterone receptor blockade. This can be done by directly blocking with prostaglandin the corpum luteum (CL) or indirectly , using dopamine agonist, which by prolactin inhibition, will arrest and finally induce functional luteolysis of the CL. This can also be tried by preventing the binding of progesterone to its receptors using a progesteron-receptor antagonist like aglepristone (*Johnston SD et al., 2001*).

Many authors have reported the use of PGF to treat pyometra and the results are generally positive, except for cases in which large doses were used (*Verstegen J et al., 2008*). The treatment with PGF dose of 10-50 mg/kg, administered 3-5 times a day for 3-7 days, either solely or in combination with other drugs has been reported to be successful. Preferably, the use of natural prostaglandine is reccomended, in the expense of the analogues.

Also, a particular attention may be payed for the dose calculation, given that the LD50 in dogs is approximately 5 mg/kg, and the side effects are quite severe in high doses.

A new method, which opens new perspectives in the treatment of pyometra is represented by intravaginal infusion of prostaglandins, once or twice daily, but this method needs further validation (*Verstegen J., 2008*). In the treatment of pyometra it has also been reported as successful the use of the dopamine agonists, as bromocriptine and cabergoline, alone or in combination with natural or synthetic prostaglandins. It seems that the best results are obtained with cabergoline, which has the fewest side effects and only requires daily administration, unlike bromocriptine requiring twice daily (*Okano S et al., 1998*).

In 2007, *England et al*. shows the results of a study in which 22 bitches with pyometra (both close and open cervix) were treated with a combination of cabergoline 5 mg/kg and cloprostenol administered every 3 days. The treatment was successful, finding a rapid clinical improvement associated with the reduction in plasma progesterone concentration, an increase in vulvar discharge and a reduction in the diameter of the uterus, in an average of 10 days.

In countries where the drug is available, progesterone-receptor antagonist are used recently, with results more or less satisfactory. Mifepristone and aglepristone binds to progesterone receptors completely blocking them (*Verstegen J, 2008*). The controversy of the treatment resulted in an inability to induce uterine contractions when used alone, so some authors recommend using progetesterone-receptor agonist in combination with PGF (*England GCW, 2007*). More recent studies have described the successful use of aglepristone in combination with cloprostenol, in the treatment of pyometra (*Gropetti D et al., 2009*).

Regardless of the medical protocol chosen, antibiotherapy should be performed. The identification and sensitivity in the vaginal discharges are preferably as soon as possible, also when administered orally along with PFG, the adverse effects as vomiting should be considered. Treatment with antibiotics is given 10-14 days even after complete resolution of pyometra (assesed by ultrasonography, physical examination and blood work) (*Verstegen J et. al, 2008*). Further, to avoid relapses, especially in bitches with degenerative age-related uterine processes (CEH), it is important to ensure the regeneration during post-treatment anestrus. By administering androgen-receptor agonist such as mibolerone, the prolongation of anestrus is obtained, allowing apoptosis and regeneration of the endometrium. Mibolerone is administered approximately one month after the drug treatment for pyometra (*England GCW et al*., 2007). The incidence of pyometra recurrence after medical treatments is still controversial, with contradictory results published, but it seems that the percentage is steadily decreasing over time with improvements in therapeutic approaches and treatments (*Verstegen J et. al*., *2008*).

After drug treatment, it appears that fertility is usually good, *Kelly Nelson* (*2001*) reported that 8 of 15 bitches treated with prostaglandins whelped at least one healthy litter after the treatment. Expected conception rates varied from 50% to 75%, depending on the age of the treated animal (the youngest animals generally being more fertile).

Conclusion and perspectives

Mucometra, hydrometra, hematometra and especially pyometra, are uterine pathological entities in unsterilized bitches with the aforementioned clinical signs and

requires identification and differentiation. The exact mechanism that triggers these pathologies are still unknown although there are several theories that require validation.

It seems that currently, the best diagnosis method (speaking in terms of economic) for the aforementioned pathologies remains the ultrasound examination, eventhough some authors have proposed some interesting alternatives, like the analysis of the PGF-metabolite or the endometrial cytology and computerized morphometric analysis of epithelial nuclei.

After numerous studies over the years, apparently, the best treatment for the pyometra involves the use of a combination of cabergoline and cloprostenol administered every 3 days, continuing with administering androgen-receptor agonists such as mibolerone for the regeneration of the endometrium. Over the last 10 years, substantial improvements in the treatement of pyometra have been made. Results are good and continuously improving with the availability of better medications.Future developments can be expected, for exemple, the authors have developed a new transcervical endoscopic catheterization technique (TECT) to treat pyometra (unpublished), allowing the resolution of the disease in 3-5 days versus 7-10 days. This new approach should further improve our ability to successfully manage pyometra (closed or open cervix).

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FACTORS INFLUENCING SPERM MOTILITY IN BOAR

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Abstract
Motility is one of the most important quality parameters of the semen and it is defined as the percentage of sperm that can normally move forward. A high level of motile spermatozoa is a prerequisite to fecundation. Therefore, motility is a good parameter in the seminal analysis of boar semen, and eliminates low quality semen if its value is lower than a certain limit. However, many variables may influence the assessment results. As the value of motility has such a great impact on the use or discharge of the ejaculate, it is important to know all the factors that can affect sperm motility. Thus, the object of the present review was to present the results of studies which evaluated the sperm motility in boars, according to certain variation factors. The study shows that the results of semen evaluation regarding the value of motility can be influenced by two categories of factors. First category affects the spermatogenesis process, while the second occurs after ejaculation, before and during assessment. First category includes breed, collecting frequency, season, epididimal integrity, microbiological burden, intoxications and can be controlled by breeding technology and growing conditions. The second one includes collecting technique, extender, type of preservation, duration of preservation, and temperature of assessment and can be controlled by semen collecting and processing techniques.
Keywords: *boar semen, motility, influence, review*

Introduction

Motility is one of the quality parameters of usual spermogramsand its determination is usually performed under active metabolism of sperm both in fresh or preserved semen. The method consists in determining the percentage of sperm within an ejaculate that present rectilinear forward movements with a speed characteristic to their species (*Bogdan AT, 1999*). Among other indicators of quality, motility is one of those thatreceive special attention, especially in semen processing centers (*Martín-Hidalgo D. et al., 2013*). It provides important information about the energy status of sperm. A high level of motility is essential for sperm to reach the fertilization site. The relationship between motility and fertility rate is strong, as shown in Table 1. When motility is at least 60%, there are no strong correlations between estimates of in vivo and in vitro fertility. In contrast, ejaculates with less than 60% motility fertilizefewer oocytes in vitro and determine fewer piglets/farrowing in vivo. Therefore,only ejaculates showing a motility of at least 60% should be used for artificial insemination

Table 1. Relationships between motility rates and fertility of boar semen (*Youngquist Robert S., Threlfall W., 2006)*

Motility (%)	In vitro fertilization rate (%)	Farrowing rate(%)	Number of piglets born alive
95,2	60,7	84,7	10,4
82,3	59,4	87,4	10,2
78,1	54,3	82,7	10,3
62,1	65,4	84,9	9,9
52,4	39,6	75,2	9,4
44,2	33,9	72,3	9,4
32,7	17,3	52,2	8,4

Considering the results of studies conducted by several authors, it seems that motility in boar semen can be influenced by a number of factors that can be divided into two categories (Table 2):

Table 2. Factors influencing sperm motility in boar

Factors influencing sperm production and maturation	Factors that intervene after ejaculation
Collection frequency	Microbiological burden
Breed	Extender
Season	Type of conservation
Age of boar	Length of storage
Epididimal integrity	Examination protocol
Intoxications	

Collection frequency

One of the main goals in a semen processing center is producing a large quantity of semen in a short time. Of course, one way to get more semen is collecting it more frequently. But one of the factors that affect sperm quality the most is actually frequent collection, because spermatogenesis is a process that needs time, and the ability of testicles to produce and maturate spermatozoa can be overcome. Studies conducted on this subjectconcluded that boars subjected to a busy schedule of collection offer semen of a lower quality, as a result of the forced passage of sperm from the caputto the cauda of epididymis, thus, their maturation process being shortened(*Bonet et al, 1991, J Strzezek et al, 1995*). For example, by collecting semen every 12 hours for 4 days, the value of motility drops to 20% (inappropriate for fertilization), while the collection at an interval of at least 4 days provides ejaculates with approximately 80% motility (*Anna Pruneda et al, 2005*).

Breed of boar

Motility values may vary by 10% from one race to another. By examining a number of 230705 ejaculates obtained from 2712 boars of 22 collection centers, Smittal J. (2008) noted that the average values of motility ranged from 71.29% in Duroc breed to 80.40% in the Czech Meat breed. On the other hand, by examining boar ejaculates from five different races, *Schulze et al. (2014)* obtained values ranged between 71.3% inDuroc breed and 74.6% in Pietrain breed (Table 3).

Table 3. Average values of sperm motility in boar, according to the breed

Breed	**Average value of sperm motility**	
	Smittal J, 2008	*Schulze et al, 2014*
Pietrain	74.46	74,60
Duroc	71.29	71,30
Hampshire	78.61	-
Landrace	74.69	74,40
Large White	75.96	73,40
Yorkshire	-	73,70
Czech Large White	74.81	-
Czech Meat	80.40	-

Different values obtained from different breeds suggest that reference values for spermogram in boar should be adapted according to race.

Besides the genetic capacity of sperm maturation, it seems that different breeds of pigs show different sensitivity of sperm motility to the action of some stress factors such as conservation *(Martín-Hidalgo et al. 2013)* or high frequency of semen collection *(Anna Pruneda et al, 2005)*.

Season

Semen quality is not the same throughout the year, as observed many years ago. Over time, various studies have been conducted attempting to specify precisely the conditions and limits of variation of semen parameters over a year, in order to find solutions for ensuringhigh quality of boar semen regardless of the season. These studies continue nowadays, resulting in more intimate details about the stress caused by seasonal climate variations on the boar sperm production and quality.

It seems that the lowest reproductive performance, both in boars and sows are obtained during the late summer and early autumn. Intimate mechanisms of seasonal fluctuations in semen quality are not fully understood. The cause may be an ancestral mechanism originated from the wild pigs, which is still present in modern, domestic breeds. For example, European wild boar shows seasonal sexual activity without mating in summer and autumn. Maybe this is the reason why the low sexual performances in pigs are observed in the summer *(Xue et al., 1994)*.

Some authors have correlated this with longer photoperiod and higher ambient temperatures specific for the summer in temperate areas *(Peltoniemi OA and Virolainen JV, 2006)*, while others say that the intimate mechanism of this control is a complex combination of endogenous circannual rhythm adjusted and synchronized by photoperiod and melatonin titer*(Chemin P et al, 2008)*.

Many researchers state that motility is one of the main semen parameters that vary from one season to another, showing lower values in summer *(Jankevičiūtė N and Žilinskas H, 2002; Janet F et al, 2005;Murase T et al, 2007; Smittal J. et al. 2008)*.

Table 4 presents some of the values obtained by several authors in recent studies.

Table 4. Seasonal variation of sperm motility in boar

Season	Average value of sperm motility				
	Cheon YM et al, 2002	*Janett Fet al, 2005*	*Smittal J, 2008*	*BarancoIsabel et al, 2013*	*KnechtDet al, 2013*
Spring	88,80	80,70	75,35	80,47	83,33
Summer	90,00	79,70	75.24	77,62	83,58
Autumn	86,30	79,90	75.48	76,21	83,83
Winter	88,80	79.90	75.21	80,93	82,19

The values vary from one study to another, probably according to the conditions of semen collection and examination (boar breed and age, frequency of collection, examination technique, etc.), but it can be observed that motility has showed different values throughout the year in all of the studies. However, the trend of variation is different between the five studies, although all were conducted in temperate climate zones (South Korea, Switzerland, Czech Republic, Poland, Spain), which may suggest that, under current conditions, there is no longer a strict influence of season on the quality of boar semen. This is probably due to the standardization and optimization of swine breeding, boars being held in approximately the same microclimate conditions throughout the year, which disturbs the ancestral system regulating reproductive function.

Age of boar

Spermatogenesis is startedalong with the onset of puberty, a phenomenon that occurs at different ages for each species depending on body development *(V. Ardelean, 2002)* and is maintained until the installation of andropause. Andropause occurs physiologically with aging and is characterized by the reduction of testicular function, both in terms of spermatogenesis and synthesis of sexual hormones.*(Drugociu D, 2009)*. After this step, the epithelium of the seminiferous tubules containonly Sertoli cells *(Runceanu L. et al., 2007)*. Therefore, the structure of seminiferous tubules varies depending on the age of the male, which will also cause a change in the production of semen, both quantitatively and qualitatively.

In general, it is recommended to use forreproduction only boars older than 8 months because below this age, motility and other qualitative indicators are unsuitable *(Schulze et al., 2014)*.

Sperm motility is one of the parameters that are affected by age. Motility values are lower immediately after puberty, then grow and remain at a higher level during adulthood and then decrease with the aging of male, as demonstrated in a study conducted by *Tsakmakidis et al. (2012)*. The authors concluded that the lowest values of sperm motility are obtained from elderly boars, aged between 51 and 61 months (66.92% sperm motility in their study), intermediate values from young boars, aged 7-10 months (68.81%), while mature boars aged 18-33 months provide semen with the best motility values (69.33%). Approximately similar results were obtained by*Jankevičiūtė and Žilinskas (2002)*, which state that after the age of 30 months, boarsprovide semen with lower motility.

Thus, although both boars younger than 1.5 years and those older than 4 years may be used in breeding, for superior results in terms of artificial insemination in pigs it is recommended to use adult boars aged between 1.5 and 2.5 years *(Jankevičiūtė N and Žilinskas H, 2002; Tsakmakidis et al, 2012*).

Epididimal integrity

Male gamete (spermatozoon) produced by the testis is an immature cell that needs to spend some time in the epididymis in order to become mobile and to acquire fertilizing capacity*(Olson GE, 2002)*. This essentialprocess involves interaction of sperm with a particular environment, regulated by specific secretory activities of epididimal epithelium.

During transit thorough epididymis, mammalian spermatozoa undergo a series of changes in order to get the capacity to move straight forward and the ability to recognize, attach to and penetrate the zonapellucida of the egg. Obtaining of movement and linear

progression are the most obvious effects of maturation occurred in the epididymis. Movements of immature sperm are characterized by erratic and asymmetric tail beats resulting in almost immobile sperm without propulsion or with irregular routes. Along the epididymis, sperm routes become more regular, but curved or circular, which later turn into linear paths. In boar, this phenomenon occurs in the proximal and middle portions of the epididimal tail *(Bassols J et al., 2005)*.

Thus, epididimal function of sperm maturation depends on the state of the epithelial cells lining the luminal surface. Any condition that disturbs the morphologic and functional integrity of epididimal epithelium leads to altered sperm maturation environment, with negative implications for their ability to move.

Intoxications

In contact with certain substances, sperm metabolism can be disturbed, resulting in a decrease oftheir motility and viability, with repercussions on fertility.

A particular case is the toxicity of the toxin produced by *Bacillus cereus*. The toxin acts as an ionophore, transporting potassium ions through the ion-transport system, to the mitochondria. The oxidoreductivefunction of damaged mitochondria will be turned off, thereby causing changes in the macroscopic behavior of the cells, expressed as a decrease in cell motility *(Andersson, MA et al., 2004)*. Very high toxicity of this compound on determined some researchers to use semen as detection system of small amounts of toxin produced by Bacillus cereus in various foods. Depending on the magnitude of impact on the motility of sperm, one can appreciate even the amount of toxin that exists in a certain environment. *(RajkovicAndreja et al., 2006;AnderssonMaria A. et al, 2007)*.

Microbiological burden of semen

The presence of bacteria in semen may be due to inflammatory conditions of the genitals or contamination of semen during its collection or processing*(Ciornei Ş, 2009)*. In case of healthy boars, the level of semen pollution by microorganisms depends on the collection methodology *(Goldberg Ana Maria G. et al, 2013)*.

Semen is not collected under aseptic conditions. The presence of microorganisms in fresh ejaculate is a common thing*(Althouse GC and Lu KG 2005;Ciornei Ş, 2009)*, and most bacteria belong to the *Enterobacteriaceae* family *(Úbeda JL et al, 2013)*. Most of thesebacterial strains are not considered pathogenic for boar semen, but the level of contamination is a parameter that must be taken into account within the quality control of semen used for artificial insemination. Otherwise, this contamination will result in lower reproductive performance in farm*(Althouse GC et al., 2000)*.

The effect of bacterial contamination on semen quality is controversial. While some researchers believe that the presence of bacterial strains usually produce no sperm deficiencies *(Cottell E et al., 2000)*, other authors demonstrated that this type of contamination can affect reproductive function in boar, causing sperm agglutination or reduced motility*(Monga M and Roberts JA, 1994, Martín LM et al., 2010)*, inhibiting the acrosomial reaction or causing alterations in cell morphology.

Motility is one of the main semen parameters affected by a high bacterial burden, its value decreasing by up to 25% compared to uncontaminated semen *(Úbeda JL et al, 2013)*. It seems that the decrease in motility is caused mainly by sperm agglutination *(Úbeda JL et al 2013)*, although other authors blame other ways, that do not require direct contact of the

sperm with the bacterial cell, but the action of a parallel mechanism through humoral factors*(Schulz M et al., 2010)*.

Contamination of semen with fungi has also been reported by several researchers. The literature cites data regarding the presence of fungi in semen and the influence they exert on the biological value of sperm*(Ciornei, Ş., 2009)*. For example, in a study carried out by *Runceanu L. et al., (2002)*, fungi represented 6% of the microorganisms isolated from semen. They were isolated within polymicrobial contamination, in association with various Gram-negative or Gram-positive bacteria.

A careful boar hygiene, a good hygiene within the farm and regular monitoring can contribute significantly to decrease bacterial burden*(Althouse G. and Kristina G. 2004)*. Also, many authors recommend the addition of antibiotics in the semen extender, while others recommend including the determination of bacterial burden among routine boar semen examination procedures, and removing the ejaculatesthat contain E. coli in a proportion higher than 3.5×10^3 CFU/ml *(Martín LM et al., 2010)*.

Extender

Semen extenders provide metabolic and nutritional support for preserved semen. In general, the ingredients used for producing these media include glucose, electrolytes, buffer substances, and antibiotics. Glucose is the predominant energy source, electrolytes help controlling the osmotic pressure, buffer substances are involved in the neutralization of metabolic wastes and pH maintaining while the antibiotics inhibit bacterial growth. As known, there is an inverse correlation between fertility and the duration of storage for all extenders but usually the decrease of fertility rate depends on the buffer system used for dilution*(YoungquistRobert S., ThrelfallW., 2006)*.

Longevity of stored sperm is determined by the interaction of certain factors, such as the individual performances of boar, semen concentration, storage temperature and type of extender.

Currently, there are many types of extenders showing small differences in their composition. Thus, their behavior and protection of sperm during storage will be slightly different.

Based on this, *Dubè Charlotte şi col., (2004)*conducted a comparative study between two extenders, respectively Androhep Plus and BTS. Examining the semen quality parameters in semen stored at 17°C every day for 12 days of storage, they observed that, among others, the values of motility differ significantly between the two solvents, being higher in case of usingAndrohep Plus. On the same principle, *Martín-Hidalgo et al. (2013)* conducted a study comparing the effects on sperm motilityof two well-known extenders: MR-A and XCell. Preserving semen at 17°C for 7 days with both extenders and assessing the motility parametersdaily, they observed that substantial differences occur, especially in terms of average path velocity, curviliniar velocity and straight line velocityandamplitude of lateral head displacement.

It seems that some environmental changes of extender regulate sperm motility. The main factors which can accomplish this are: temperature, pH and ionic composition. For example, an increase in temperature from 30°C to 40°C results in the inactivation of avian sperm motility. On the other hand, alkalizing the extender as well as addition of calcium ions increase the percentage of motility *(BonatoM.et al, 2012)*.

An important thingwithin boar semen processing is represented by temperature of extender while performing dilution. In case of storage at 17°C, it is better if the initial temperature of the extender isisothermic (37°C) than hypothermic (20°C). As shown by*Schulze M et al. (2013)*sperm boar are sensitive to sudden temperature reductionafter ejaculation. A rapid drop of semen temperature as it happens when using a hypothermic extender can alter the molecular organization of the sperm membrane, with consequences on some properties, includingmotility. These negative effects are visible from the early days of storage (Table 5) but, according to the authors, are more evident in a later stage of storage, such as day 6, when the difference in total motility reaches 8 %, and the difference in progressivity reaches 9%. Thus, the sperm will live longer and will be more and resistant to stress when performing isothermal dilution. *(M. Schulze et al, 2013).*

Table 5. The influence of extender temperature during dilution on the motilityparameters of sperm during storage*(Schulze et al. 2013)*

Parameter	Extender	Storage time		
		Day 1	Day3	Day6
Total motility (%)	Isothermic	89.0	85.9	73.9
	Hypothermic	86.6	84.2	66.0
Progressivity (%)	Isothermic	84.0	76.8	64.2
	Hypothermic	81.1	74.4	55.5
VAP (µm/s)*	Isothermic	68.8	60.1	63.7
	Hypothermic	69.7	60.5	58.9
VCL (µm/s)**	Isothermic	118.7	104.0	111.5
	Hypothermic	120.8	103.3	105.6
VSL (µm/s)***	Isothermic	51.1	46.2	47.6
	Hypothermic	51.7	46.6	43.3

*VAP = averagepathvelocity; **VCL = curviliniarvelocity; ***VSL = straight line velocity

Type of conservation

Semen preservation method has a major impact on sperm quality. The main objective of semen preserving is to maintain the viability of the sperm for a long time, and this can be achieved by reducing the metabolism of the sperm cells, by reducing the temperature or by adding substances thatprolong the viability. However, higher sensitivity of boar sperm to hypocaloric shockcauses the need of storage at temperatures above 15°C, which limits the reduction of sperm metabolism.

Therefore, the main method used for boar semen preservation is storage at a temperature of about 17°C of in a liquid state, which preserve the fertilizingability of sperm for about 7 days. On the smaller scale,freezingis used as a method of storage, but it has a more pronounced negative effect on sperm viability in boar.

Because of some particularities in the composition of their membrane, the boar spermatozoa are very sensitive to cooling, freezing and thawing. This sensitivity to low temperatures causes the need of preserving boar semenat moderate temperatures (15-20°C), which restricts the capacity of semen storage, because on the one hand cellular metabolism

cannot be reduced enough and on the other hand microbiological conditions cannot be controlled as efficiently as inlower temperatures. Lesions of sperm membrane and changes in cellular function occur also at 20°C, if storage is not performed properly, resulting in alterations of membrane permeability and decreased energy production which willdecrease sperm motility*(Tejerina et al., 2008)*.

Length of storage

As well known, the storage time is a factor influencing motility values decisively .Sperm motility decrease gradually during the storage, due to "exhaustion" of sperm cells. Many researchers have described this phenomenon, which is one of the reasons why the storage of boar semen is usually limited to a maximum of 7 days.

In case of preservation at 17°C, the motility of sperm is relatively constant for the first three days and then decreases gradually (*de Ambrogi M. et al., 2006*). Other authors state that the value of motility begins to decrease even after the first day, while after four days of storage it reaches about half of the initial value *(Kumaresan et al., 2009)*.

Examination protocol

Apart from factors that reallyinfluence sperm motility,one must take into account a number of factors which could influence the results within the examination of motility, and which in reality present a "false" value of motility. These factors are basically examiner errors, especially from lack of experience or failure of examination protocol.

Usually, the percentage of motility in boar semen is evaluated subjectively by an expert examining semen under a microscope. The technique consists in placing a drop of semen on a slide heated at 37°C, then covering it with a coverslip followed byits microscope examination (usually at the of magnitude 200) with an overall assessment of the proportion of spermatozoa showing forward movement. In the microscopic field, sperm must be arranged in a single layer. Of course, the accuracy of this method depends largely on the sample preparation, the quality of the microscope, and especially the experience and skill of the examiner. Thus, the method is not standardized and is subjective, having alimited degree of confidence *(Verstegen J et al., 2002; Broekhuijse et al., 2011)*.

Therefore, many efforts were made to find a solution for providing an easy, objective, and efficient method for determination of sperm parameters. In this respect, H. Dott and G. Foster proposed for the first time computerized semen analysis (CASA system - Computer Assisted Sperm Analysis), in 1979 *(Broekhuijse MLWJ, 2011)*. It was available for semen processingcenters in 1985 and currently has become the widely preferred method in many andrologycenters, both veterinary and human, due to its ability of rapid and accurate analysis of many sperm indices (motility and its characteristics, concentration, morphology, pH, fructose level) for a large number of samples. Developing CASA has helped increase the accuracy and confidence in assessing sperm motility, providing more information than classical evaluation. The availability of information recorded by the computer facilitates comparison of results and enables the detection of possible differences in motility according to different situations *(I. Palacín et al., 2013)*.

Many researchers have demonstrated that CASA method is more accurate, therefore more objective *(Davis DO and Katz DF 1993; Krause W. 1995;Broekhuijse et al, 2005; Didion BA 2008;Broekhuijseet al., 2011; Amann RP and Waberski D, 2014)*.

However, incorrect results may occur even when computerized determination of motilityis used *(Broekhuijse et al., 2011; Amann RP and Waberski D, 2014)*. Results can be influenced by the settings of software, image capture settings, the number of fields analyzed, concentration and dilution of the semen sample, adjusting of microscope chamber, slide and sample temperature, time between ejaculation and examination etc*(Broekhuijse et al., 2011)*.
In conclusion, the determination of sperm the motilitywithin a boar semen sample represents an examination which must be performed with special attentionbecause its results decide the use or the removal of the entire ejaculate. Also, the obtained values should always be interpreted taking into account the particularities of the individual, the conditions of semen collection and the technique of examination and processing.

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MONTHLY VARIATION OF SEMINAL PARAMETERS IN BOAR

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Abstract

Sperm production in boar can be influenced by many factors, including breed, age, nutrition, climate, health status and collection frequency. These factors will determine a variation of seminal parameters. A better knowledge of the factors that influence the quantity and quality of semen can increase the efficiency of swine reproduction units. The aim of this study was to observe the monthly variations of the main parameters in boar semen and the correlations between these variations, in order to detect the factors altering semen quality in pigs. The study was conducted on a number of 537 ejaculates, from 31 healthy Pietrain boars, on a period of 12 months. Semen was collected by manual method, and examined using the beaker, the ACCUCELL photometer, and CASA system (CEROS II). Briefly, results were as following: Volume showed higher values in June (222.4 ml) and lower in the February (157.2 ml); Total number of spermatozoa/ejaculate was higher in June (107.5×10^9) and lower in February (71.7×10^9); Semen concentration was higher in November (600.4×10^6/ml) and lower in July (419.6×10^6/ml); Total number of motile spermatozoa/ejaculate showed higher values in June (89.1×10^9) and lower in February (62.9×10^9); % of Motility was higher in January (89.5%) and lower in July (80.6%); Total number of progressive spermatozoa/ejaculate was higher in June (58.9×10^9) and lower in September (40.3×10^9); % of Progressivity was higher in January (63.5%) and lower in April (49.0%). The best month for reproduction (based on the number of progressive sperm/ejaculate) was June. On the other hand, September was the weakest month in terms of semen production, showing the lowest values for the number of progressive sperm/ejaculate. The results contradict those of others studies. However, the variation of seminal parameters from month to month was not high, and this fact suggests that the standardization of exploitation conditions of boars can reduce the heat stress usually observed in summer, offering a great solution against seasonal infertility.

Keywords*: boar, sperm, variation*

Introduction

The best results in swine reproduction can be obtained only using semen of a high quality for artificial insemination. However, quality of semen obtained from boars is not always at the highest standards. It seems that spermatogenesis in domestic pigs is conducted respecting an ancestral model, inherited from the wild boar (*Xue, 1994;Tast et al., 2001*) but is also influenced by a number of factors, including: race, age, nutrition, environmental factors, health status, frequency of collection. Thus, any variation of the factors mentioned above will affect the values of the semen parameters with consequences over sperm fertilization capacity and also over the economic efficiency of production units.

In such circumstances it is essential to detect the conditions that favor obtaining semen of good quality and those that adversely affect spermatogenesis process, in order to optimize breeding in pigs.

Over time, many authors have noted that semen parameters vary by season (*Cheon YM et al, 2002; Janett F. et al, 2005; Koziorowska-Gilun Magdalena et al, 2011*) due to changes in microclimate. However, according to other authors, values of semen indicators vary not only from one season to another, but also from one month to another (*Lawrence JA et al., 1970; Murase T. et al, 2007 Zervos IA et al 2010, Knecht et al., 2013*). As within a season there may be significant variations from one month to another, we think that monthly monitoring of semen is more effective compared to the seasonal monitoring.

Nowadays, the global trend in industrial-scale pig farms is to provide standardized conditions of life for animals with minimizing variations in microclimate and maintaining the microclimate between optimum limits in order to avoid stress action of some factors such as extreme temperature, high humidity and strong air currents. Therefore, the seasonal variations in semen parameters that depend on the microclimate could be minimized.
The aim of this study is to observe the monthly variations of the main parameters in boar semen and the correlations between these variations, in order to detect the factors altering semen quality in pigs.

Material and method

The study was conducted by examining a number of 537 ejaculates from 31 clinically healthy Pietrain boars, aged between 8 months and 2.5 years. Duration of the study was 12 months, between December 2012 and November 2013.
Semen was examined within a commercial unit for swine breeding, located in north-eastern Romania. The farm provides modern housing conditions for boars, with a standardized microclimate.

Semen collection was performed using manual method with double glove (*Bogdan AT, 1999; Ciornei SG, 2012*) on a dummy, with an interval of at least 7 days between two successive collections from the same boar.

Following parameters were determined within semen examination: - Volume (using the beaker); - concentration (using the ACCUCELL photometer produced by IMV Technologies), total motility, progressivity, total number of sperm within the entire ejaculate, total number ofmotile sperm within the entire ejaculate,and totalnumber of progressive sperm within the entire ejaculate (using CASA system, the CEROS II device, produced by IMV Technologies). Ejaculates showing motility lower than 70% have been discarded.

The values obtained within the semen examination were introduced in Microsoft Office (Excel). Statistical analysis was performed with IBM SPSS Statistics version 21. In order to verify the relations among the variables, Pearson correlation was used.

Results and discussions

The mean values for every month within the study as well as the average values for the entire period (12 months) are shown in Table 1.

Table 1. Average values of main seminal parameters obtained between December 2012 and November 2013

Month	Volume (ml)	Total no. of sperm/Ej. (x 10^6)	Conc./ml (x 10^6)	Total no. of motile sperm./Ej. (x 10^6)	Motility (%)	Total no. of progressive sperm./Ej. (x 10^6)	Progressivity (%)
Dec. 12	167.68	72844.78	449.27	63195.59	86.76	42690.76	58.32
Jan. 13	170.69	77912.22	488.25	69798.33	89.50	49542.42	63.58
Feb. 13	157.22	71704.07	467.49	62943.85	87.67	45182.70	63.22
Mar. 13	177.58	85294.06	491.65	73360.45	86.26	52022.65	61.32
Apr. 13	197.96	85988.49	454.48	72897.76	84.35	42583.29	49.00

May 13	205.91	92357.33	462.10	78567.00	84.97	51659.52	55.39
Jun. 13	222.48	107575.93	499.62	89103.59	82.59	58989.86	54.48
Jul. 13	222.24	89491.61	419.59	72758.45	80.66	44786.55	49.24
Aug. 13	211.86	93209.43	456.14	78896.83	84.11	56203.69	59.17
Sep. 13	149.50	75120.37	537.61	61861.03	80.90	40362.60	52.40
Oct. 13	152.50	87404.64	589.22	73712.86	83.50	43346.14	51.14
Nov. 13	167.50	95082.86	600.40	82286.73	86.55	50291.27	53.36
Annual average	183.59	86165.48	492.99	73281.87	84.82	48138.45	55.89

Monthly variation of volume

As shown in Table 1, the average monthly values of volume ranged from 149.5 ml in September to 222.48 ml in June. The difference between the two limits is not very high (72.98 ml). The variation of ejaculate volume is illustrated in Figure 1.

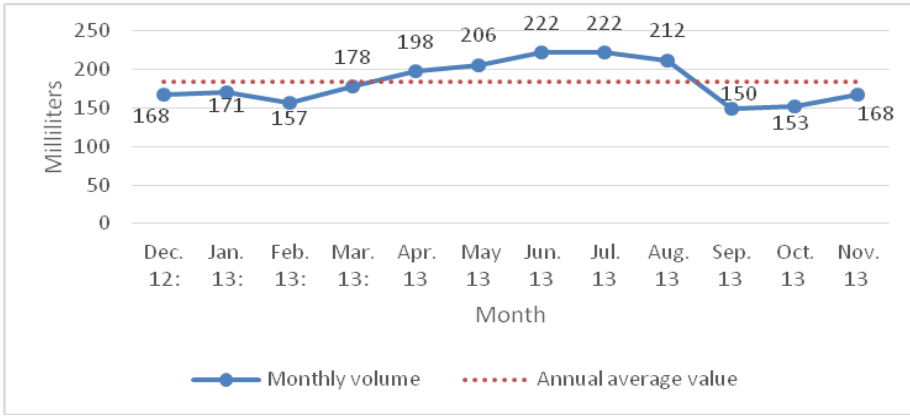

Fig. 1. Monthly variation of ejaculate volume

It can be observed that the volume presented an oscillating evolution, with different values from one month to another, which is consistent with the results of other researchers (*Lawrence JA et al. 1970, Murase T. et al, 2007 Zervos IA et al, 2010, Knecht et al., 2013*). Approximately 93% of semen volume is represented by seminal plasma (*Ciornei ŞG, 2012*). Thus, any increase or decrease in semen volume, would be due to an increase or decrease in the activity of accessory glands, which produce seminal plasma.

The relatively small difference between the maximum and minimum values obtained within the present study suggest that the activity of accessory glands is not affected by the semen collection month.

Monthly variation of semen concentration

Semen concentration ranged from 419.59×10^6/ml in July and 600.40×10^6/ml in November with a relatively big difference (180.81×10^6/ml) between the two extremes. However, the values were close to the annual average value (492.99×10^6/ml) for the first nine

months of study and showed a significant increase in the last three months, which can be correlated with the decrease of ejaculates volume recorded in that interval, probably due to a decline of accessory glands activity. This oscillation is illustrated in figure 2.

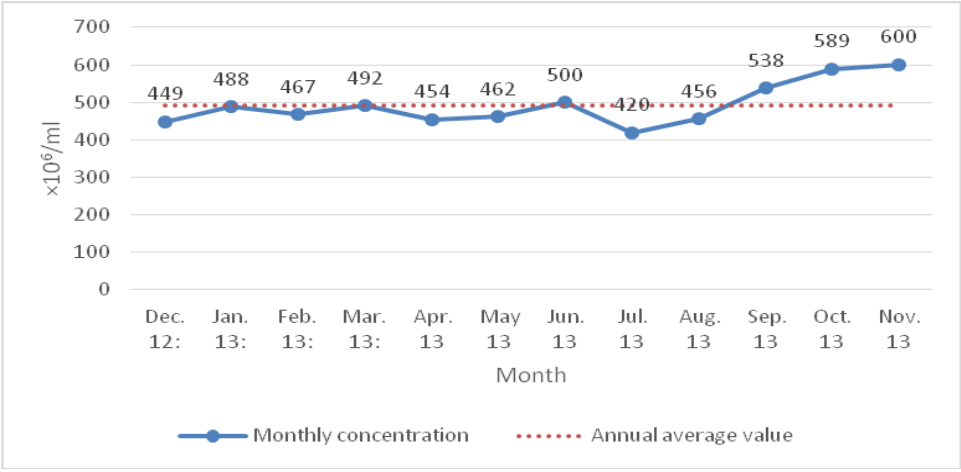

Fig. 2. Monthly variation of semen concentration

Monthly variation of motility and progressivity

As is well known, one of the main characteristics to be taken into account when examining the sperm is motility, which reflects the fecundant capacity of ejaculate. If the value of motility is situated under certain standard limits, the whole ejaculate is discarded.

The current trend in terms of semen examination is to pay attention also to sperm progressivity, defined as the percentage of sperm that show forward movement. In case of semen examination using CASA system, "motility" represents the percentage of sperm showing any kind of movements, while "progressivity" means the percentage of spermatozoa showing progressive, forwarding movements.

In addition to reflecting the fecundant capacity of semen, these two parameters may also indicate the presence of some epididimal affections, if their values are very low (*Solís et al., 2007*).

The percentage of motility (%Mot) ranged between 80.66% in July and 89.50% in January with a moderate difference (9.16%), while the annual average value was 84.82%. The chart of motility does not show significant variations, with low increases and decreases from one month to another (figure 3). The values were higher in December and January, when they reached the highest limit and then decreased smoothly, until September, when they began to increase progressively until the end of the study. The differences between consecutive months were small, almost insignificant, and it can be observed in Figure 3 that, overall, motility showed a regular variation, with gradual increases and decreases, reaching the upper limit in January and lower in July and September.

On the other hand, the mean values of progressivity (%Prog) varied between 49.00% (in April) and 63.58% (in January), with an annual average of 55.89%. The chart of %Prog shows irregular variation with successive increases and decreases, as well as maintaining

relatively constant. There was a sharp decrease in April, which is hard to explain, taking into account that the other parameters were above average during thismonth. A very low value was recorded also in July, in this case the results being consistent with those reported for motility.

Figure 3 shows that %Mot and % Prog had an approximately similar variation, with lower values in July and high values in January, which means that these two parameters are under the influence of the same factors.

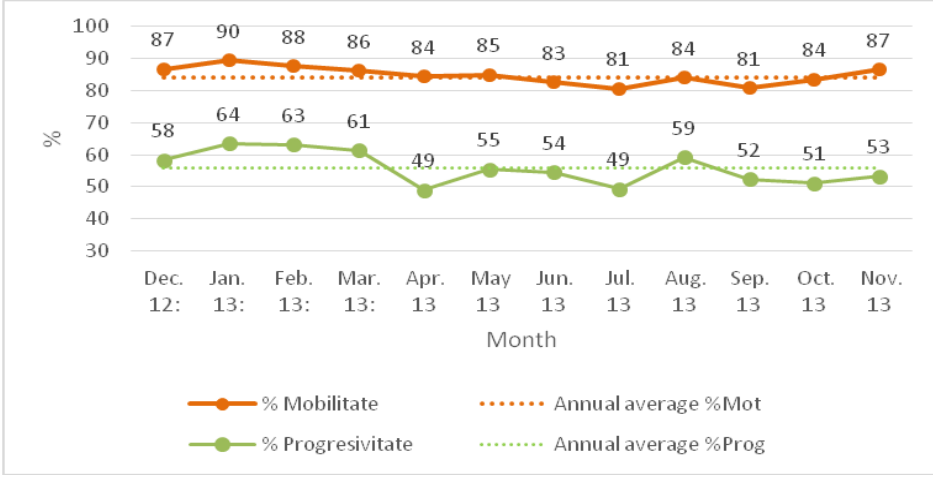

Figure 3 Monthly variation of average values for sperm motility and progressivity

Considering the low values of motility and progressivity that were obtained in July, it can be stated that the process of sperm maturation is affected by high temperatures (specific to this month), but it is not negatively influenced by low temperatures, as in January and February the recorded values were above average.

Monthly variation of total number of sperm within the entire ejaculate, of total number of motile sperm within the entire ejaculate, and of total number of progressive sperm within the entire ejaculate

One of the most important parameters of semen is the total number of spermatozoa/ejaculate (TSE). It accurately reflects the intensity of spermatogenesis. Using Table 1 it can be observed that TSE ranged from 71.70×10^9in February to 107.57×10^9 in June, showing a relatively highrange of variation (35.87×10^9).

The variation of TSE was relatively harmonious (Figure 4), with a gradual increase until June when it reached the maximum value, followed by a decrease to a minimum value in September and then another gradual increase until November.

Regarding total number of motile sperm/ejaculate (TMS), a parameter that reflects both the intensity of sperm formation and efficiency of their maturation, the variation limits were 61.86×10^9 in September and 89.10×10^9 in June.

Although the number of motile spermatozoa is related to the percentage of motility, the chart of TSM shows a different trendline than the chart of % Mob, because TSM is

directly dependent of TSE. For this reason, the highest value was in June, while January showed a value only close to average (Figure 4). Nevertheless, the minimum value was reached in September, following the variation of % Mot.

During semen examination, CASA system also calculates the total number of progressive sperm/ejaculate (TPS). This parameter reflects accurately the fecundant capacity of the ejaculate, showing the exact number of sperm within an ejaculate which have the ability to reach the place of fertilization. TPS values ranged between 40.36×10^9 in September and 58.98×10^9 in June. The annual average was 48.13×10^9.

One can observe the same aspect as in the case of TSM. Although % Prog presented the lowest values in April and July, TPSshowed the lowest values in September, similar to TSE. Also, TPS presented the highest values in June, although in this month % Prog showed a lower mean value than the annual average. This fact suggests that the dependence of TPSon TSE is bigger than the dependence of TPS on %Prog.

The strong relationship between TSE, TPS and TSM is illustrated in Figure 4 in which one can observe the similar trendlines.

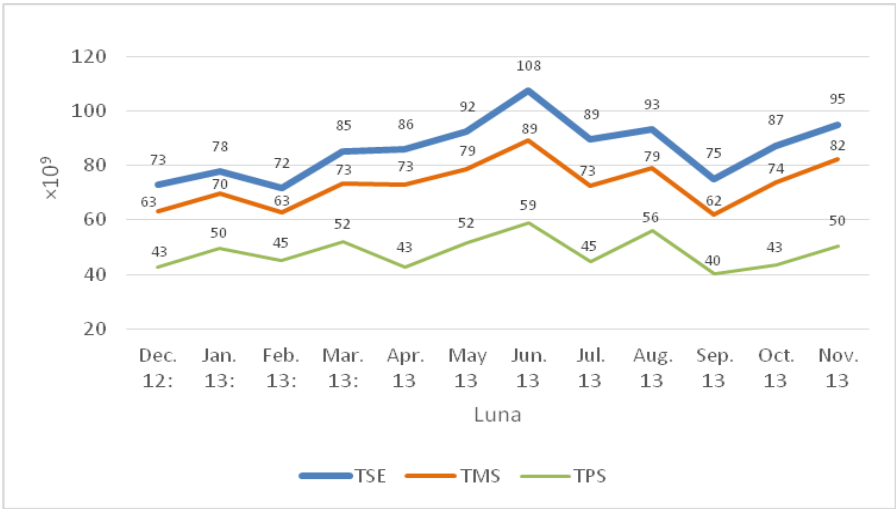

Figure. 4 Monthly variation of TSE, TMS and TPS

Based on the performed analysis and obtained results, it can be stated that the most favorable months for boar semen collection are June in terms of quantity and January in terms of quality. At the opposite side, the less favorable months are February in terms of quantity and July in terms of quality.

Statistical interpretation

The statistical analysis of the values obtained within the study is presented in table 2.

Table 2. Statistical analysis of the main seminal parameters in boar

	Conc.	Volume	TSE	TMS	TPS	Motility	Progressivity
Conc.	-	-.57*	.15	.19	-.04	.02	-.18
Volume		-	.70**	.64*	.58*	-.37	-.25
TSE			-	.97**	.73**	-.37	-.38
TMS				-	.80**	-.17	-.23
TPS					-	.12	.33
Motility						-	.76**
Progressivity							-

* Correlation is significant at the 0.05 level

** Correlation is significant at the 0.01 level

Volume and concentration correlate significantly, but negatively: the higher the volume, the lower the concentration. As stated before, the increase in semen volume, is due to an increase in seminal plasma quantity, resulting in an actual higher dilution ratio of sperm within the semen. Thus, the concentration decreases.

TSE correlates significantly with volume, indicating that the higher the volume, the higher the TSE. This fact suggests that the intensification of spermatogenesis is usually accompanied by an increase in the activity of accessory glands. However, TSE does not correlate with concentration, which means that an increase in the value of concentration does not necessarily mean an intensification of spermatogenesis.

TMS and TPS correlate significantly with TSE and do not correlate with %Mot and %Prog. This indicates, as stated before, that, although the number of motile spermatozoa is related to the percentage of motility and the number of progressive spermatozoa is related to the percentage of progressivity, these two parameters (TMS and TPS) are modulated rather by the total number of spermatozoa/ejaculate.

Motility and progressivity do not correlate with any variable, except with each other. They correlate positively, which means that they are modulated by the same factors.Also, the fact that the percentages of motility and progressivity do not correlate with the total number of spermatozoa/ejaculate indicates that the process of sperm maturation is independent of the process of sperm production (spermatogenesis).

Conclusions

By analyzing the results, the following conclusions can be drawn:

1. The best month in terms of formation and maturation of sperm (based on the number of sperm within an ejaculate with progressive movements) was June. At the opposite pole was September;

2. Total number of motile sperm/ejaculate and total number of progressive sperm/ejaculate are influenced rather by the total number of sperm/ejaculate than by the percentages of motility and progressivity;

3. Semen concentration is modulated rather by the activity of accessoryglands (by increasing or decreasing the volume of ejaculate) than by the spermatogenesis;

4. The activity of seminiferous tubules and the activity of accessory glands are influenced by the same factors, as the number of sperm/ejaculate and the volume of semen are positively correlated;

5. Spermatogenesis and epididimal sperm maturation are influenced by different factors, as total number of sperm/ejaculate and the percentage of motility do not correlate;

6. Housing conditions have a great influence on the variation of the main semen parameters in boar. Thus, the standardization and optimization of the microclimate within the farm can reduce the heat stress on spermatogenesis in the summer.

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SEASONAL DYNAMICS OF IXODID TICKS IN IAȘI URBAN AREA

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Abstract

Ticks are arthropods with huge medical significance that can harbor a great variety of microorganism including pathogens as Borrelia burgdorferi, Babesia spp., CCHF-Virus, Rickettsia spp., Dirofilaria spp., which they can transmit to humans and animals. A better knowledge of ticks ecological boundaries and seasonal activities bring new insights on their spatial and temporal distribution and help preventing the acquisition of such diseases. Herein we analyzed the seasonal dynamics of host-seeking Ixodid ticks in four outdoor leisure zones from Iași urban areas: CA Rosetti, Ciric, Bucium, Cetățuia. Starting October 2013 until September 2014, ticks were monthly collected by dragging method, over a surface of 1.5 ha[2] in each collection site. Collection was not performed during the winter season or in rainy days. Data regarding daily mean temperature (^oC) and relative humidity (%) were obtained from the Regional Centre of Meteorology Moldova. Over 1500 ticks were collected during the study period and identified as Dermacentor reticulatus, Haemaphysalis punctata , Ixodes ricinus and Ixodes redikorzevi. Dermacentor reticulatus was found mainly at CA Rosetti and Bucium recreational areas, and his peak activity was recorded during March. Haemaphysalis punctata was found at three recreational areas with a high prevalence (30%) at Cetățuia, having a peak activity in April. Ixodes ricinus was the dominant tick species found in all recreational areas. At CA Rosetti, Ciric and Cetățuia Ixodes ricinus had the peak activity during April, instead at Bucium the peak activity was recorded in May. Correlations of seasonal dynamics of ticks and meteorological data were made. A decrease of the relative humidity together with an increased temperature will strongly reduce nymphs and adult ticks activity but will have no effect on larvae. This study underlines that Ixodes ricinus is very well adapted to environmental conditions of the studied areas, with a delay of his peak activity at Bucium recreational area due to the climatic factors.

Key words*: Ixodid Ticks, Iași, Seasonal dynamics, , Romania*

Ticks are hematophagous arthropods parasitizing a large variety of hosts such as reptilians , birds and mammals. Out of 896 ticks in the world (7) less than 20% of them attach to humans (4) and are vectors for various pathogens (viruses, bacteria, protozoans, nematodes). In Romania, exist 25 species of hard ticks (6, 12) 11 possess a questing behavior (13), and 6 species frequent attach to humans (1, 9). Ixodid ticks spend 99% of their lives off the host, and climatic factors such as temperature and relative humidity have a major impact on their questing activities (14). Studying their seasonal activities and their host-seeking behavior may provide useful data regarding the risk of acquiring tick-borne diseases.

Material and Method

Starting October 2013 until September 2014, excluding the winter season, ticks were monthly collected, by dragging method in four collection sites from Iași urban area. Dragging was performed with a white flannel ($1m^2$), over 5 lines of 300m long in each location. The line was split into ten transects and the flag was examined after every 10 meters of dragging, 20 meters being the gap distance between each transect. Surface covered at each recreational zone was 1,5 ha. After collection, ticks were preserved in ethanol 70%

until identification. No tick sampling was performed during rainy days or when the leaf cover was to wet. Ticks were separated by developmental stages and identified on the basis of their morphological features using Feider identification keys.

Data regarding daily mean temperature (^{o}C) and relative humidity (%) were obtained from the Regional Centre of Meteorology Moldova.

The four sampling areas: C.A. Rosetti, Ciric, Cetățuia and Bucium were chosen for this study for the reason that they are suitable habitats for ticks and the main outdoor leisure zones from Iași. Vegetation is mainly formed by deciduous trees as *Quercus spp., Carpinus spp., Fraxinus spp., Fagus spp*., and fauna represented by birds, small rodents as rabbits (*Lepus europaeus*), squirrels (*Sciurus vulgaris*), hedgehogs (*Erinaceus romanics*), bank voles (*Myodes glareolus*), field mouse (*Apodemus agrarius*) large and medium herbivores (*Ovis aries, Bos taurus, Capreolus capreolus*). Coordinates and altitude of each location are presented in Tabel 1.

Table 1. Iași sampling areas

Collection site	Latitude(◦)	Longitude (◦)	Altitude (m)	Vegetation Type
C.A. Rosetti	47.19	27.57	52.28	Deciduous forest
Ciric	47.17	27.61	80.79	Deciduous forest
Cetățuia	47.13	27.59	81.38	Deciduous forest
Bucium	47.08	27.65	375.97	Deciduous forest

Results and discussions

A total of 1526 ticks were collected under this study: *Ixodes ricinus* was the most abundant tick species (92,39%) with 35 males, 26 females 220 nymphs and 1129 larvae, followed by *Haemaphysalis punctata* (6,94%) with 1 male, 12 females, 16 nymphs and 77 larvae and *Dermacentor reticulatus* (0.59%) with 2 males and 7 females. One adult specimen of *Ixodes redikorzevi* was found at C.A. Rosetti recreational area. For a better observation of results data regarding tick collection, temperature and relative humidity of October 2013 were placed at the end of charts.

In Iași, the seasonal distribution of host-seeking ticks, shows mainly the pattern of *Ixodes ricinus* activity, representing over 90% of ticks collection (Fig. 1). *Dermacentor reticulatus* had the first peak-activity, registered in March, decreasing in April and May and ceasing during the summer. Instead *Haemaphysalis punctata*, had the peak activity in April and *Ixodes ricinus* in April-May. All three species had a second peak in autumn, presenting a bimodal pattern of their seasonal activity. This pattern is particular for countries with temperate continental climate, with dry and hot summer and wet and cold winter, where *Ixodes ricinus* is the dominant species (3, 8), whereas in countries with Mediterranean climate, *Ixodes ricinus* can have his peak activity during autumn, as a consequence of climate change (2, 5).

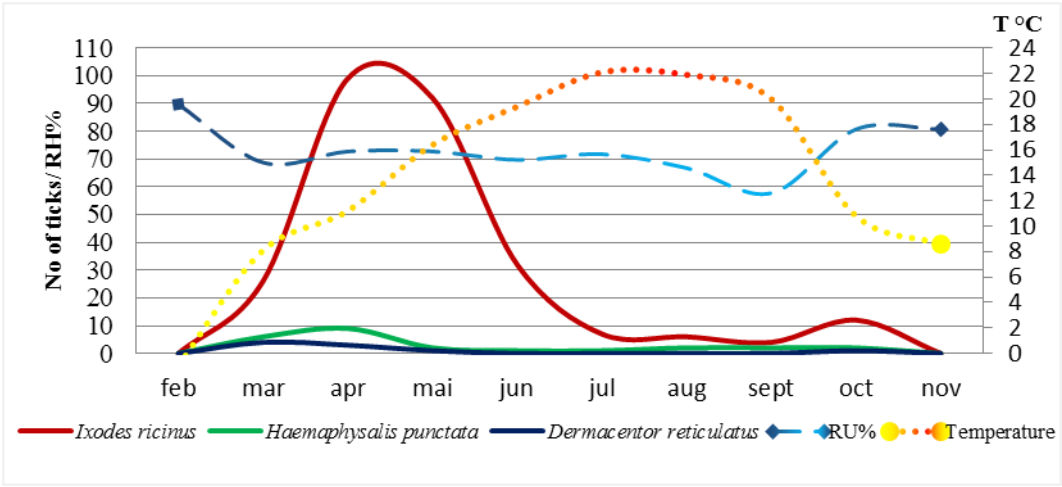

Fig.1 Ticks seasonal dynamics in Iași

There was a negative correlation between high temperatures along with a decrease of air humidity registered during summer months (July - August) and adults activity. We performed analysis regarding ticks activity compared with monthly rainfall, however we found no correlations (data not shown). Studies regarding ticks seasonal activity carried out in other European countries found correlations between adults seasonal activity and monthly rainfall, with a increase of adults in the wettest months (10).

Nymphs of *Ixodes ricinus* had nearly the same seasonal activity with adults (Fig. 2), nevertheless their abundance was considerably higher. The peak activity of nymphs of *Ixodes ricinus* was registered in April at CA Rosetti, Ciric and Cetățuia, and in May at Bucium.

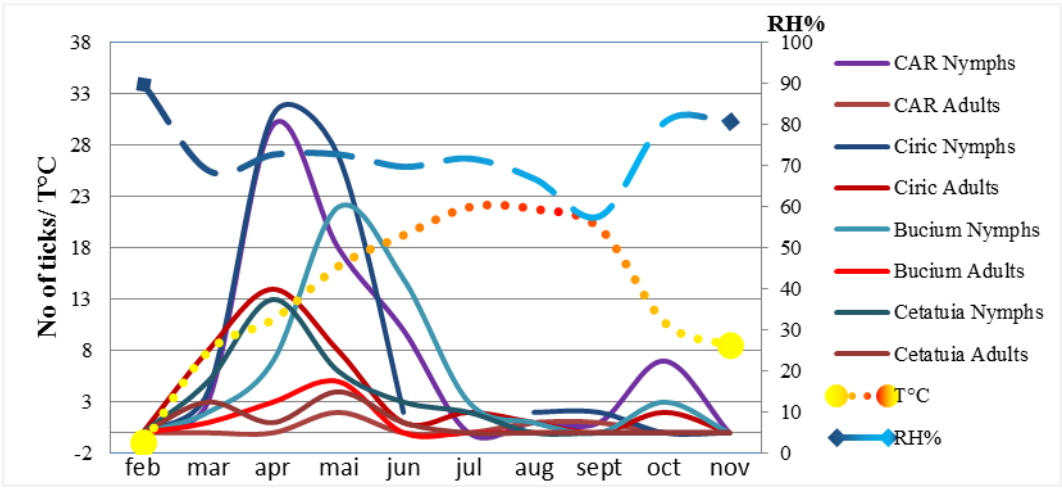

Fig.2 Seasonal activity of *Ixodes ricinus* nymphs and adults in Iași

Larvae had an allochronic activity in comparison with nymphs and adults, having their peak activity during the summer, positively correlated with temperature, at three out of four collection sites (Fig.3). These findings are in concordance with other European studies regarding *Ixodes ricinus* larvae seasonal activity, temperature being the most important abiotic factor influencing their host-seeking behavior (10).

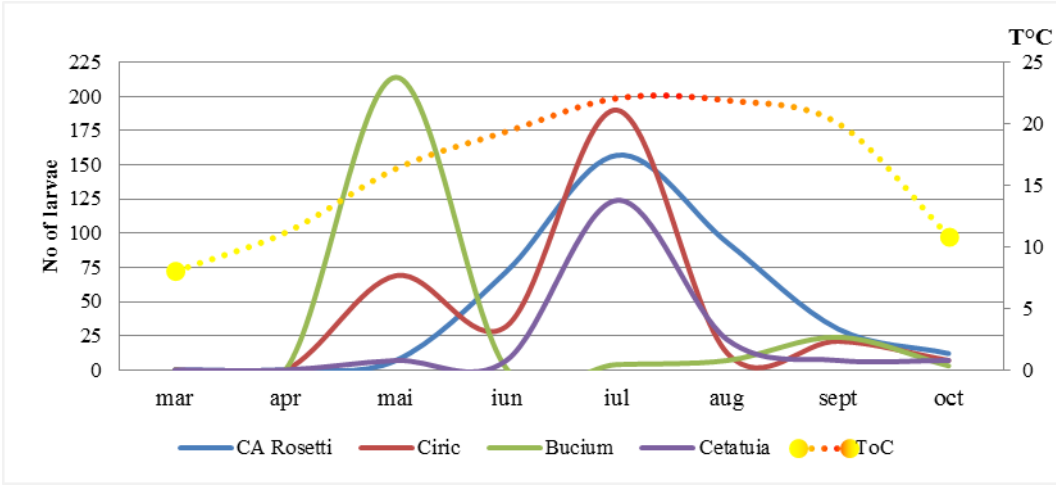

Fig.3 *Ixodes ricinus* larvae activity in Iași

The allochrony between immature and adult stages of *Ixodes ricinus* was not observed at Bucium collection site. All stages had their peak activity in May. Bucium collection site is located at a much higher altitude than the other collection sites. Altitude will increase the duration of *Ixodes ricinus* life cycle and has a negative correlation with abundance of ticks (11).

Conclusions

Ticks community in Iași urban area is comprise predominantly of three species *Ixodes ricinus*, *Haemaphysalis punctata* and *Dermacentor reticulatus* carrying out a bimodal seasonal distribution.

Ixodes ricinus is the dominant species in the region. Nymphs and adults begin host-seeking activity in March and peak in April and May, a high temperature along with a decrease of relative humidity will stop their quest.

Larvae of *Ixodes ricinus* have an allochronic activity in comparison with nymphs and adults, having their peak activity during the summer, positively correlated with temperature, however at higher altitude their host-seeking behavior is synchronical.

Haemaphysalis punctata is the second dominant species in Iasi, beginning host-seeking activity in late February and peaking in April. *Haemaphysalis punctata* had a high prevalence (35%) at Cetățuia recreational area probably due to the local ecological factors.

Dermacentor reticulatus has an early spring host-seeking activity, starting in February and peaking in March.

Acknowledgements

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THE ACAROLOGICAL RISK IN IAȘI RECREATIONAL AREAS

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Abstract

Tick-borne disease studies should include the vector community, reservoir hosts, pathogens prevalence, in both, vectors and reservoir hosts and the probability of a sensitive host encounter. Among those, knowledge of vector community is essential to assess the acarological risk of any tick-borne disease. Our aim was to characterize host-seeking tick communities in four outdoor leisure zones from Iasi urban area. Starting October 2013 until September 2014, ticks were monthly collected by dragging method over 1.5 ha^2 for each collection site. Collection was not performed during the winter season. Ixodes ricinus was the dominant tick species found in all four recreational areas - the recorded prevalence of Ixodes ricinus counted for over 90% of the tick species for three recreational areas under study (CA Rosetti, Ciric, Bucium) and 70% for the fourth one (Cetățuia). The high prevalence (30%) of Haemaphysalis punctata at Cetățuia recreational area is worth mentioning, probably due to local ecological factors.Tick density was measured, with the highest values during the spring season. We consider these data to have public health significance as Ixodes ricinus is the main vector for Lyme Borreliosis and Haemaphysalis punctata can transmit diseases as Babesiosis, Tick-borne encephalitis or Crimean-Congo disease.

Key words*: Acarological risk, Iași, Recreational areas, Ticks*

The epidemiology of tick-borne diseases comprise many factors as the vector community, reservoir host, pathogens prevalence, in both, ticks and reservoir host and the possibility of a sensitive host encounter. All of these can be defined as the acarological risk.

Ticks are increasing their habitats at higher spatial scale and higher altitude (7, 9, 17) and maintaining their vectorial capacities even at the border of their distribution (12). These obligate hematophagous arthropods can transmit pathogens as *Borrellia burgdorferi, Rickettsia spp., Babesia spp., Theileria spp., Anaplasma spp., Ehrlichia spp., Neoehrlichia mikurensis,* (4, 1, 13, 20,). The hard ticks fauna of Romania comprises 25 species in 5 genra: *Ixodes* (11), *Dermacentor* (2), *Haemaphysalis* (5), *Rhipicephalus* (4), *Hyalomma* (3) (8, 10). *Ixodes ricinus* is the most abundant and widespread tick species in region of Moldavia, Romania (18, 19,). Beside that, *Ixodes ricinus* is the vector of *Lyme Borreliosis* and the main tick species attaching to humans (2, 3). Under these circumstances a better knowledge of tick communities in areas where humans are exposed to ticks will help preventing the acquisition of tick-borne diseases.

The aim of this study was to describe tick communities for four recreational zones in Iași urban area and to assess human risk exposure to ticks by evaluating their density at each location.

Material and Method

Starting October 2013 until September 2014, excluding the winter season, ticks were monthly collected, by dragging method in four outdoor leisure zones from Iași urban area.

Dragging was performed with a white flannel ($1m^2$), over 5 lines of 300m long in each location. The line was split into ten transects and the flag was examined after every 10 meters of dragging, 20 meters being the gap distance between each transect. Surface covered at each recreational zone was 1,5 ha. After collection, ticks were preserved in ethanol 70% until identification. No tick sampling was performed during rainy days or when the leaf cover was to wet. Ticks were separated by developmental stages and identified on the basis of their morphological features using Feider identification keys.

The four sampling areas: C.A. Rosetti, Ciric, Cetățuia and Bucium were chosen for this study for the reason that they are the main outdoor leisure zones from Iași. Coordinates and altitude of each location are presented in Tabel 1. Vegetation type, usually was formed by deciduous trees as *Quercus spp., Carpinus spp., Fraxinus spp., Fagus spp*.

Table 1. Tick collection areas

Recreational zone	Latitude(◦)	Longitude (◦)	Altitude (m)	Vegetation Type
C.A. Rosetti	47.19	27.57	52.28	Deciduous forest
Ciric	47.17	27.61	80.79	Deciduous forest
Cetățuia	47.13	27.59	81.38	Deciduous forest
Bucium	47.08	27.65	375.97	Deciduous forest

Results and Discusions

A total of 1526 ticks were collected under this study: *Ixodes ricinus* was the most abundant tick species (92,39%) with 35 males, 26 females 220 nymphs and 1129 larvae, followed by *Haemaphysalis punctata* (6,94%) with 1 male, 12 females, 16 nymphs and 77 larvae and *Dermacentor reticulatus* (0.59%) with 2 males and 7 females. One adult specimen of *Ixodes redikorzevi* was found at C.A. Rosetti recreational area. Tick communities for each outdoor leisure zone studied are presented in Table 2.

Table 2 Tick communities and their abundance at the four recreational areas

Species	CA Rosetti		Ciric		Bucium		Cetățuia	
Ixodes ricinus	448	96,55%	435	99,77%	315	97,83%	212	69,96%
Haemaphysalis punctata	13	2,80%	0	0%	2	0,62%	91	30,03%
Dermacentor reticulatus	3	0,65%	1	0,23%	5	1,55%	0	0%

In three out of four recreational areas *Ixodes ricinus* had over 96% abundance, but a big part of this percentage are due to the big number of larvae. Even so, excluding larvae, *Ixodes ricinus* had the lead, with 88,59% at CA Rosetti, 99,04% at Ciric, 89,85% at Bucium and 65,51% at Cetățuia (Fig1).

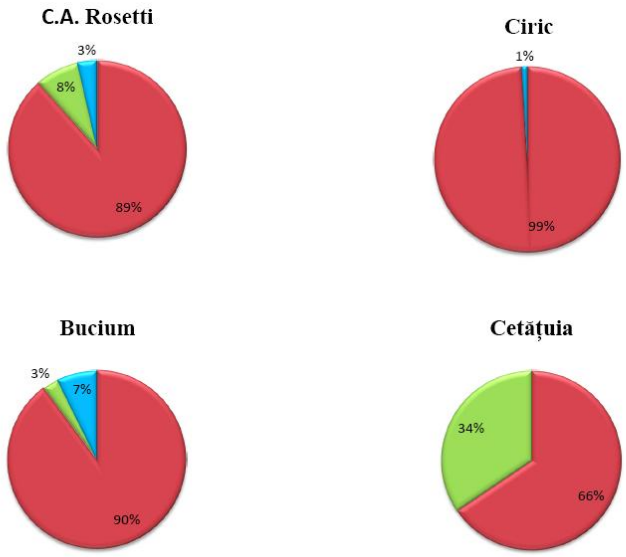

Fig 1: Tick communities for each recreational zone

Ixodes ricinus is the most abundant and widely distributed tick species in Moldavia region of Romania (19) and also the main tick species attaching to humans (3). *Ixodes ricinus* is the main vector for *Lyme Borreliosis* in Romania with an overall pathogen prevalence of 1.4%, *Borrelia afzeli* being the most frequent genospecies found in the region (14). *Ixodes ricinus* is also vector for other important diseases such as tick borne encephalitis, human granulocytic anaplasmosis and other rickettsiosis. Numerous of these etiological agents were already reported in ticks from Romania 4, 13) some of them collected from humans (1, 3).

Haemaphysalis punctata was the second most abundant species found in three recreational areas, with a prevalence of 3% at Bucium 8% at CA Rosetti and 34% at Cetățuia. The high prevalence of *Haemaphysalis punctata* at Cetățuia recreational area may be due to the local ecological factors such as slope exposure, temperature and relative humidity or plant community , this species being adapted to more arid environmental conditions. *Haemaphysalis puctata* is a specialist of ruminants but imature stages can attach to birds , especially on passeriformes, and rodents (8) and occasionally on dogs and humans (2). *Haemaphysalis punctata* is the main vector for ruminants babesiosis but is vector for tick-borne encephalitis and for Crimean-Congo hemorrhagic fever (6).

Dermacentor reticulatus was the third most abundant species with a prevalence of 1% at Ciric, 3% at CA Rosetti and 7% at Bucium recreational area. This species has an early spring activity, with a peak activity during March and April and a second in autumn and just adults quest (8). *Dermacentor reticulatus* is increasing his spatial distribution in the last decade (22, 23, 24) is the main vector for *Babesia canis* (21), *Rickettsia raoultii* (11) and *Anaplasma phagocytophilum* can be harboured with a high prevalence within this tick (11, 15).

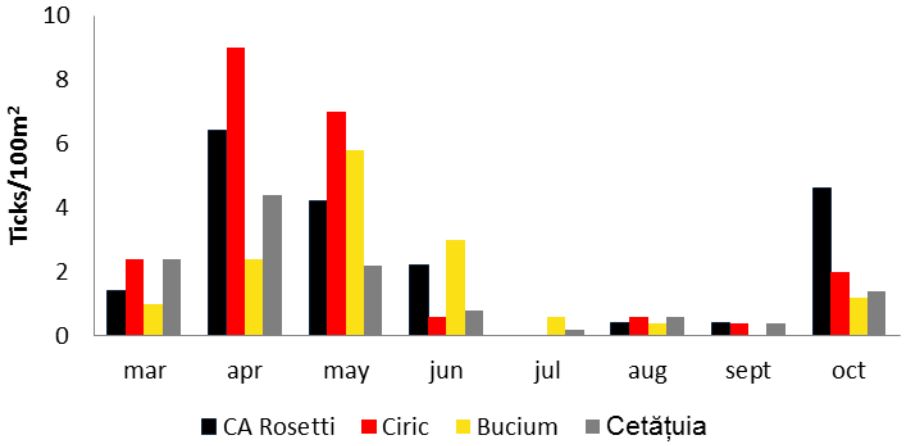

Figure 2: Ticks density per $100m^2$ for each reacreational zone

For assessing ticks density and evaluate the risk of exposure to ticks for human hosts, larvae were excluded from the analysis, being known the fact larvae are less infected by pathogens, as *Borrelia burgdorferi*, and usually they do not attach to humans. The graph below is showing ticks density for each location, measured monthly beginning October 2013 until September 2014, except for the winter season.

During the study, host-seeking ticks were always found at the four recreational areas, except for the warmest months when we collected predominantly larvae. The highest peak density was registered in April for CA Rosetti (6,9 ticks/$100m^2$), Ciric (9 ticks/$100m^2$) and Cetățuia (4,4 ticks/$100m^2$) and in May for Bucium (5,8/$100m^2$). The delay of the peak activity at Bucium recreational area is due to the climatic conditions, the location altitude is 375 meters above sea level. It is known that temperature and relative humidity play a major role in seasonal activity of ticks. The overall ticks density registered was lower compared with ticks density registered in other European countries (5, 11, 16), continental climatic conditions of Romania, with very high temperatures during the summer and very low in winter, pause ticks activity during these seasons, force them to enter in quiescence respectively to hibernate. Females of *Haemaphysalis punctata* and nymphs of *Ixodes ricinus* are more difficult to be detected than *Ixodes ricinus* adults or *Dermacentor reticulatus* adults when attached to the human host, and they are more likely to transmit pathogens, and spring season – when ticks are most abundant – can be considered the period with the highest risk to ticks exposure in Iași recreational areas.

In Romania, the incidence of tick-borne diseases in humans appears to be underestimated maybe due to poor surveillance of these zoonoses, misdiagnosis and asymptomatic cases. This study provide useful information regarding the level of tick infestation in the main recreational areas from Iasi. To reduce tick exposure, prevention efforts should focus on controlling grass and brush particularly along paths and in pic-nic areas, providing information for visitors about the risk of tick bites and recommend appropriate behavioral measures to be taken, including protective clothing, use of insect repellent, tick checks and early tick removal (5).

Conclusions

Spring season is the period with the highest risk to ticks exposure in Iași and the highest tick abundance was registered at Ciric recreational area , where questing ticks density reached 9 ticks/100m^2 in April.

Ixodes ricinus is the dominant species in the region, with over 90% and 65% prevalence at CA Rosetti, Ciric, Bucium respectiveley at Cetățuia recreational areas.

Measures of prevention are needed to reduce tick exposure by controlling the grass height and providing information to visitors about the tick bites and recommend use of protective clothing, use of insect repellent, tick checks and early tick removal.

Acknowledgments

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THE DISTRIBUTION OF THE PARASITIC COMPLEX OF *EIMERIA, GIARDIA* AND *CRYPTOSPORIDIUM* IN HOUSED CALVES FROM MOLDAVIA

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Abstract
In this paper we present the data about constant identification of Giardia spp. and Cryptosporidium spp. with their zoonotic potential in the farms from region of Moldavia, Romania. Eimeria spp., contained in the parasitic complex could produce also economically losses. The researches were made through specific methods of identification for Eimeria spp., Giardia spp. and Cryptosporidium spp. The purpose of the work was to evaluate the epidemiology of parasitic protozoa infections in calves. For counting Eimeria spp., has been used flotation methods, with hyper saline solution. Giardia spp. and Cryptosporidium spp. has been identified and counted using a modified Immunofluorescence method with the commercial kit from Merifluor©. Between July 2013 to September 2014, 140 samples from 16 different farms were collected and analyzed. The samples have been collected in farms from the departments of Moldavia, Romania: Botoşani, Iaşi, Suceava, Neamţ, Vaslui, Bacău, and Galaţi. The farms breed cattle's for different purpose, mainly replacement calves for dairy farms, beef cattle and hybrids between dairy and beef breeds. We noticed differences in terms of hygiene, accommodation and feeding between farms, and housing systems. The feeding is specific for each farm, but in specially is made with milk, milk substitutes and concentrates. The data obtained show the presence of parasitic loads vary depending on the system of maintenance and feeding, hygiene as well as depending the season. Colder seasons the prevalence is higher and the fact that in higher farms, with an efficient management, the parasitic load is reduced.
Key words:*calves, Moldavia, protozoa, Romania, zoonotic.*

The protozoan parasites such as *Giardia spp.*, *Cryptosporidium spp.*, and *Eimeria Spp.* are very important factors in the early development of young calves and can cause the death or significant depreciation of real economically value of future cow (4). Also loses appear as a higher consumption of medicaments and milk or milk replacements. Another risk is the zoonotic characteristic of *Giardia spp.* and *Cryptosporidium spp.* (3, 9).

The prevalence of *Cryptosporidium spp*. is higher in calves with ages between 5 days and 1 month. In young calves the *C. parvum* predominates and is considered as highly pathogenic (3).

Giardia spp. has also a high clinical significance, but the pathogenesis of giardiosis is not clearly understood. The prevalence of giardiosis is higher in calves with ages between 1 to 3 months. The most significant alteration is an increase in epithelial permeability which appears to result from enterocyte apoptosis (3) and from cytoskeletal reorganization induced by trophozoite toxic products (3).

Bovine coccidiosis is a disease affecting calves all over the world resulting in considerable economic losses to farmers (1*)*. The prevalence of the infections was between 20-100%, with variations of seasons. (7). In cattle is known to be found over 20 different species, with

differences of virulence and pathogenicity. The most prevalent species identified were *E. bovis* and *E. zuernii*, wich are also the most virulent. (2)

Materials and methods

Materials

Sample collection was done between July 2013 and March 2014 with a total of 159 samples from 16 different farms. The samples were collected from calves with age between 3 weeks and 5 months by rectal stimulation straight into the glove. After collecting the samples are transported to the laboratory at temperature between 4°and 8° C and examined in maximum 72 hours. The farms are located in Suceava, Botosani, Iasi, Bacau, Vaslui and Galati County.

Sample collection farms

Sampling was made in randomly chosen locations to cover farms from entire Moldavia region. The farms were chosen to have different conditions of hygiene, accommodation and feeding. The farms studied are both beef cattle breeds, replacement calves for dairy farms and hybrids between dairy and beef breeds.

Table 1. Sample collection farms

No	**Date**	**Farm**	**Number of Samples.**	**Type**	**Housing System**	Feeding
1	15 07 2013	Ferma 1	10	Dairy	SP	MR
2	28.07.13	Ferma 2	8	Dairy	CP	MR
3	02.12.13	Ferma 3	9	Dairy	SP	Milk
4	05.12.13	Ferma 4	10	Dairy	SP	MR/Milk
5	06.12.13	Ferma 5	10	Dairy	SP	MR/Milk
6	15.12.13	Ferma 6	9	Dairy	CP	MR/Milk
7	28.12.13	Ferma 7	8	Dairy	CP	Milk
8	09.01.14	Ferma 8	8	Dairy	SP	MR/Milk
9	14.01.14	Ferma 9	8	Dairy	CP	Milk
10	21.01.14	Ferma 10	8	Dairy	CP	Milk
11	24.01.14	Ferma 11	8	Dairy	SP	MR/Milk
12	31.01.14	Ferma 12	8	Dairy	CP	Milk
13	21.03.14	Ferma 13	8	Dairy	CP	MR/Milk
14	26 05 14	Ferma 14	9	Dairy	CP	MR/Milk
15	26 05 14	Ferma 15	9	Dairy	CP	MR/Milk
16	26 05 14	Ferma 16	10	Dairy	SP	MR/Milk

Legend: CP – collective pen, SP – single pen, MR – Milk replacements

The housing systems are either single pen for young dairy calves or in collective pens for older ones. Also the feeding is made differently with milk, milk substitutes and concentrates. In some farms the tea of alfalfa and hay is also used for hydrating the calves.

Method

For identification of parasite two different techniques has been used: for identification of fecal egg or oocysts a modified Mc Master technique by analyzing 4 grams

of faeces. The sensitivity of the test is 50. The calculation of fecal egg or oocysts counts per gram (EPG or OPG) was done the following formula:

$$\frac{4 \text{ (grams)} \times 50 \times \text{number of counted eggs or oocysts}}{\text{Actual amount of feces (grams)}}$$

For the quantitative detection of *Cryptosporidium spp.* and *Giardia spp*. in faeces we searched the oocysts and cysts by Immunofluorescence. The assay was done with a commercial kit from Merifluor© by analyzing 1 g feces following the manufacture indications.

The slides were examined under an Immunofluorescence microscope at 400X: (oo) cysts were counted and the number of cyst was multiplied by fifty to obtain the number of (oo) cysts per gram of faeces. The calculation of fecal egg or oocysts counts per gram (EPG or OPG) was done the following formula: the number of oocysts/cyst counted is multiplied by fifty to obtain the number of (oo) cysts per gram of faeces.

Results and discussions

From the total of 140 samples collected from all 16 locations in Moldavia, Romania were identified, using specific identification methods, both *Eimeria spp.*, *Giardia spp.* and *Cryptosporidium spp.*. Each farm presents differences in terms of breed, production, housing and feeding system of young calves and also in terms of microclimate (influenced by the season's change of temperature and humidity). The farms with single pen for each calf can provide better condition also for hygiene and disinfection but also for counting the amount of intake of milk/milk replacement. Is also more facile to identify and treat the calf with diarrhea. The maximum numbers of recontamination calves are the left and right neighbors, comparing with the entire herd kept in the same collective pen.

Table 2. Results

No	Date	Farm	No. of Samples.	Housing System	Eimeria spp.		Giardia spp.		Crypto. spp.	
1	15 07 13	Farm 1	10	SP	0/10	0%	0/10	0%	0/10	0%
2	28.07.13	Farm 2	8	CP	3/8	37.5%	0/8	0%	0/8	0%
3	02.12.13	Farm 3	9	SP	1/9	11,11%	9/9	100%	2/9	22,22%
4	05.12.13	Farm 4	10	SP	1/10	10%	6/10	60%	2/10	20%
5	06.12.13	Farm 5	10	SP	1/10	10%	7/10	70%	1/10	10%
6	15.12.13	Farm 6	9	CP	7/9	77,77%	6/9	66,66%	0/9	0%
7	28.12.13	Farm 7	8	CP	2/8	25%	6/8	%	1/8	%
8	09.01.14	Farm 8	8	SP	0/8	0%	1/8	12,5%	0/8	0%
9	14.01.14	Farm 9	8	CP	5/8	62,5%	4/8	50%	0/8	0%
10	21.01.14	Farm 10	8	CP	8/8	100%	4/8	50%	0/8	0%
11	24.01.14	Farm 11	8	SP	0/8	0%	6/8	75%	1/8	12,5%
12	31.01.14	Farm 12	8	CP	7/8	87,5%	4/8	50%	0/8	0%
13	21.03.14	Farm 13	8	CP	0/8	0%	0/8	0%	0/8	0%
14	26 05 14	Farm 14	9	CP	6/9	66,66%	5/9	55,55%	2/9	22,22%

15	26 05 14	Farm 15	9	CP	3/9	33,33%	3/9	33,33%	1/9	11,11%
16	26 05 14	Ferma 16	10	SP	3/10	30%	2/10	20%	2/10	20%

Legend: CP – collective pen, SP – single pen,

Is well known the fact that the prevalence of *Cryptosporidium spp*. is higher in calves with ages between 5 days and 1 month and also that after 2 weeks the oocysts shedding drops. The prevalence of *Giardia spp.* is higher in calves with ages between 1 to 3 months. **(3)**

Eimeria infections occur frequently in calves and thus can cause monetary losses. The most prevalent species of coccidia in calves is *Eimeria bovis* and *Eimeria Zuernii*. The overall prevalence is in young calves(40%) and until they reach the age of one year old.(**2)**

Thus, an important aspect in our study is the calf's age; the sample has been taken from calves with the age in between 3 weeks old and 5 months, without any consideration about sex or other criteria. The EPG was influenced also by housing and feeding condition.

In the farms were the calves are kept in collective pens the *Eimeria spp.* EPG is higher. From the 9 farms analyzed, using collective pens, on 8 of them we found positive samples. From a total of 75 samples 41 (54. 66 %) of them has been positive. In contrary, in the farms were the calves are maintained in individually pens, from 65 samples, only 6 (9.23 %) of them have been positive.

For the *Giardia spp.* the results show that from a total of 75 sample collected from farms with collective pen 32 (42.66%) have been found positive. In contrast from 65 sample (individual pen farm) 31 (47.69%) have been positive. That suggests the high contamination of the *Giardia spp.* The *Cryptosporidium spp*. positive samples are the fewest, 12(8.57%), but mainly because of the age of calves, being more prevalent in calves aged 8–14days (5).

The positive samples are found mostly in the farms with individual pens. From a total of 75 samples collected from farms using collective pens, 4 was positive (5.33%) for *Cryptosporidium spp.* The farms were the calves are kept in single pens the number of positive sample was 65 samples revealed 8 (12.03 %) positive. Autoinfection could be also a factor for higher number of calves with cryptosporidium infection. The calves maintained in individual pen are also much younger than the one from collective ones. Regarding the studies already done, the low prevalence in calves from individually pens demonstrates that the dam is an important source of contamination. The prevalence of calves with mixed infection is 56 (40%) from 140 samples.

The sample collected from farm 1 and 12, has been taken during summer and spring. We noticed that the EPG for the parasite complex is zero or very low. The high prevalence in spring and autumn coincide with the calving period planned in these periods.

Conclusions

The highest prevalence for *Eimeria spp* .and *Giardia spp.* is found in the farms were the calf are housed in collective pens, in wich one the hygiene and feeding contions can't be always respected inoptimal conditions. More than 50% of the calf from this farms are infested by different species *Eimeria*.

The research suggests that the higher risk of infection with *Cryptosporidium spp.* is found in younger calves. Also the calf categories most often housed in individually pens are new born until couple of week's age. Using a good management, a disinfection and prevention program can avoid the financial loses (5) produced by diarrhea in housed calves.

There must be noted the presence of *Giardia spp.* and *Cryptosporidium spp.* as species found in Moldavian and the hazardous of this prevalance, taking in consideration the zoonotic risk (8).

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THE PREVALENCE OF *EIMERIA* SPECIES IN DAIRY FARMS FROM ROMANIA

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Abstract

This paper presents the data about the prevalence and the economical risk caused by the infection with Eimeria spp. in the farms from Romania. The research has been done through specific method for the identification of Eimeria species. The purpose of this work was to evaluate the distribution of the parasitic infections with pathogenic Eimeria species in calves from commercial farms. Our study also aim to justify the treatment with substances against Eimeria spp. especially toltrazuril in those farms. For identifying Eimeria spp., has been used the Willis flotation method, with hyper saline solution and an optical microscope. Starting October 2010 until April 2011, 354 samples from 36 farms have been collected and analyzed. The samples have been collected from dairy farms located in different departments of Romania and shipped on ice to the laboratory of Parasitology from Faculty of Veterinary Medicine, Iasi. The age of the subjects was between one to five months. Between farms exists several differences in terms of hygiene, accommodation and feeding of the calves. The housing systems are either single calve pen either in collective pens. The data obtained show that the parasitic loads vary depending on the system of maintenance and feeding, hygiene conditions. The results show that Eimeria bovis is the most prevalent. Also Eimeria bovis var. elipsoidalis has been identified. In some cases Eimeria bovis is occurred in the same sample with the more pathogenic Eimeria zuernii.

Key words: *calves, eimeria, farms,management, pathogenic, Romania.*

The objective of our study is to show that *Eimeria spp.* is a constant presence in the housed calves from dairy and beef in Romania, as same as all over the world.(2***).*** The prevalence of the infections was between 20-100%, with variations of seasons. (6). In cattle is known to be found over 20 different species, with differences of virulence and pathogenicity. The most prevalent species identified were *E. bovis* and *E. zuernii*, wich are also the most virulent. (*2*).

Bovine coccidiosis is a disease affecting calves all over the world resulting in considerable economic losses to farmers. Due to the economical losses caused by this disease (*1*) the benefit in treatment and prevention of different active substances (3, 4) and because in region of Romania the studies in the last years are missing we propose to renew the data. This study tries to demonstrate the necessity of using an anti coccidian substance either as a metaphylactic or as treatment. The high resistance of oocysts on the soil and the risk of infestation recommend also a good farm management. (5*)*

Materials and methods

Sample collection farms

Sample collection was done between October and April with a total 354 of samples from 36 different farms. The samples were collected from calves with age between 1 and 5 months by rectal stimulation straight into the glove. After collecting the samples are shipped to the laboratory at temperature between 4°and 8° C and examined in maximum 72 hours.

The farms are located in the departments of Romania. Conditions of hygiene, accommodation and feeding are different in each farm. The farms studied are beef, dairy and hybrids between dairy and beef breeds.

Method

For identification of parasite the Willis technique has been used. Since our objectives was to determin the presence of any pathogenic species we used this qualitative technique.

A small part of faecal sample (about 5g) is placed in a Berzelius glass with a small quantity of hypersaline solution. The obtained solution is thoroughly mixed and more hypersaline solution is added. The mix is poured through wire mesh. An Erlenmeyer glass is filled with the mix and hyper saline is added until a convex meniscus is formed at the surface. On top a slide is placed and left for 25-30 minute. With a fast movement the slide is turned upside-down, without losing the drop. Another slide is placed on top of the drop. The slides are examined under the microscope. Oocysts were measured using Motic measurement system.

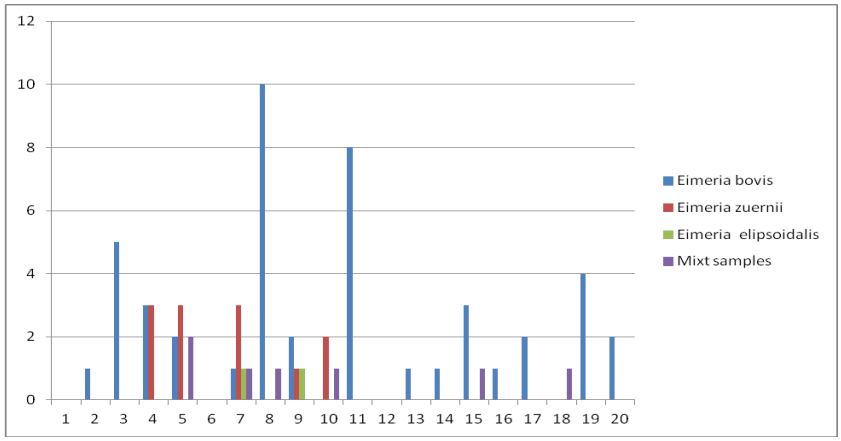
Results and discussions

From the total of 354 samples collected from 36 locations in Romania were identified 46 calves with *Eimeria bovis, 12* with *E. zuernii, 2* with *Eimeria elipsoidalis,* and 7 with mixt infections. Each farm presents differences in terms of breed, production, housing and feeding system of young calves and also in terms of microclimate.

Table 1

No.	Department	Number of farms	Number of sample	*Eimeria bovis*	*Eimeria zuernii*	*Eimeria elipsoidalis*	*Mixt samples*
1	Giurgiu	1	12	0	0	0	0
2	Sibiu	2	12	1	0	0	0
3	Vaslui	3	27	5	0	0	0
4	Cluj	5	21	3	3	0	0
5	Neamt	2	20	2	3	0	2
6	Timis	1	15	0	0	0	0
7	Bistrita Nasaud	3	31	1	3	1	1
8	Botosani	2	20	10	0	0	1
9	Teleorman	2	31	2	1	1	0
10	Hunedoara	1	10	0	2	0	1
11	Arad	3	45	8	0	0	0
12	Prahova	1	15	0	0	0	0
13	Satu Mare	2	18	1	0	0	0
14	Braila	1	6	1	0	0	0
15	Brasov	2	12	3	0	0	1
16	Mures	1	8	1	0	0	0
17	Ilfov	1	15	2	0	0	0
18	Covasna	1	15	0	0	0	1
19	Iasi	1	11	4	0	0	0
20	Dambovita	1	10	2	0	0	0
Total		36	354	46	12	2	7

A total of 354 sample recolted from 36 different farm from wich:
283 negatives(79,94%) and 71 positives (20,06%)
Eimeria bovis 46 (12,99), Eimeria zuernii 12 (3,38%),
Elipsoidalis 2 (0,56%), Mixed sample 7 (1,97)



Eimeria infections occur in 20.06% calves and thus can cause monetary losses. The most prevalent species of coccidia in calves is *Eimeria bovis* (12,99%) and *Eimeria Zuernii(3.38%)* wich are the most pathogenic one for calves. Thus, an important aspect in our study is the calf's age; the sample has been taken from calves with the age in between 1 and 5months old, without any consideration about sex or other criteria.

The high number of sample, collected in various farms from different department of Romania, provide a real prevalence of *Eimeria spp*. infections.

Conclusions

The highest prevalence is for *Eimeria bovis.* 71 of the calf from this farms are infested by different species *Eimeria*.

79.94% from examined samples has been negatives, 20.06% were positive, from wich *Eimeria bovis (12,99%) and Eimeria Zuernii(3.38%)*

A good farm management with measures as desinfection, metaphilactic treatment with toltrazuril helps avoid any economical loses.

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ELECTROENCEPHALOGRAPHIC FINDINGS IN JUVENILE MYOCLONIC EPILEPSY IN DOG

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Abstract
Background*: Juvenile epilepsy represents about 7% of the cases of epilepsy in dogs. The syndrome is defined by myoclonic seizures (jerks) with and without tonic-clonic seizures.The* ***aims*** *of this study were to describe a short time EEG recording (30 minutes) using Redding's model and to determine the diagnostic value of electroencephalographic recordings in dogs suffering from juvenile myoclonic epilepsy.* ***Methods****: Electroencephalograms were performed on 7 puppies, between 3-6 months old, suffering from juvenile myoclonic epilepsy. EEGs were obtained via five subdermal needle electrodes, recorded with sensitivity: 70µV/cm; time constant: 0.3 seconds; notch filter inserted; impedance of all electrodes < 10 kΩ.* ***Results*** *– Brain electrical activity in 6 of 7 patients was characterized by the presence of interictal epileptiform discharges. Exclusive presence of single spikes and polyspikes were recorded in 2 patients (33.33%) and sharp wave and multiple sharp wave complexes were encountered in other 4 dogs (66.66%).* ***Conclusion*** *– In epileptic puppies from this study, the EEG interictal epileptiform discharges had similarities with juvenile myoclonic epilepsy in humans. In these dogs, EEG was a valuable examination in confirming the diagnosis of canine epilepsy, excluding other non-epileptic movement disorders.*

Keywords*: juvenile myoclonic epilepsy, electroencephalogram, dog, diagnostic*

Introduction

Idiopathic epilepsy (IE), also called primary epilepsy is chronic recurring seizures with no underlying structural brain lesion or other neurological or clinical signs (Engel, 2006; Chandler, 2006). The term idiopathic epilepsy is not applied simply to any patient in whom the cause of the seizures is unknown. Instead, it refers to recognized clinical syndromes with typical clinical features, such as age of onset and lack of other neurological abnormalities. In this disease the typical age of seizure onset is between 1 and 5 years. Nonetheless, IE is commonly suspected in dogs younger than this age (occasionally start before 6 months), and similarly in dogs whose first seizure occurs after five years of age (Patterson et al, 2005; Licht et al, 2007). Results of the studies on epileptic dogs can be translated to humans and vice versa because canine seizures exhibit a remarkable resemblance to human seizures regarding clinical, epidemiological features, prognosis and mortality (Berendt et al, 2004). In humans, juvenile epilepsy is a common type of genetic (idiopathic) generalized epilepsy, comprising 5–10% of all epilepsies (Vollmar et al, 2011) and has been the subject of intensive research over the past 25 years (Genton et al, 2013). The outcome of juvenile epilepsy is good, 71% of children becoming free of seizure for 1 to 4 years and discontinued antiepileptic drug treatment (Camfield and Camfield, 2005).

Until now, in dogs there are few papers describing seizures in puppies younger than one year old (Coates and Bergmam, 2005; Arrol et al, 2012; Lavely, 2014), none of these studies describing EEG findings. Furthermore, in these studies, seizures had different etiologies (idiopathic, symptomatic, and reactive). Only one study presented familial juvenile

epilepsy in Lagotto Romagnolo dogs. (Jokinen et al, 2007), electroencephalographic examination being included in the diagnostic protocol.

Considering this, the aims of current study is to compare EEG findings in puppies between 2 and 6 month old with juvenile epilepsy in humans and to determine the value of EEG diagnostic of this disease in dogs.

Material and methods

The study was made on 7 dogs that were presented to Internal Clinic of Faculty of Veterinary Medicine Iasi, and after neurological and imagistic (CT/MRI) examinations were diagnosed with epilepsy. These dogs, 2-6 months old, were not littermates, and none of their brothers or parents exhibited seizures (Table 1).

Table no.1. Signalments and clinical signs of dogs included in the study

No.	Breed	Sex	Age	Clinical signs
1	German Shepherd	Male	12 weeks	Generalized tonic-clonic seizures, loss of consciousness, urination during the seizure
2	German Shepherd	Female	12 weeks	Generalized tonic-clonic seizures, consciousness preserved
3	Boxer	Female	18 weeks	Generalized tonic-clonic seizures, consciousness preserved
4	Bichon	Male	24 weeks	Generalized tonic-clonic seizures, loss of consciousness
5	Crossbreed	Male	15 weeks	Generalized tonic-clonic seizures, loss of consciousness,
6	Crossbreed	Male	16 weeks	Generalized tonic-clonic seizures, loss of consciousness, urination and defecation during the seizure
7	Crossbreed	Female	14 weeks	Generalized tonic-clonic seizures, loss of consciousness

EEGs were made with Neurofax S device (MEB 9400K, Nihon Kohden), after general anesthesia that was induced with medetomidine hydrochloride (Domitor, Pfizer), 0.05 mg/kg inj. EEGs were obtained via five subdermal stainless steel needle electrodes (F3, F4, O1, O2 and Cz) as described by Redding (1978) with reference electrode placed on the bridge of the nose (Fig. 1). The area on which the electrodes were placed was trimmed and degreased with alcohol and Skin Pure (Nihon Kohden). In EEG recoding the parameters used were: sensitivity = 70μV/cm; time constant = 0.3 seconds; Hf = 70 Hz; Lf = 0.5 Hz; notch filter inserted; impedance of all electrodes < 10 kΩ.

Visual examination of all EEGs was performed registering the EEG pattern (any characteristic EEG activity) background activity (any EEG activity representing the setting in which a given normal or abnormal pattern appears and from which such pattern is distinguished), all paroxysmal activities (spikes, sharp wave, spike-wave complex), as well as possible artifacts.

Recording sections were visually selected for analysis of background activity using Fast Fourier Transformation (FFT). Spectral bands were, 8.0-13.0 Hz for alpha, 13.0-30.0 Hz

for beta, 0.5-4.0 Hz for delta and 4.0-8.0 for theta activity. In order to minimize errors through different skull sizes, forms and thicknesses, the relative power of the spectral bands was calculated for every lead.

Results and discussions

In all dogs included in the present study, EEG background activities were dominated by the presence of theta and delta waves, while alpha and beta waves were less frequent. Similar results were obtained in dogs with idiopathic epilepsy in Brauer et al (2012) and Pakozdy (2012) studies when anesthesia was induced with propofol and by Jeserevics et al (2007) who used medetomidine like anesthetic. Suppression of alpha and beta waves in dogs under anesthesia with medetomidine has been described by Itamoto et al (2001), being observed after the use of other anesthetics too: xylazine (Pellegrino and Sicca, 2004) and a combination of medetomidine with propofol (Srenk and Jaggy, 1996). The results of the above mentioned studies, allow us to consider that this type of background activity observed by us is a normal one in anesthetized dogs. For performing an EEG in children, general anesthesia is not usually used, but just like in the present study, in juvenile epilepsy a normal EEG was obtained.

Interictal EEG analysis in 6 of 7 affected puppies indicated epileptiform discharges. In 2 patients were recorded the exclusive presence of single spikes and polyspikes (Fig. 1; Fig. 2) and in other 4 dogs were encountered sharp waves, and multiple sharp wave complexes (Fig. 3). These paroxysmal activities had a generalized appearance in 5 dogs, only in one patient being observed the focal aspect of sharp wave complexes. The same EEG aspects were registered in human juvenile epilepsy: spike or polyspike and wave complexes which are commonly assumed to occur without lateralizing or localizing features (Nordli, 2005; Lee et al, 2014).

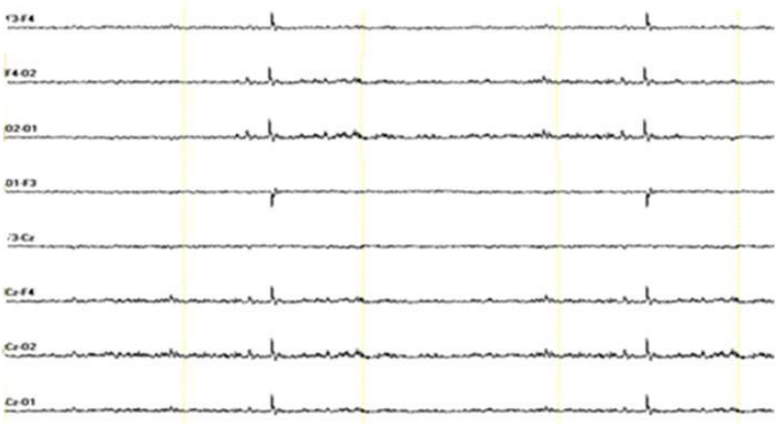

Fig.1. EEG recording in a German Shepherd bitch, 12 week old with juvenile epilepsy. Fast spikes encountered during cluster associated with epilepsy

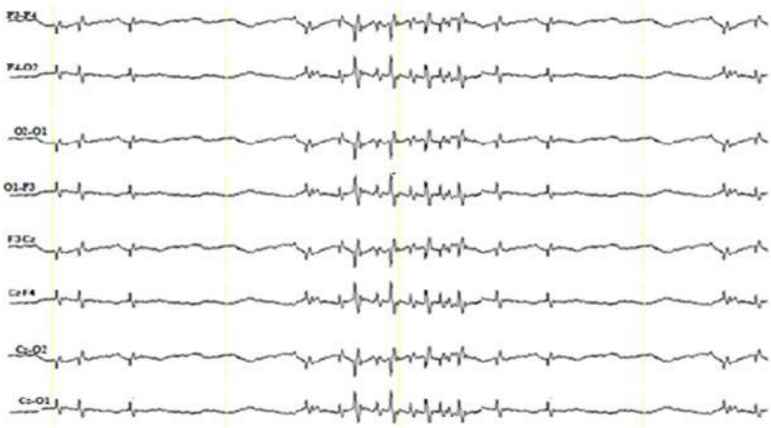

Fig. 2. EEG recording in a mixed breed dog, 15 week old with juvenile epilepsy. Spikes and polyspikes

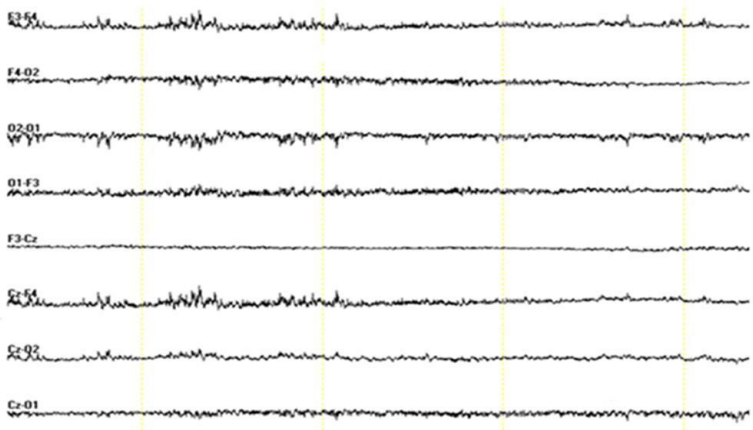

Fig. 3. EEG recording in a mixed breed bitch, 14 week old with juvenile epilepsy. Monopolar recording with proeminent wave and multiple sharp-wave complexes

In dogs from our study predominated generalized EEG activity (83.3% of dogs with abnormal EEG). In contrast to our results, Jokinen et al (2007) claimed that from 14 Lagotto Romagnolo dogs with EEG abnormalities, 13 puppies had focal changes (92.8%) and in only 1 generalized activity (7.2%) was seen. A possible cause may be that in Jokinen et al (2007) study the majority of puppies experienced milder form of epilepsy with focal seizures and consciousness preserved that was in contrast with our study where all dogs had generalized seizures and 4 puppies lost their consciousness during the seizures.

In 2 dogs of our study (one Boxer and one German Shepherd) consciousness was preserved and generalized tonic-clonic movements were clinically difficult to distinguish between paroxysmal movement disorders and seizure activities. Furthermore, Ramsey et al (1999) described in Boxer puppies episodic involuntary skeletal muscle activity with

preserved consciousness during episodes. In movement disorders (neurologic conditions characterized by abnormalities in movement and posture, usually attributable to disorders of the basal ganglia or extrapyramidal system) interictal EEG should be normal (Tarsy, 2003; Beaumanoir et al,1996; Nakahata et al,1992). The two dogs from our study exhibited changes in interictal EEG, and consequently, episodes were interpreted as seizures instead of paroxysmal movement disorders.

Regarding the interictal EEG changes, the present study found that they were present in 6 puppies (85.7%). Similar results obtained Jokinen et al (2007) who observed epileptiform activity in 87.5% (14 of 16) of affected Lagotto Romagnolo puppies that suffered from benign familial juvenile epilepsy. The literature thus far investigating diagnostic value of EEG in idiopathic epilepsy, observed that epileptic animals exhibit EEG abnormalities during seizures, but interictally EEG can be normal (Klemm, 1989; Jaggy et al, 1998). The same situation is encountered in children with epilepsy where in spite of using of different activation techniques to enhance the diagnostic value of EEG (photic stimulation, hyperventilation, sleep deprivation), the rate of EEG detection is lower. Betting et al, (2006) observed that up to a half of patients do not show any epileptiform EEG abnormalties and Labate et al (2007) using EEG recording in the morning and intermittent photic stimulation, obtained a superior percentage (69% of children) in detecting generalized epileptiform discharges in patients with juvenile myoclonic epilepsy.

Conclusions

1) EEG findings of juvenile epilepsy of dogs were similar with childhood epilepsy, consisting in single spikes, polyspikes, sharp waves and multiple sharp wave complexes.

2) EEG can be valuable examination in confirming the diagnosis of canine epilepsy, especially when clinically it is not possible to differentiate a seizure from movement disorder.

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EFFICIENSY OF PUERPERAL ENDOMETRITIS THERAPY IN COW, BY USING THREE DIFFERENT PROTOCOLS

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Abstract

In the reproduction field, it is known that the puerperium represents a critical stage, because in this period most reproductive disorders occur, that can subsequently cause temporary or permanent infertility. It requires a focus on this period. The aim of this paper is the efficiensy of puerperal endometritis therapy by using three different protocols. All therapeutic variants are applicable and have shown effectiveness in combating endometritis in cow, in proportions ranging from 69.23% clinical cure for the PG and antibiotics protocole and 92.86% clinical cure in eficur and hipophysin protocole.

Key words*: cow, endometritis, puerperium, economical efficiency.*

Puerperal disorders occur due to predisposing and determinant factors. The predisposing factors can be dietary factors, metabolic (by weakening the body's defense system), hormonal disorder that can cause infections: hiperestrogenia, hiperprogesteronemia, estrogen-progesterone imbalance (4,8).

Inflammatory disorders of female genital tract represent a determinant factor in the profitability of all cattle farms, an important factor that can hinder the achievement of every farmer objectives to obtain a viable product (calf) each year, respectively a CI of maximum of 365 days (1,3).

Endmetritis as systemic disorder, has negative effects on production and reproduction in dairy cows. These diseases of the reproductive system in the puerperium period constitutes an important risk regarding the efficiency of breeding. The risk of clinical endometritis increases with the dystociccalvings and with the affections occurring in the puerperium, a clinical endometritis has negative effects on the effectiveness of reproductive indices, and a high risk for slaughter, even if it does not have a major effect on metabolic hormones (2,5,9).

Material and Methods

The research was conducted in a dairy cow farms breed Holsthein for Vaslui County, in the period 2013-2014 , on a group of 60 heads recently calved in February, March, April. Basically, a large number of cows on this farmdevelop various forms of puerperal endometritis. To solve this problem it has been designed a protocol for the evaluation of the streamlining of the injectable treatement with Epicur (cephapirine) SC associated with hypophysinLA(retard ocytocine), for the prevention, or at least, amelioration of the systemic illness associated with metritis.

Animals in which the diagnosis of acute endometritis was established were identified, and the diagnosis was recorded in the register of treatments and gynecological records.

Table 1. Experimental groups and the therapy of different puerperal endometritis in cows

Group	Treatement	Number of treated animals
E1	EFICUR + HYPOPHYSIN	14
E2	OZONE SOLUTION	15
E3	PG + ANTIBIOTICS	13
E4	CONTROL GROUP	13

Four groups of 15 heads of animals recently calved have been formed, for the application of three different therapeutic choises. Animal groups were numbered from1 to 4, setting a specific therapeutic protocolfor each group. Not all the animals taken in this study have completed lactationdue to various diseasesthat generated their removal from the herd.

For the E1 group, allanimals recently calved have had their temperature taken from the first day till the 5th day after calving, and the ones that had more than 38,8 °C have been treated for 3-4 days with eficur SC (cephalosporine) and hypophyzin*LA IM (carbetocine) daily, untill the dropping of the temperature.

For the E2 group, the therapeutic protocol comprises administering IM a dose of PG (Dinolitic) at the begining of the treatement and intrauterine with the insemination pipette 80-100 ml of antibiotic solution (repeated at 48 hours). The final treatement contains: Negamicin 2.0 gr, 2.0 gr Enrofloxacin, Metronidazole 2.0 g, glucose 33% 10 ml,Water-soluble vitamin AD3E 10 ml, 100 ml saline ad, Mf Suspension, D.S. intrauterine.

Lot E4 shunt consists of cows with prolonged puerperium.

Results and discussion

The results of application of the therapeutic protocol for each group are shown in Table. 2.

Group E1: was diagnosed with genital disease and were treated with Eficur and Hypophysin, a number of 15 cows. However, none of the 15 animals studied were not systematically genital ill post partum. A bovine was effectively eliminated from the hip due to desmorexie. It took a number of medicinal applications 3.71 per cow.

Following treatment with eficur and hypophysin all 14 cows remained pregnant being recorded the following results: interval calving - first insemination 58.42 days; SP of 79.14 days, number IA 1.92 / gestation, CI 363.3 days.

Regarding the cost of treatment for the 14 animals in group E1 returned a cost of 98 USD / animal treated.

E2 group: genital diseases have been diagnosed and treated with ozone, a number of cows 15. It took a number of medicinal applications 3.2 per cow.

Following treatment with ozone all 15 cows remained pregnant being recorded the following results: 62.26 days interval calving first insemination; SP 85.8 days, number IA 2.13 / gestation, CI-369, 2 days.

Regarding the cost of treatment for the 15 animals treated in group E2 returned a cost of 20 USD / animal treated. The good results achieved in this group can not fully explain.
E3: was diagnosed with genital disease and were treated with antibiotics respectively intrauterine PG and 15 cows, two heads were slaughtered due to metabolic disorders.
It took a number of drugs per cow 3.5 applications.

Following treatment with antibiotics and PG 13 cows remained pregnant, being recorded the following results: 66.76 days interval calving first insemination; SP 102.46 days, number IA 2.61 / gestation, CI-386, 6 days.

E4: a number of 15 cows were diagnosticated with genital disorders, two heads were slaughtered because of the parameter.

Following results were recorded: interval calving first insemination 109.07 days, SP 153.92 days, number of IA 3.15 / gestation, CI-437 days (table 2).

Table 2. Reproductive indices and applying different treatment effectiveness, in acute endometritis in cow

Specification	Animals studied	Animals treated	Clinical healing		Pregnant IA1-IA3.		SP	IG	Repeted mating		Cost	Time
			After 21 days								animal	waiting
			total	%	total	%	zile	No.	total	%	RON	Hours .
E1	**15**	**14**	**13**	**92,86**	**12**	**85,71**	**79,14**	**1,92**	**2**	**14,29**	**98**	**0**
E2	**15**	**15**	**12**	**80**	**12**	**80.00**	85,8	**2,13**	**3**	**20**	**20**	**0.**
E3	**15**	**13**	**9**	**69,23**	**8**	**61,53**	102,46	2,61	**5**	**38,46**	**55**	**144**
E4	**15**	**0**	**5**	**38,46**	**51**	**49,04**	**153,92**	**3,15**	**4**	**30,76**	**X**	**X**
TOTAL	**60**	**42**	**39**	**70,13**	**81**	**69,07**	**105,33**	**2,45**	**14**	**28,95**		

Conclusions

1. All therapeutic options used are applicable and effective in combating endometritis in cows, in proportions ranging from 69.23% PG protocol clinical cure and clinical cure antibiotics and 92.86% for protocol with Hipophysin and Eficur.

2. The best results were obtained following the protocol with Eficur and Hipophysin (92.86%) clinically cured and 85.71% pregnant after the 3rd IA.

3. Even if the protocol applied with prostaglandins and antibiotics worked (69.23%) clinically cured and 61.53% were pregnant after the 3rd IA, the impediment created by delivery ban milk obtained from animals treated due the waiting time does not justify making this type of protocol.

4. Is observed a significant increase of pregnant animals remained after the performed treatments.

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RESEARCH ON THE EFFECTIVENESS OF OZONE THERAPY IN CHRONIC INFLAMMATORY DISEASES OF THE ENDOMETRIUM

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Abstract

*Chronic endometritis are occult forms and remittances, therefore they are particularly important since the disease may not be discovered until the time of oestrus, when it is recomended to performe the treatment too.Chronic inflammatory pathologies of the uterus are usually treated with antibiotics or hormones, alone or in association, and coupled with a flare phenomenon.The purpose of this study is to apply the new formula for treatment of chronic endometritis, based on the local action of ozone.Research focused on the application of comparative therapy in chronic endometritis in cows.The groups were homogeneous groups and treated as: G1 Lotagen + metricure, G2 Jodofoam + antibiotics, G3 Ozonated saline + marigold extract. Based on the results obtained after applying the three therapeutic protocols, all variants used are applicable and effective in combating endometritis in cows.. The results obtained with Ozone Protocol open new perspectives in the treatment of chronic uterine disorders***.***

Keywords: *Cow, chronic endometritis therapy, ozone*

Maternal changes during development of the conceptus (the major adaptive systems, metabolic, morphological), parturition with all the changes it produces,require, in post-natal period, numerous restructurings. (2,4,8) This period is called the puerperium, when it can manifest and develop gynecological disorders that can become permanent and that are difficult to treat (7).

Endometritis are inflammatory processes of the uterine lining, that occur frequently after birth,leading to a delay in the return of females to normal breeding activitythat have an effect on milk production too (1). Chronic endometritis have as symptomthe character of estrous leaks. Vaginal secretions are whitish and have formed from debris of ribbed tongue. These discharges occur during oestrus and disappear after heat. In the next cycle, the symptoms are similar, aspect of the sexual cycle that occurs more than one in a row. The specification of the seat of leakage is done by internal vaginal speculum exam, by observing the uterine neck ajar from which catarrhal secretions drain (3,5,9).

Due to its high oxidation potential, ozone oxidizes compounds of bacterial cell walls and thus penetrate into cells.After he came inside bacterial cells oxidize all its essential elements (enzymes, proteins, DNA). By these reasons, we try to apply new forms of therapy for endometritis in cows (3,7,10).

Material and methods

The research was conducted at a dairy farm in October 2012 - August 2014on a total of 43 cows inseminated with various forms of chronic endometritis.

It has beendeveloped a therapy protocol that included several different types of medicationLotagen and metricur, jodofoam, ozonated saline solution and masterly treatment with antibiotics.

Parameters measured morbidity, number of cows pregnant after the 3rd AI, seed number, servicing, number of cows to repeated matings, costs or expenses for treatment of cows for milk waiting time.Pregnancy diagnosis was performed using an ultrasound score Tring; 30 days after sowing.

Four groups of animals with chronic endometritis were formed for the application of four different therapeutic protocols. The groups of cows with chronic endometritis have been numbered from 1 to 4, each group being assigned with a particular treatment protocol.

For the group G1 therapeutic protocol consisted of administration by intrauterine insemination pipette attached to a syringe of 100 mL, an amount of 50-80 ml lotagen(Composition: 36% solution of sulfonated cresol with methanol)solution 4% based on the ability of the uterus rebound effect aimed at chronic disease, and 48 hours later received a dose of metricur IUD (500 mg cephapirin).

For the group G2 treatment protocol consisted on administration by intrauterine insemination pipette to a dose of spray depending on capacity jodofoam uterus aimed rebound effect of chronic disease, and 48 hours was given a dose of antibiotics in utero.

For the group G3 treatment protocol consisted on the administration by intrauterine insemination pipette attached to a syringe of 100 mL, a volume of 50-80 mL of solution of Calendullaofficinalis extract (marigold) in 10 ml ozonated saline solution + 90 mL repeated at 48 hours.Ozone was produced with an H CLEAN unit 203, the oxygen was taken from a cylinder of medical oxygen. Group G4: Control group consisting of cows in puerperium without therapy.

Results and discussion

In Table 1 are numerically lots constituted , therewere studied in total43 cows with chronic endometritis.

Table no. 1

Group	Treatment	No of treated animals
G1	Lotagen+metricure	10
G2	Jodofoam+antibiotics	10
G3	Ozonated saline solution+marigold extract.	11
G4	Control group	12
Total animals seeded =43		31treated.

The animals were examined during estrus gynecological year, those with induced estrus and those with physiological estrus as well. On this occasion, it was appreciated the presence or absence of genital secretions and their characteristics, volume, consistency and responsiveness of the uterus and ovarian activity. Inseminated animals were clinically cured at the entrance of heat, only when the estrous mucus examination did not reveal any pathological aspect.

The results of applying therapeutic protocol every batch are shown in tables No. 2, 3, 4, 5.

Group G1: was diagnosed with genital disease and 10 cowswere treated with lotagen and metricure. It has been used in an average1,2 medications per cow (Table No. 2. Group.-1).

After the treatement was taken, 9 cow remained pregnant after 1-3 AI, and a cow at the 4th AI, interval calving- first insemination was 59.4 days, 79.1 days SP average number of AI on gestation 1.9, CI - 363 days.

Regarding the cost of treatment for the 10 animals in group G1 returned a cost of 35 lei / treated animal.

Group G2: was diagnosed with genital disease and 10 cows were treated with antibiotics jodofoam. It took an average of 1.8 applications of drugs per cow (Table 3. Group-2).

After the treatment, 5 cows remained pregnant after 1-3 AI and 3 cows at the 4th AI, interval calving- first insemination was 89.3 days, SP 124.2 days, average number of AI on gestation 2.7, CI-408.5 days.

Regarding the cost of treatment for the 10 animals in group G2, 28lei/animal was the cost oftreatment.

G3: was diagnosed with genital disease and 11 cows were treated with ozonated saline solution. It took on average 2.9 applicationsbofdrug per cow (Table 3. Group.-3).

After taking the treatment, nine cows remained pregnant after 1-3 AI, and 2 cows in the 4th IA, calving- first insemination interval was 78.72 days, SP 102.2 days, the average number of AI per pregnancy 2 .3, Cl - 386.2 days.

Regarding the cost of treatment for the 11 cowsfrom G3it could not be calculated. The good results achieved in this group can notbe fully explained.

Group G4: was diagnosed with genital disease 12 cows (Table No.4. Group.-4)

After treatment all 8 cows remained pregnant after 1-3 AI and 4 cows at the 4th-AI, the first seed calving interval was 95.6 days, the SP 143.16 days, the average number of BI gestation 3.16, CI - 427.1 days.

Table 1. breeding indices obtained by treatment of group G1 (LOAGEN AND METRICURE)

No	Reg No.	Parturition Date	No of treatements	Date of first AI	Calving interval first AI (days)	Date of ferile AI	Calving interval Fertile AI (days)	Gestation Index (n)	Gestation period (days)	Calving Interval (days)
1	4717	02.11.2012	1	01.01.2013	61	01.01.2013	61	1	281	342
2	6525	06.11.2012	1	05.01.2013	62	26.01.2013	84	2	288	372
3	6823	06.11.2012	1	03.01.2013	59	03.01.2013	59	1	287	346
4	302	09.11.2012	1	02.01.2013	55	22.01.2013	76	2	280	356
5	2750	12.11.2012	2	04.01.2013	54	12.03.2013	122	4	279	401
6	5976	17.11.2012	1	04.01.2013	59	06.01.2013	81	2	284	365
7	4720	22.11.2012	1	25.01.2013	65	25.01.2013	65	1	291	356
8	5975	27.11.2012	2	25.01.2013	60	28.03.2013	103	3	282	385
9	4516	03.12.2012	1	30.01.2013	58	20.02.2013	79	2	285	364
10	6105	05.12.2012	1	04.02.2013	61	04.02.2013	61	1	283	344
total			1,2		59.4		79,1	1,9	284	363

Table 2. Indices of reproduction obtained by batch G2 JODOFOAM therapy and antibiotics

No.	Registration No.	Parturition Date	No of treatements	Date of First AI	Calving interval first AI (days)	Date of ferile AI	Calving interval Fertile AI (days)	Gestation Index (n)	Time of Gestation (days)	Calving Interval (days)
1	7447	**06.12.2012**	**1**	**02.03.2013**	**87**	**23.03.2013**	**108**	**2**	**281**	**389**
2	4600	**06.12.2012**	**3**	**07.03.2013**	**92**	**10.05.2013**	**156**	**4**	**285**	**441**
3	6829	**10.12.2012**	**2**	**09.03.2013**	**90**	**01.04.2013**	**113**	**2**	**287**	**400**
4	3290	**10.12.2012**	**1**	**06.03.2013**	**87**	**28.03.2013**	**107**	**2**	**283**	**390**
5	6819	**12.12.2012**	**3**	**21.03.2013**	**100**	**15.05.2013**	**155**	**4**	**279**	**434**
6	1883	**15.12.2012**	**1**	**13.03.2013**	**89**	**02.04.2013**	**109**	**2**	**287**	**396**
7	6766	**24.12.2012**	**1**	**15.03.2013**	**82**	**06.04.2013**	**104**	**2**	**290**	**394**
8	5852	**29.12.2012**	**3**	**07.04.2013**	**100**	**05.06.2013**	**159**	**4**	**281**	**440**
9	3297	**03.01.2013**	**1**	**27.03.2013**	**84**	**19.04.2013**	**107**	**2**	**286**	**393**
10	1884	**04.01.2013**	**2**	**26.03.2013**	**82**	**07.05.2013**	**124**	**3**	**284**	**408**
total			**1,8**		**89,3**		**124,2**	**2,7.**	**284,3**	**408,5**

Table 3. Reproductive indices produced by ozone therapy for group G3

No	Reg. No	Date Of Parturition	No. of treatments	Date of first AI	Calving interval First day of AI (days)	Date of Fertile AI	Calving interval Fertile AI(days).	Gestation index (n)	Time Of Gestation (days)	Calving Interval (days)
1	**3300**	**06.01.2013**	**3**	**18.03.2013**	**72**	**07.04.2013**	**92**	**2**	**288**	**380**
2	**2227**	**09.01.2013**	**3**	**01.04.2013**	**83**	**22.04.2013**	**104**	**2**	**279**	**383**
3	**5897**	**12.01.2013**	**2**	**03.04.2013**	**82**	**03.04.2013**	**82**	**1**	**284**	**366**
4	**5896**	**16.01.2013**	**3**	**11.04.2013**	**86**	**02.05.2013**	**107**	**2**	**280**	**387**
5	**4508**	**18.01.2013**	**4**	**02.04.2013**	**75**	**08.06.2013**	**142**	**4**	**285**	**427**
6	**6136**	**18.01.2013**	**3**	**04.04.2013**	**77**	**25.04.2013**	**98**	**2**	**284**	**382**
7	**6827**	**28.01.2013**	**2**	**22.04.2013**	**85**	**22.04.2013**	**85**	**1**	**290**	**375**
8	**6117**	**28.01.2013**	**4**	**14.04.2013**	**77**	**19.06.2013**	**143**	**4**	**283**	**426**
9	**5212**	**03.02.2013**	**3**	**19.04.2013**	**76**	**10.05.2013**	**97**	**2**	**282**	**379**
10	**6113**	**04.02.2013**	**2**	**21.04.2013**	**77**	**21.04.2013**	**77**	**1**	**283**	**360**
11	**8920**	**07.02.2013**	**3**	**23.04.2013**	**76**	**15.05.2013**	**98**	**2**	**286**	**384**
total			**2,9**		**78,72**		**102,2**	**2,3**	**284**	**386,2**

Table 4. Reproductive indices obtained in the control group

No.	Reg. No.	Date of parturition	Date of first AI	Calving interval first AI(days)	Date Of Fertile AI	Calving interval Fertile AI(days).	Gestation Index (n)	Time Of gestation (days)	Calving interval(days)
1	**5977**	**26.10.2012**	**04.02.2013**	**101**	**16.03.2013**	**142**	**3**	**287**	**429**
2	**8921**	**06.11.2012**	**05.02.2013**	**91**	**08.04.2013**	**164**	**4**	**278**	**442**
3	**4529**	**10.11.2012**	**06.02.2013**	**88**	**29.03.2013**	**140**	**3**	**283**	**423**
4	**8924**	**27.11.2012**	**26.02.2013**	**91**	**28.04.2013**	**153**	**4**	**280**	**433**
5	**6765**	**12.12.2012**	**28.03.2013**	**107**	**28.05.2013**	**168**	**4**	**281**	**449**
6	**322**	**15.12.2012**	**06.03.2013**	**82**	**26.04.2013**	**133**	**3**	**284**	**417**
7	**4528**	**23.12.2012**	**01.04.2013**	**100**	**13.05.2013**	**142**	**3**	**292**	**434**
8	**329**	**02.01.2013**	**07.04.2013**	**96**	**09.06.2013**	**159**	**4**	**283**	**442**

9	**9858**	**03.01.2013**	**06.04.2013**	**94**	**18.05.2013**	**137**	**3**	**284**	**421**
10	**321**	**04.01.2013**	**17.04.2013**	**104**	**29.05.2013**	**146**	**3**	**283**	**429**
11	**331**	**16.01.2013**	**23.04.2013**	**98**	**14.05.2013**	**119**	**2**	286	**405**
12	**332**	**27.01.2013**	**01.05.2013**	**95**	**21.05.2013**	**115**	**2**	**287**	**402**
TOTAL				**95,6**		**143,16**	**3,16**	**284**	**427,1**

Conclusions

There is a significant increase in SP for pregnant cows after iodofoam and antibioticsperformed treatments and for those untreated.

Based on the results obtained after applying the three therapeutic protocols in cows with endometritis the following conclusions can be drawned:

1. All therapeutic options used are applicable and effective in combating endometritis in cows.
2. The best results were obtained following the protocol with lotagen and metricur.
3. Even if the protocol applied with jodofoam and antibiotics gave results , it has generated impediment by prohibiting the delivery of milk obtained from animals treated with the type of waiting does , this does not justify performing this type of protocol.
4. The results obtained with Ozone Protocol open new perspectives in the treatment of chronic uterine disorders.

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PREVALENCE OF INTERNAL PARASITES IN WISENTS (*BISON BONASUS BONASUS*) FROM TWO ROMANIAN RESERVATIONS - PRELIMINARY RESULTS

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Abstract

The wisent (Bison bonasus bonasus) or European bison is a Eurasian species of bison. At present, the wisent is listed as a vulnerable species. In Romania there are several natural reserves in Neamț, Brașov, Dâmbovița, Hunedoara or Caraș-Severin counties which tries hardly to preserve this species. This study was carried out on 23 faeces samples collected from Hațeg (Hunedoara County) and from the newest reservation Armeniș-Plopu (Caraș-Severin County). 17 samples came from wisents of different European countries (9 from Sweden, 4 from Germany, 2 from Italy, and 2 from Belgium) which were moved to the new reservation Armeniș-Plopu from Caraș-Severin County and 6 samples from wisents from Hațeg, respectively. 13/23 (56.52%) wisents excreted Eimeria spp. oocysts, 9/23 (39.13%) harboured gastrointestinal nematodes, 6/23 (26.09%) were infested by Strongyloides spp. and 2/23 (8.69%) expelled tapeworm eggs. 8/23 (34.78%) wisents were parasites free, but 10/23 (43.48%) of them were infested by at least 2 species of parasites. All samples collected from Hațeg were positive.

***Key words**: Romania, wisents, internal parasites, prevalence.*

The wisent or European bison (*Bison bonasus bonasus*), the largest herbivorous animal in Europe, very common in the Carpathian region during Middle Ages, finally disappeared there by late XVIIIth century.

Nowadays, the wisent is protected both by international and national laws, because it is a vulnerable species. The most individuals are living in Poland, in wild or enclosed, but also in other European countries such as Spain, Denmark, Germany, Sweden, Ukraine or Russia (Bauer, 2009; Moran, 2013; Perzanowski and Olech, 2013; Moskwa et al., 2014).

In Romania, wisents returned in 1958 from Poland. Now, there are several natural reserves in Neamț, Brașov, Dâmbovița, Hunedoara or, more recently, Caraș-Severin counties.

Being grazing animals, wisents are exposed to gastrointestinal nematode or other parasite infestations, but there is a lack of information on internal parasites of these herbivores in Romania.

This paper aims to identify the endoparasitic fauna of wisents from two Romanian reservations: Hațeg-Slivuț and Armeniș-Plopu.

Materials and methods

Approximately 200 grams of fresh faeces samples were collected individually from transporting trucks, when wisents arrived in Armeniș-Plopu reservation. For wisents took into account from Hațeg-Slivuț, fresh faeces samples were collected from the ground, after observation of defecation. Wisents aged between 1 and 12 years.

Samples were collected in plastic bags and stored at 4ºC till they were processed in the parasitic diseases lab of Faculty of Veterinary Medicine Timișoara. Willis, modified McMaster, Baermann and sedimentation methods were used for samples analyze. Results are listed in Table 1.

Table 1. Endoparasitic fauna in wisents from Armeniș-Plopu and Hațeg-Slivuț reserves

Crt. no.	Name	Sex	Country	Coproscopic exam
1.	Egomundis	F	Germany	*Eimeria* spp.
2.	Eggemadel	F	Germany	*Eimeria* spp.
3.	Spomenka	F	Germany	*Eimeria* spp.
4.	Spiderwoman	F	Germany	-
5.	BBB029	M	Italy	*Eimeria* spp.
6.	BBB028	M	Italy	-
7.	Swan	F	Sweden	GIN eggs
8.	Isabell	F	Sweden	GIN eggs; *Eimeria* spp.
9.	Birk	M	Sweden	GIN eggs
10.	Sussie	F	Sweden	-
11.	Isa	F	Sweden	-
12.	Hilda	F	Sweden	-
13.	Mildred	F	Sweden	-
14.	Terese	F	Sweden	-
15.	Sabina	F	Sweden	-
16.	Zwitcher	M	Belgium	*Eimeria* spp.; tapeworm eggs
17.	Zwanda	F	Belgium	*Eimeria* spp.; tapeworm eggs
18.	Romanița	F	Romania	GIN eggs; *Eimeria* spp.; *Strongyloides* spp.
19.	Wisent 1	F	Romania	GIN eggs; *Eimeria* spp.; *Strongyloides* spp.
20.	Wisent 2	F	Romania	GIN eggs; *Eimeria* spp.; *Strongyloides* spp.
21.	Wisent 3	M	Romania	GIN eggs; *Eimeria* spp.; *Strongyloides* spp.
22.	Wisent 4	F	Romania	GIN eggs; *Eimeria* spp.; *Strongyloides* spp.
23.	Wisent 5	F	Romania	GIN eggs; *Eimeria* spp.; *Strongyloides* spp.

GIN = gastrointestinal nematodes

Results and discussions

Results recorded in Table 1 shown that all wisents (100%) from Haţeg-Slivuţ reserve were infested, while only 52.94% (9/17) of wisents moved to Armeniş-Plopu one were infested.

Parasites belonging to three classes (Protozoa, Cestoda and Nematoda) were identified. No parasitic elements were noticed by Baermann or sedimentation techniques.

13/23 (56.52%) wisents excreted *Eimeria* spp. oocysts, 9/23 (39.13%) harboured gastrointestinal nematodes, 6/23 (26.09%) were infested by *Strongyloides* spp. and 2/23 (8.69%) expelled tapeworm eggs. 8/23 (34.78%) wisents were parasites free, but 10/23 (43.48%) of them were infested by at least 2 species of parasites.

The most intensive studies on wisent endoparasitic fauna were carried out in Poland during the last decades, but also in Russia or Czech Republic (Drożdż, 1961; 1967; Drożdż et al., 1998; 2002; Kotrla et al., 1976; Kotrla and Kotrly, 1977; Osinska et al., 2010).

During these studies, in Poland were identified 3 protozoan species, 4 trematodes species, 6 tapeworm species (both metacestodes and adults), and 38 nematodes species. Nematodes of the *Trichostrongylidae* family were most prevalent (98%), followed by *Fasciola hepatica* (53.8%), *Paramphistomum* spp. (45.1%) and *Dictyocaulus viviparus* (30.7%).

In the last few years, the *Ashworthius sidemi*, a blood-sucking nematode of the Trichostrongylidae family, emerged as highly pathogenic in wisents (Moskwa et al., 2014). This infestation was possible because wisents are grazing on same pastures with sheep and cattle, but also with red deer and roe deer, hereby facilitating the interspecific transmission of parasites.

This study on diagnosis of gastrointestinal nematodes in wisent from Romanian reserves stands mainly on coproscopical examination, but different strongylid parasite species require different interpretations of faecal egg counts. More than that, faecal egg counts cannot be directly correlated with worm burdens, as they could not be identified to genus or species (Irvine et al., 2006).

Because of the scarcity of information concerning endoparasitic fauna in wisents from Romania, further studies are required.

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THE SIGNIFICANCE OF TECHNOLOGICAL FLOW IN TRANSMISSION OF SALMONELLA AT PIGS

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Abstract
The present study aimed the microbial load of raw materials from the combined feed, from the FNC (mixed fodder factory) and those from the feeders. In parallel, fecal samples were collected from sows and piglets of the second week of life to the finish. The recorded values which ranged between 10% and 14% for raw material analysed, but for all the finished feed samples the results was negative, being similar to those obtained from samples taken from bunkers. For the feed taken from the farm (feeder) the values ranged between 5% to 7.1% due to the presence of bearing animals and the vectors. Regarding the results obtained from the examination of faces samples it was found that the number of positive samples increased after pigs transfer to the fattening shelters, noting a number of 44% positive samples from sows and their piglets, an easy growth at pigs for weaning (55%) and 66% in fattening pigs.
Key words: *faces, feeding, raw material, pigs, samples.*

Introduction

In animal production, feed is a potential pathway by which *Salmonella spp.* may be introduced into farm livestock (3). For pigs, contaminated feed and newly introduced pigs are considered the most significant sources of infection (11). Young weaning pigs are most often affected by *Salmonella spp*. infection and in general, the infection causes disease. Adult pigs are however also susceptible to infection (15). Berends et al. (1996) estimated that about 15–30% of all infections in the finishing period may be attributed to (re)contaminated feed (2). Adult pigs rarely show clinical signs, thus undetected *Salmonella* carriers can enter the slaughtering process (10). It was shown that live animals carrying *Salmonella* are three to four times more likely to result in contaminated carcasses than *Salmonella-free* animals (2). The production of safe feed is thus the first step for ensuring safe food (5). *Salmonella* can be isolated regularly from vegetal feed ingredients for farm animals (10), as well as in the finished feeds (13). Since all raw feed components must be considered as a potential source of *Salmonella*, process control and decontamination steps are essential to avoid spread of contaminated feed to herds (14). Diverse process steps aimed at reducing or eliminating a contamination with *Salmonella* are available, mainly: the implementation of a heat treatment, the use of organic acids (1).

Materials and methods

An assessment of the situation was made, with reference to carrier status by collecting and analyzing the data about the microbial load of the feed in various stages of the technological process. 100 samples were taken, raw material (maize, soybean meal, sunflower meal, barley) before processing.

To highlight the importance of HACCP system and last but not least, the simplest possibility to achieve an effective control of biosecurity in the compound feed production sector was achieved by using Sal Curb Dry (product based by using various combinations of

organic acids and salts of these acids) directly in the mixed fodder (doses of 4 kg/tonne) after a well established manufacturing program of such nature that could "cover" the entire farm.

Later it was followed the frequency and level of recontamination of finished feed in the feeding bunkers, by taking the same number of samples (n=100). At the same time from the pigs farms were collected (n=50) faecal samples from sows and piglets of the second week of life to the finish.

Samples were collected in sterile containers and transported to the laboratory of Hygiene and analyzed by method SR EN ISO 6579:2002.

Results and discussion

After analyzing the materials before placing them in the technological flow, were found values that ranged between 10% and 14%.

These values are in line relative to those obtained by other similar studies (7, 9). By applying a technological flow to the product Sal Curb Dry, product that favors decontamination of both combined feed and later the bunkers of finished product, transportation vehicles, it was found that the feed is free of *Salmonella spp*.(figure 1)

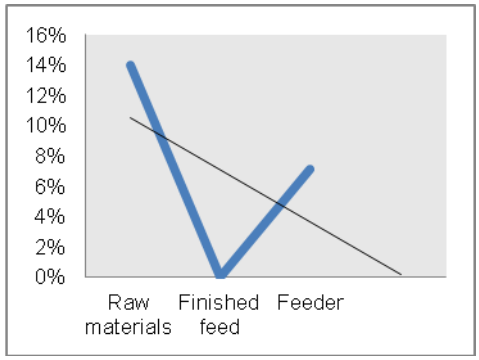

Fig 1. The presence of *Salmonella spp.* in feed

Based on observations regarding the cargo of *Salmonella* microorganisms in raw materials, Cooke (2002), believes that decontamination methods resulting a reduction of 3 log by load will eliminate the presence of *Salmonella* in forage (4).

For the decontamination of the feed to be a practical method for reducing the risk of *Salmonella* and the state of carrier, eliminating the contamination degree during the processing of the feed, must be supported during transport and delivery to the farms. In the case of Salinpork project that involved five European countries (12), before the distribution of feed in pigs farms, it was found a prevalence of 6.9% (4)

Points of recontamination are: dispensers and place of distribution (7, 9), to which is add the transportation (8), and into the farm the manner and type of storage(6).

The results obtained in the samples of the finished feeding from the bunkers showed positive samples between 5% and 7.1%, which is atributable to the state of carrier to the vectors (rats).

In farms, after analyzing the colected samples from sows and piglets to weaning, was found a prevalence of 44% positive samples.

Continuing the analysis on other stages of growth, at piglets after weaning, there was a prevalence of *Salmonella spp*. of 55%, and finally before delivery the same herd reached 66% positive samples.

Conclusions

- Raw materials had a microbial load between 10% and 14%;
- The using of Sal Curb Dry with HACCP procedures, ensures finished feed free of germs;
- After the storage, depending by time, the finished feeding were positive between 5% and 7.1%;
- After the allotments, cross-contamination were made which determined values of positive samples from 44% to maternity, 55% at weaning and 66% at the end.

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PREVALENCE OF PARASITISM WITH *CYATHOSTOMUM SPP.* FROM WESTERN ROMANIA

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Abstract

The cyathostomins (small strongyles) are the most common parasite found in horses. They were identified in the majority of horses examined, in proportion of 70% to 100%, each horse having more than one species of Cyathostomum. First of all the species of parasite must be accurately identify prior to evaluate the prevalence of cyathostomins. The study was performed on 50 horse faeces samples from Western Romania. Larval cultures were performed to identify Cyathostomum subtypes. The larvae were identified with key proposed by Madeira de Carvalho. Five subtypes (Cyathostomum A, B, C, D, F) were found and the most prevalent subtype was Cyathostomum A (56%), followed by subtypes D (40%), C (24%), B (14%), and F (6%).

Key words: *prevalence, cyathostomins, horses.*

Introduction

The cyathostomins (small strongyles) are the most common parasite found in horses. They were identified in the majority of horses examined, in proportion of 70% to 100%, each horse having more than one species of Cyathostomum.

The importance of this parasites is given by larval cyathostominosis, a syndrome associated with weight loss and severe diarrhoea, caused by the mass reactivation of inhibited cyathostomin larvae residing in the instestinal mucosa (Giles et al., 1985, Kelly and Fogarty, 1993, Osterman Lind et al., 2003).

The aim of the study was to find the cyathostomins prevalence in horses from western Romania.

Materials and methods

The study was conducted from December 2013 to April 2014 on 50 horses with age ranged 1-28, from seven farms in western Romania. All 50 horses have been dewormed one month previously the faecal samples were collected. Anthelmintic treatment included use of ivermectin exclusively, fenbendazole exclusively or ivermectin with praziquantel.

First of all the species of parasite must be accurately identify prior to evaluate the prevalence of cyathostomins. Faecal samples were collected from 50 horses, as freshly expelled faeces off the ground. A McMaster test was used to determine strongyle egg counts. The larval cultures procedures were performed according to Roberts and O'Sullivan (1950). After seven-ten days the larvae were collected by the Baermann technique and identified according to Madeira de Carvalho (1991, 1999).

Results and discussion

Data on examination of faeces samples for parasite eggs, eggs per gram of faeces (EPG) and the identification of the subtypes of *Cyathostomum* by larval cultures, are listed in Table 1. Five horses out of 50 had negative counts on McMaster test, but at the qualitative examination strongyle eggs were present.

The larvae found and identified were: *Cyathostomum* A, B, C, D, F and *Gyalocephalus capitatus.*

The prevalence of the *Cyathostomum* subtypes was: *Cyathostomum* A-56%, D-40%, C-24%, B-14% and F-6%. *Gyalocephalus capitatus* prevalence was 8% (Fig. 1).

Table 1. The number of EPG and the *Cyathostomum* subtypes found in faeces samples from horses.

Farm	Horse	EPG	*Cyathostomum subtypes*
Farm 1	1	150	A
	2	150	D
	3	550	C
	4	1100	A, D
	5	1200	A
	6	1850	D
	7	3750	A
	8	1700	D
	9	2150	C
	10	500	A
	11	1050	A
	12	950	C
	13	700	A
	14	750	A, D
	15	1200	A
Farm 2	1	1950	D
	2	700	A
	3	550	D
	4	550	A
	5	1250	A
	6	2500	D
	7	1350	D
Farm 3	N 3249-7	1095	A
	3241	945	C, D
	3218	270	D, F
	2763	345	A, D, *Gyalocephalus capitatus*
Farm 4	Dove	420	A, C, D
	Tsunami	480	A, B
Farm 5	1	240	A, C
Farm 6	1	150	A, D
	2	300	A, D
	3	1100	A, D, C
	4	350	A, C, B
	5	1050	A, D, C, B
	6	1600	D, B, F, *Gyalocephalus capitatus*
	7	1150	A, C, *Gyalocephalus capitatus*
	8	50	A, C
	9	500	A, B
	Janett	50	A, F
	Derby	-*	-
	Vera	150	A, C

Farm 7	Maxim	-*	-
	Tiganca	-*	-
	Donna	50	B, D
	Destin	50	C
	Tora	50	A, D
	Xena	150	B, C
	Lucky	-*	-
	Mendoza	-*	-
	Bella	50	F, *Gyalocephalus capitatus*

* - Positive on qualitative examination

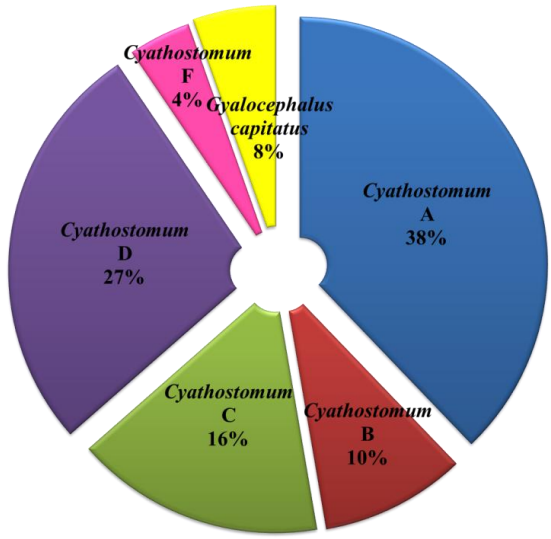

Fig. 1. The prevalence of *Cyathostomum spp*. in western Romania

In subtype A are included the species: *Cylicocyclus insigne, C. nassatus, C. radiatus, Cylicostephanus minutus, P. poculatus, Cyathostomum catinatum, C. Pateratum*; subtype B: *Cylicocyclus brevicapsulatus, C. ultrajectinus, Cylicondontophorus bicoronatus*; subtype C: *Cylicostephanus calicatus, C. Hybridus*; subtype D: *Cylicocyclus ashworthi, C. insigne, Cylicostephanus longibursatus, Coronocyclus coronatus*.

In Romania, at Bazosul Nou, Morariu et al., (2007), have identified the following cyathostomins: *Cyathostomum catinatum* 25%, *Cyathostomum pateratum* 7.30%, *Cyathostomum tetracanthum* 2.08%, *Cylicocyclus brevicapsulatus* 12.50%, *Cylicocyclus insigne* 8.33%, *Cylicocyclus leptostomum* 1.04%, *Cylicocyclus nassatus* 7.30%, *Cylicocyclus radiatus* 2.08%, *Cylicostephanus calicatus* 3.12%, *Cylicostephanus goldi* 5.21%, *Gyalocephalus capitatus* 2.08%, *Parapoteriostomum mettami* 2.08% and *Petrovinema poculatum* 3.12%.

Osterman Lind et al. (2003) have found 15 cyathostomins species and the six most prevalent species were: *Cylicostephanus longibursatus, Cylicocyclus nassatus, Cyathostomum catinatum, Cylicocyclus leptostomum, Cylicostephanus minutus* and *Cylicostephanus calicatus*; they represents 91% of the total worm burden.

In another study Traversa et al. (2010) have identified the five most prevalent cyathostomins species in Italy, United Kingdom and Germany: *Cylicocylus nassatus* (87.2%), *Cylicostephanus longibursatus* (86.2%), *Cyathostomum catinatum* (81.3%), *Cylicostephanus goldi* (78.4%) and *Cyathostomum pateratum* (75.5%), with the exception of *C. insigne* , which had the same prevalence with *C. pateratum* in Italy and *C. longibursatus in UK*, and of *C. ashworthi* which was more prevalent than *C. catinatum* in Germany.

Kuzmina et al. (2011) have identified the prevalence for cyathostomins in Poland and Ukraine. In Poland five species were prevalent: *Cyathostomum catinatum*, *Cylicocyclus nassatus*, *Cylicostephanus longibursatus*, *C. goldi* and *Cyathostomum pateratum*. In Ukraine the five most prevalent species were: *Cylicocyclus nassatus*, *Cylicostephanus longibursatus*, *Cyathostomum catinatum*, *Cylicostephanus calicatus* and *Coronocyclus coronatus*.

In Central Kentucky, Lyons and Tolliver (2013) have identified 12 species of small strongyles in four weanling horses treated with ivermectin. The first three prevalent species were: *Cyathostomum catinatum, Cylicocyclus nassatus, Cylicostephanus calicatus*, and the other nine species: *Coronocyclus coronatus, C. labiatus, C. labratus, Cyathostomum pateratum, Cylicocyclus ashworthi, C. leptostomum, Cylicostephanus goldi, C. longibursatus, C. minutus*.

Conclusions

The prevalence of the *Cyathostomum* subtypes was: *Cyathostomum* A-56%, D-40%, C-24%, B-14% and F-6%. *Gyalocephalus capitatus* had 8% prevalence.

Cyathostomum catinatum, Cylicocylus nassatus, *Cylicostephanus longibursatus* are among the most prevalent species worldwide, species identified also in this study.

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SENSITIVITY VALUES OF MICROFLORA CARCASSES OF SHEEP AND PIGS TO SOME ANTIBIOTICS

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Abstract

Globalization of the food chain causes constant new challenges and risks for health and consumer interests. The main objective of food safety is currently achieving the highest possible level of health protection human consumer interests in relation to food. For this reason the study aimed at assessing the presence and diversity of microorganisms in pig and sheep carcasses during trading and the sensitivity of isolated microbial flora to some of frequently used antibiotics for animal treatment. As a result of investigation it was determined that the risk of carcasses contamination is more common bacterial flora of the external order and takes place immediately after the slaughter of the animal. A higher sensitivity of isolated micro flora from sheep carcasses was observed against enrofloxacin and oxytetracycline, which resin area was more than 25mm.

***Key words** : contamination, insemination, antibiograma, antibiotics, sample, carcasses.*

Introduction

Global food security policy has increased considerably following the maximum level of safety of products of animal origin intended for human consumption. Due to the current trend of consumption of fresh foods, nutritional and sensory properties constant close to those of natural products, there is concern at the industrial level for obtaining products with minimal processing, the application of heat treatment reduced or even eliminate them. Instead, these products can be microbiologically unstable. Food quality and safety is based on the efforts of all those involved in the complex chain that includes agricultural production, processing, transportation and consumption [3].

Basic principles of food safety is an integrated approach, such as "farm to fork", including all sectors of the food chain as: feed production, plant and animal health, animal welfare, primary production, food processing, storage, transportation, marketing and imports and export them. Globalization of the food chain causes constant new challenges and risks for health and consumer interests. Main objective now on food safety is to achieve the highest possible degree of protection of consumers' interests humane on the food.

The risk of food being contaminated with chemicals or microorganisms exist throughout the food chain, from these considerations, namely intake veterinary food control along the entire technological flow and trading up to increase considerably and requires a mandatory step in maintaining innocuousness food and ensuring public health [2,7].

Food quality and safety is based on the efforts of all those involved in the complex chain that includes agricultural production, processing, transportation and consumption. According to the European Union and the World Health Organization - food safety is everyone's responsibility begins at their origin until the time they reach the table. To maintain food quality and safety throughout the chain reminded both procedures needed to ensure that food is integral and monitoring procedures to ensure outworking operations smoothly. A special role in maintaining food quality has hygienic status of trading venues, namely maintaining sanitation halls to achieve carcasses and meat products to prevent and minimize the risk of infections and food-borne toxins [3,5]. The purpose of the investigation

that we conducted was to determine the dynamics of the microbial load of pig and sheep carcasses and determine how isolated micro flora sensitivity to some antibiotics commonly used to treat sick animals with infectious diseases of bacterial origin.

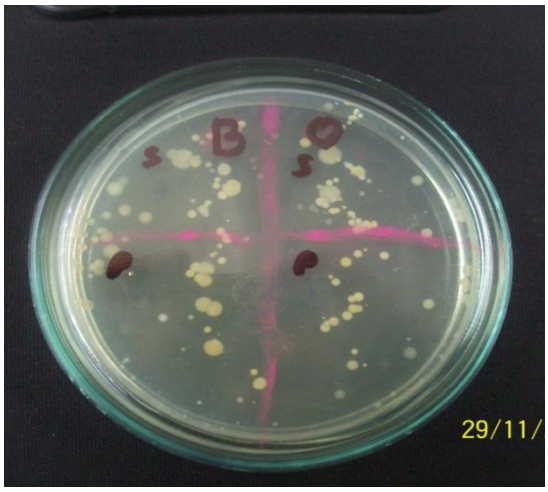
Material and methods

The investigations were carried in the laboratory of microbiology of the Department Epizootology, faculty of Veterinary Medicine; SAUM. The samples were collected from the central agricultural market m. Chisinau. Basic material investigations were the pig and sheep carcasses. The meat samples were collected to study the microorganisms load over different time periods of the selling. Samples of pig and sheep carcasses were randomly collected from the carcasses delivered in hall nr. 3 and examined in the laboratory using bacteriologic and microbiologic investigations.

Seeding was done on the following nutrient medium: peptone agar, peptone broth, medium Endo, medium Saburo, bismuth sulphite agar, which had been prepared in accordance known standards, then autoclaved and kept in the refrigerator until insemination carried out. Differential staining was performed by Gram and Ziehl-Neelsen methods. Subsequently, the plates inoculated samples were visually checked within 24 hours, and the interpretation of the final result were carried out after 48 hours.

RESULTS AND DISCUSSIONS

From the results it can be said that after 48 hours of incubation in thermostat at +37^{o}C on nutrient media peptone agar and peptone broth it seen the colonies growth with gray or white colors located at all space of Petri plates with varying sizes with round or oval form. These colonies are characteristic for streptococcus bacterial form. (Fig. 1 and 2).



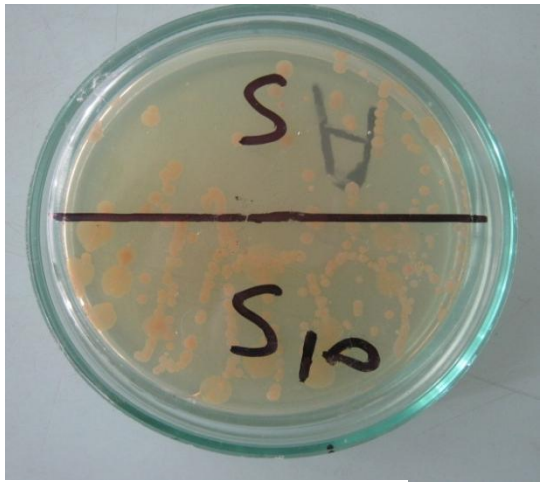
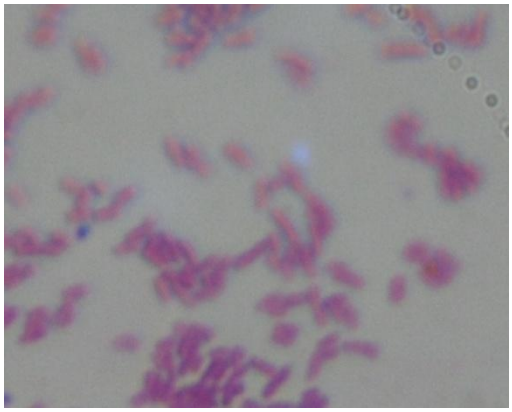


Fig. 1 and 2. Streptococcus colonies on peptone agar and peptone broth (samples collected from the surface of sheep carcasses).

Colonies of microorganisms isolated from samples of pig and sheep carcasses were fosr prepared smears that were painted by the classical method and examined on biological microscope, size 10x 20; 10x40. In the fig. 3 and 4 is shown the image micro flora isolated from colonies obtained from carcasses examined random samples. From these samples, especially samples of sheep carcasses were isolated E. coli microorganisms, presented in image like oval or round pinkish color.



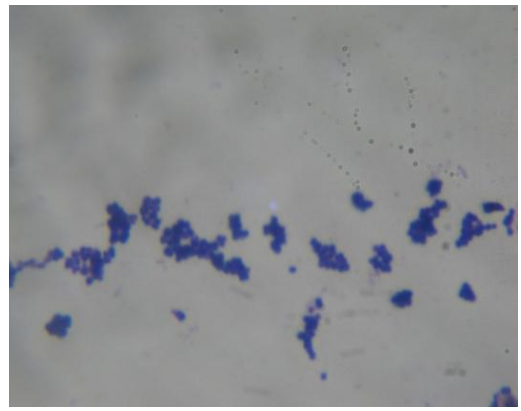
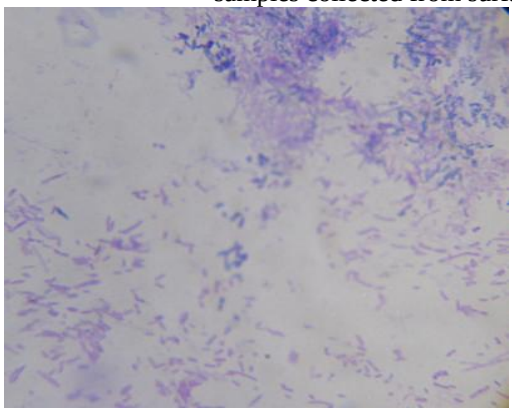
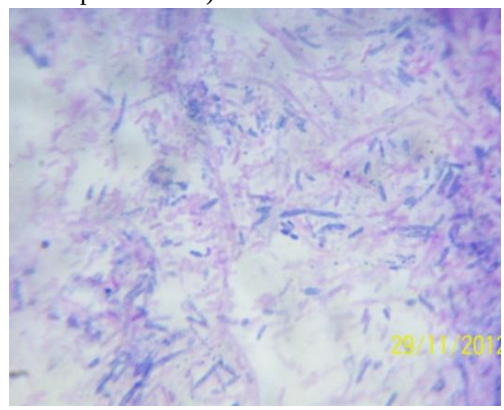


Fig. 3 and 4 microscopy smears prepared from the colonies by Endo medium (microorganisms E. coli) and from peptone agar (in piles colony of streptococcus), samples collected from surface of sheep carcasses). size 10x40.





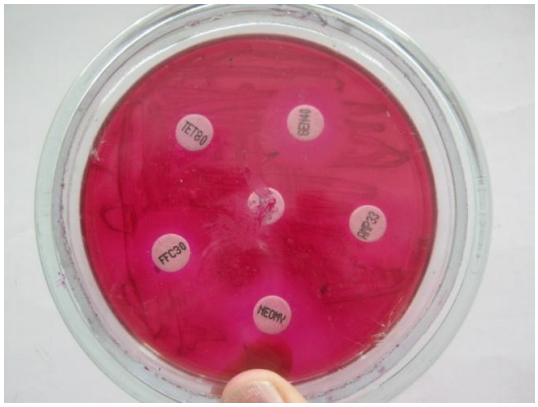
a) b)

Fig. 5 (a şi b) microscopy smears prepared from the colonies by Endo medium (microorganisms E. coli) and from peptone agar (in piles colony of streptococcus), samples collected from surface of pig carcasses), size 10x40.

In fig.5 a) and b) are shown images that reveal the presence of microbial associated flora combined with E. coli and streptococcus microbial forms isolated from colonies grown in sowing result in samples from carcasses of pig, after 48 hours of incubation at + 37oC.

The purpose of next investigations was to establish the sensitivity of colonies of microorganisms isolated from pig and sheep carcasses to some antibiotics that are used frequently in veterinary medical practice as: enrofloxaciny, neomyciny, florfenicol (flovet

5%), lincomyciny, oxytetracycline. The results of these investigations are shown in fig. 6 and 7 samples taken from sheep carcasses and on the figures 8 and 9 samples collected from the surface of pig carcasses.



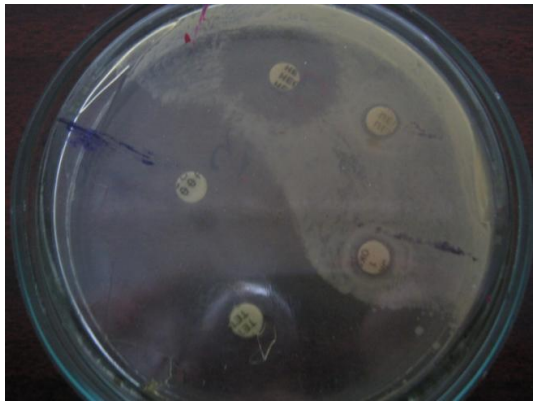


Fig. 6 and 7. Antibiograma. Sensitivity of bacterial flora of isolated colonies on sheep carcasses samples.

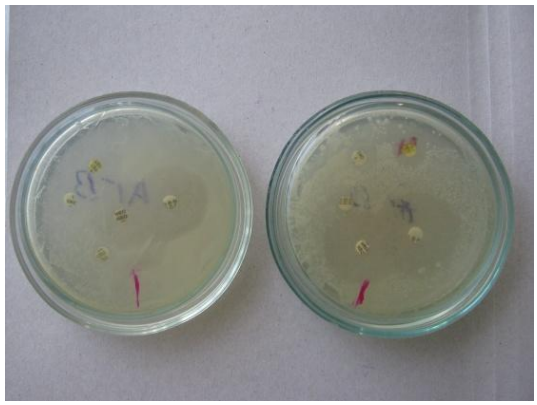


Fig. 8 and 9. Antibiogram result of bacterial flora isolated from media "peptone agar" of samples from pig carcasses.

Properties of sensitivity to antibiotics were after diffusion method by using disks containing the antibiotic. With a clamp or a mechanical device shall be deposited discussed the nutritional safety distance of 30 mm between them and 15 mm from the edge and incubate at 37 ° C for 48 hours.

Interpretation is based on the inhibition zone diameter measured with a ruler, including the block diameter. Readings (in mm) is compared with the tables of interpretation according M.O.Биргер (1982) appreciating bacterial strain as sensitive, intermediate or resistant to the antibiotic in question: - lack of inhibition zone - resistant strain; - up to 10 mm Ø area - low sensitivity; - Ø 11 to 15 mm area - intermediate sensibility - an area 15 to 25 mm - sensitive strain; - Ø ≥ 25 mm area - increased sensitivity.

Figures 6 - 9 are presented images demonstrate the effectiveness of antibacterial mentioned girl strains of streptococcus microbial firm isolated colonies on media - agar and peptone broth. Analyzing data on antybiosensitivity micro flora against some microorganisms may be noted that cultures of microorganisms (E. coli and staphylococci) are so sensitive to antibiotics such as oxytetracycline, neomycin, enrofloxacin and lincomycin. A sensitivity of the particular streptococcal culture was estabilished to antibacterial preparats as - oxytetracycline and enrofloxacine that the containment exceeded the colonies of more than 25 mm .

Conclusions

1. Microbiological examination of samples from the carcasses of pigs and sheep has shown the risk of contamination with bacterial micro flora, which is evident on the carcasses of sheep with prevailing types of microorganisms such as E. coli and streptococci.
2. Usually the microbial flora of the carcass surface is presence after 6-8 hours of placing in the hall, while the depth microorganisms may be present only after 16 to 20 hours.
3. Prevention of contamination carcasses with bacterial flora during the trading can be carried out by observing the temperature regime and to minimize contact with the environment vectors.
4. A higher sensitivity of micro flora isolated from sheep carcasses on antibiogramă investigation was observed on enrofloxacin and oxytetracycline, which resin area was more than 25mm.

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EVOLUTION OF *TRICHINELLA*-INFECTION INCIDENCE IN PIGS IN SOUTHERN ROMANIA OVER A 20-YEARS PERIOD (1994-2013)

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Abstract

Trichinellosis is a parasitic disease that in the past has not always been recognised for its importance. However, it is becoming increasingly clear that higher priority should be given to this zoonosis because of its health and economic impact, particularly in resource-poor countries. Romania is recognized for its high incidence of Trichinella-infections in pigs, as well as the human trichinellosis. Thus, according to European Food Safety Authority, our country has reported the highest number of Trichinella infections in pigs in the European Union, during the last years. According to the same report, Romania, Latvia and Lithuania have got the most cases of human trichinellosis in E.U.The aim of this study was to investigate the extent of Trichinella-infection in slaughtered pigs in Romania, over the period of 1994–2013, and to identify possible differences of the incidence among the counties from Southern Romania. Another purpose of this study was the analysis of Trichinella infections in pigs raised in households and slaughtered for own consumption compared to the farms-raised pigs. The analysis of incidence data showed a geographical heterogeneity, as well as significant differences between Trichinella-incidence rates in pigs raised in households and in pigs originating from an intensive system.

Key words: *Trichinella, pigs, incidence, Romania*

Introduction

Zoonoses, particularly those caused by parasites, represent a large group of diseases with diverse host assemblages and transmission patterns. Trichinellosis is a widely distributed zoonosis caused by parasitic nematodes of the genus *Trichinella* (5). Trichinellosis in humans is caused by consumption of infected raw or undercooked meat from *Trichinella*-infected domestic animals or game. Animals become infected by feeding with *Trichinella*-infected muscles. Ingested infective larvae mature and mate in the small intestine of host species including humans, pigs, rats, bears, horses and many other mammals, and birds and reptiles (28).

The parasite has a direct life cycle. Within hours following consumption of infected muscle by a suitable host, first stage muscle larvae are released by digestion into the small intestine (16). They develop rapidly and survive for less than 2 months. During this time, copulation takes place and the ovo-viviparous females release new-born larvae, which migrate via venules and lymphatics into the general circulation. New-born larvae are distributed throughout the body where they invade striated muscles, showing predilection for specific muscle groups (19, 28).

Currently, nine species and three genotypes are recognised in the genus, namely *Trichinella spiralis*, *T. nativa* and its related genotype *Trichinella* T6, *T. britovi* and its related genotype *Trichinella* T8, *T. pseudospiralis*, *T. murrelli* and its related genotype

Trichinella T9, *T. nelsoni*, *T. papuae*, *T. zimbabwensis*, and *T. patagoniensis* (2). Four of the species, *T. spiralis, T. britovi, T. nativa* and *T. pseudospiralis*, are known to be circulating in Europe.

All species can develop in mammals. *T. pseudospiralis* develops also in birds and *T. papuae* and *T. zimbabwensis* also infect some reptile species. Although no clear morphological differences exist between species and genotypes, they can be distinguished by biochemical or molecular analysis (14).

T. spiralis is the first species discovered and the most characterized because is the most frequently identified in human outbreaks and is a model for basic biological research investigations, due in large part to its relatively high frequency in both domestic and sylvatic animals and to its high infectivity for laboratory animals (10).

Materials and methods

Epidemiological data-sources

This study retrospectively reviewed the pigs slaughtered in 20 counties from Southern Romania during 1994-2013 period. The number of tested animals and the analysis results were collected separately for abattoir- and home-slaughtered pigs.

To collect the information on the *Trichinella*-infections in pigs, the following sources were used: (i) from 2005 to 2013 - the official reports of the Institute of Hygiene of Veterinary Public Health Bucharest in relationship with European Food Safety Authority (EFSA), based on the official reports of the selected counties; (ii) from 1994 to 2005 - the official reports of County National Sanitary Veterinary and Food Safety Laboratories.

Currently, the Institute of Hygiene and Veterinary Public Health Bucharest is responsible for centralization of passive surveillance data for *Trichinella*-infections in pigs received from national county and private laboratories, and for reporting these data to European Food Safety Authority.

In this study, we present dynamics of incidence of *Trichinella* infections in pigs from farms, slaughtered in official abbatoirs, and on the other hand, the *Trichinella* infections in pigs from households, where the pigs are raised for familial consumption and slaughtered at home in a traditional system (18). The data obtained were analyzed in time and space, taking in consideration the trend of incidence depending on areas. In order to obtain information regarding the geographic variations of the incidence rate of *Trichinella*-infection in pigs in Southern Romania, the collected data were compared.

Testing methods used during 1994-2013

The identification of *Trichinella* larvae in muscle samples is limited to post-mortem inspection of carcasses. In Romania, in order to prevent human trichinellosis, the examination of muscle samples from pigs is part of routine slaughter inspection.

In our country, until 2006, *Trichinella* meat inspection in pigs was conducted according to MAA Order no. 45/1995 and Veterinary Sanitary Law 215/2004, using the trichinoscopy method (29, 31). This method involves the compression of multiple 2×10 mm pieces of muscle tissue between two glass plates (compressorium) until they become translucent, followed by examination using a microscopic technique.

From January 1st, 2006, the legislation for *Trichinella* meat inspection in the E.U. has been replaced by Regulation (EC) No 2075/2005 laying down specific rules on official

controls for *Trichinella* in meat (23). This new Regulation (EC) No 2075/2005 on *Trichinella* meat inspection mainly refers to Regulation (EC) No 853/2004 (24) that stipulates specific hygiene rules for food of animal origin. Furthermore, Regulation (EC) No 854/2004 (25) which describes specific rules for the organisation of official controls on products of animal origin intended for human consumption and Regulation (EC) No 882/2004 (26) on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules have to be considered.

According to these regulations, the recommended method for the examination of pigs' carcasses for *Trichinella* is the artificial digestion. This technique involves enzymatic digestion of individual or pooled muscle tissue samples incorporating mechanical homogenisation or grinding, stirring, and incubation (7). This procedure is followed by filtration and sedimentation procedures to recover and concentrate any larvae that are released from muscle during digestion. Samples processed by these methods are examined under a stereomicroscope for the presence of larvae. As digestion assays is more sensitive than trichinoscopy, this method is the only one recommended by the International Commission on Trichinellosis for the routine examination of carcasses (9).

Results and discussion

During the period 1994–2013, from a total population of 25.726.271 industrial raised pigs, 17.362 were infected with *Trichinella* (approx. 68 infected animals/10^5 tested pigs), whereas from 11.459.590 tested animals from households, 15.378 were infected (approx. 134 infected animals/10^5 tested pigs). This incidence is the highest of all countries in the E.U.; according to European Food Safety Authority, Romania accounted for 51.5 % of all the *Trichinella*-positive findings in pigs from E.U. in 2012, as well as in 2010 and 2011 (22).

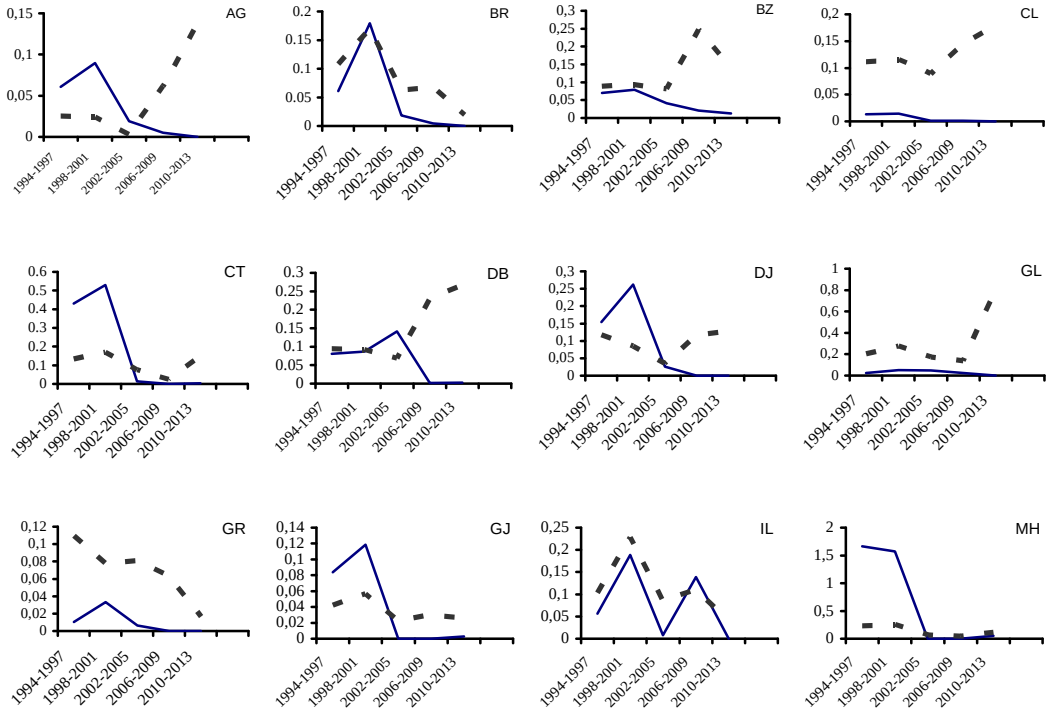
The dynamics of *Trichinella*-infection incidence in pigs in Southern Romanian counties during 1994-2013 is presented in Fig. 1. Taking into account the large analyzed period, we clustered each four chronological years, and in the graphical representation we used the average of each period.

Geographic variations of the incidence rate showed differences in the incidence between different counties. Thus, the incidence rate was heterogeneous, with higher values in counties as Mehedinti, Galati whereas in Vrancea, Giurgiu, Gorj and Arges counties the incidence was reduced. This conclusion is based on the statistical data, which was influenced by the number of slaughtered pigs. The geographic analysis can be considered only if the animals tested in a given county have been raised in the same county as they were tested.

Overall, the incidence of *Trichinella*-infection in pigs originating from households was higher compared to the one of pigs from farms. This aspect, regarding *Trichinella*-infection in pigs raised in outdoor farming systems, is clear in all parts of the world (11, 12, 13). Nevertheless, risk degree of pigs raised outdoors depends mainly on the infection levels in local wildlife, and this degree of risk is of substantial importance for 'organic' or 'green' pig producers, who provide products to consumers seeking meat from animals raised under natural conditions, without any compliance with hygienic rules. This observation points out the importance of understanding infection pressure from the wildlife population, in situations where pigs are otherwise at risk for exposure (30).

Regarding *Trichinella*-infections incidence in farms, a sharp peak was recorded during 1998-2001, in almost all counties, respectively in 16 counties of the total number involved in this study. After this period, the incidence decreased. A particular situation was met in Ialomita county, where it was also recorded a high incidence between 2006 and 2009. In farm-raised pigs, the risk of infection is mainly related to the lack of compliance with rules on the treatment of animal waste. In such farms, infection could occur due to the breakdown of the biosecurity barriers around the farm, allowing the introduction of infected rodents (6, 27). However, in the last period analyzed (2010-2013), *Trichinella*-infection incidence in pigs raised under controlled conditions was very low, being recorded 34 positive cases (0.19% of all *Trichinella*-positive findings in farms during 1994-2013), with a high incidence in Olt county (17 positive cases) and Prahova county (10 positive cases).

Cumulative incidence rates for *Trichinella* infection were determined for the 1994–2013 period and expressed as number of slaughtered and infected pigs for each county. The correlation between the number of slaughtered and *Trichinella*-infected pigs is presented in Fig. 2 and Fig. 3.



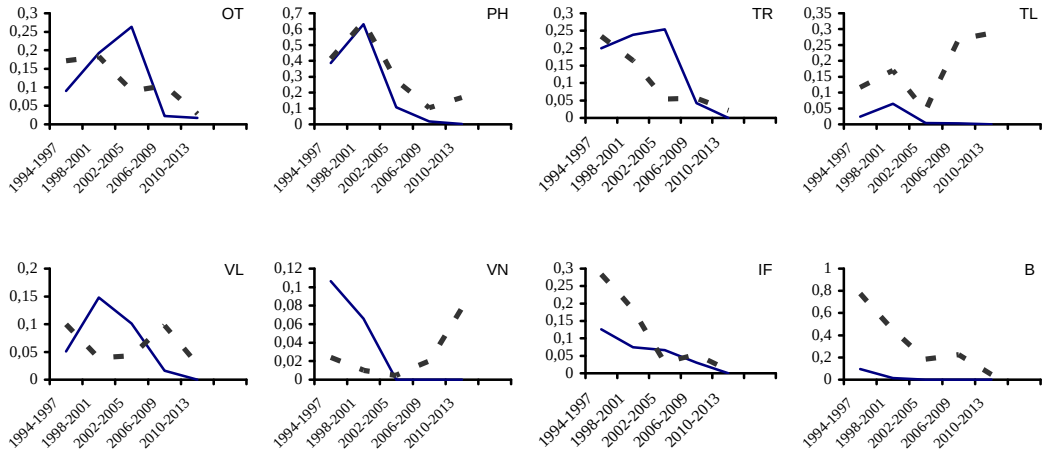


Fig. 1. The dynamics of *Trichinella*-infection incidence rates in pigs in Southern counties of Romania during 1994-2013 (farm-raised pigs – continuous line; households pigs – dotted line)

Statistical analysis is influenced by the number of tested pigs from the number of slaughtered pigs. For abattoir-slaughtered pigs, *Trichinella* surveillance can be considered exhaustive, since all animals are tested. This is not the case for the other categories of animals (pigs not raised under controlled conditions), which are tested at the request of the carcass owner; for this reason, the corresponding data may thus be biased.

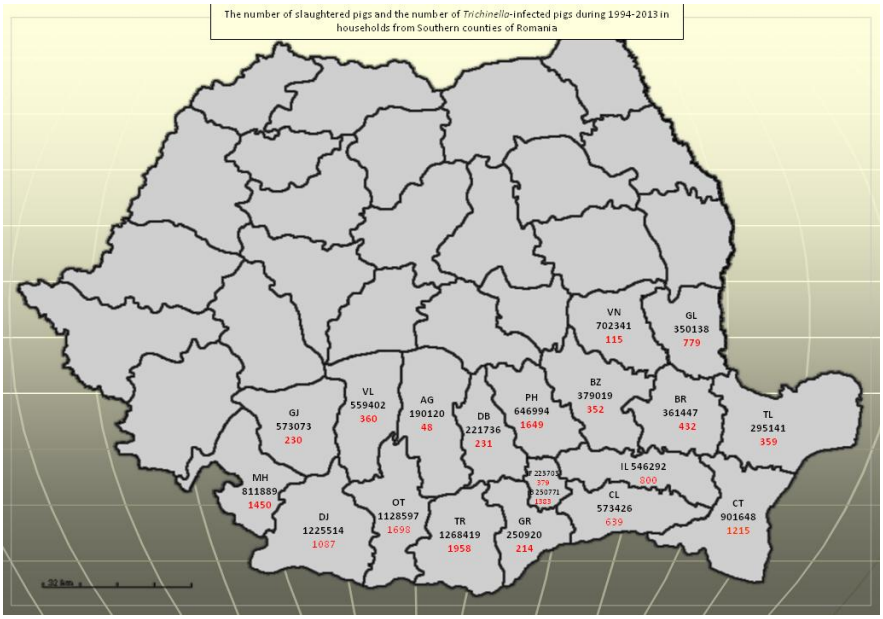


Fig. 2. The number of *Trichinella*-infected pigs compared to the total number of pigs slaughtered in Southern counties of Romania, between 1994-2013 (pigs raised in households)

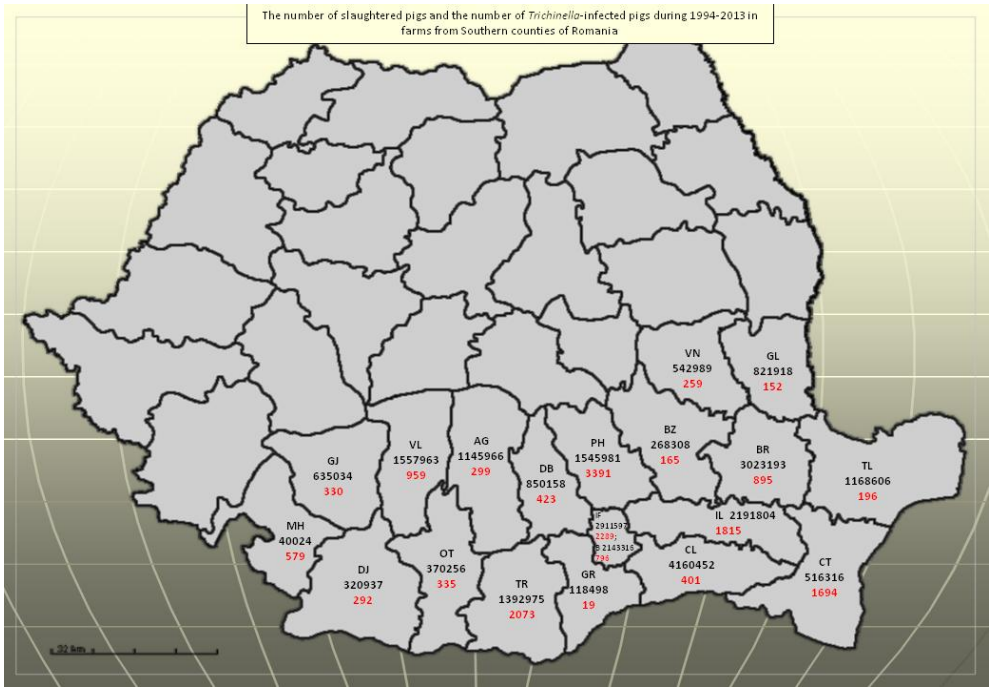


Fig. 3. The number of *Trichinella*-infected pigs compared to the total number of pigs slaughtered in Southern counties of Romania, between 1994-2013 (pigs raised in farms).

Another particular aspect is that *Trichinella*-incidence was analyzed between 1994 and 2006 by trichinoscopy, which was the official method for detecting *Trichinella* infection in Romania (3, 20). This method has got a significant lower sensitivity as compared with artificial digestion, the current recommended method for the detection of *Trichinella* infection in domestic and wild animals (1, 8, 15, 21). Therefore, it is likely that some *Trichinella* infections were not detected by this technique; for this reason, the incidence rates reported here are likely under-estimates of true incidence.

The incidence of *Trichinella*-infection in pigs is closely correlated with the number of human trichinellosis cases recorded in Romania (4). Thus, during 1994-2007, human trichinellosis incidence ranged between 2 and 15.9 cases per 100,000, whereas during 2008-2013, human trichinellosis incidence ranged between 0.51 and 2.51 cases per 100,000 (17, 22). Although the number of human trichinellosis cases decreased in the last years, compared to other countries of European Union, Romania is placed among the first member states in terms of the incidence of this zoonosis. Taking in account all these aspects, trichinellosis is still a major public health issue in Romania, which requires the improvement of sanitary conditions, implementation of a more reliable collaboration between veterinarians and pig breeders/consumers, education of the population, and a more careful supervision by the public health services.

Conclusions

1. Maps of individual county *Trichinella*-infections incidence rates showed important geographic heterogeneities. The highest incidence rates were found in counties as Mehedinti, Galati, whereas in other counties as Arges and Valcea the incidence rates were low.
2. With a incidence rate of 68 infected animals/10^5 tested pigs from farms and approximately 134 infected animals/10^5 tested pigs from households, Romania is a country with extensive *Trichinella*-infection in pigs.
3. The incidence of *Trichinella*-infection in pigs originating from households was approximately two times higher compared to the one of pigs raised in farms, this situation complying with the situation reported in many other countries from the European Union.
4. In Romania, trichinellosis represents a serious public health and ecological concern because of the favorable social, economic, cultural, and geographic conditions of this territory.

Acknowledgments

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PROSPECTIVE STUDY CONCERNING THE INCIDENCE OF METICILLIN-RESISTANT STAPLYLOCOCCI ISOLATED FROM DIFFERENT DERMATOLOGICAL DISORDERS IN PETS

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Abstract

Purpose: *Staphylococcal infections represent a major problem in veterinary dermatology, both in the terms of their increased frequency and the development of antibiotic resistance.* ***Study objective****: to achieve a prospective study about the involvement of different species of Staphylococcus and especially of methicilin-resistant strains in the dermatology of pets from the nortf-east of Romania.* ***Methods:*** *A total of 60 samples were collected from pets-dogs, cats and horses, who were clinically diagnosed with dermatitis of various etiologies, pyodermitis and pseudopyodermatitis. All strains were studied and analyzed in terms of cultural, morphological and hemolytic activity, also catalase activity. The strains classified as Staphylococcus were tested biochemically in order to identihy the species.To highlight the presence of the highly pathogen enzymatic equipment it were tested the coagulase activity – wich was correlated with the clinical picture. Methicilin-resistance was detected by testing the susceptibility to cefoxitin (10 µg) and oxacilin (1µg).* ***Results****: From all 60 samples, 40 strains were represented by Staphylococcus spp. Species distribution was as follow: 67,5% S. intermedius (27 strains); 22,5% S. aureus (9 stains); 2,5% S. delphiny (1 strains); 2,5% S. saprophyticus (1 strain); 2,5% S. chromogenes (1 strain); 2,5% S. epidermidis (1 strain). From those nine S. aureus strains, 6 were detected as methicilin-resistant, representing a percentage of 66,66%.* ***Conclusions:*** *In the analyzes group it was observed a high prevalence of MRSA - 66,66%. That Justihy further study of molecular biology to confirm the presence of specific genes and identify the source. The 9 strains of Staphylococcus aureus were isolated from animals with dermatitis, recurrent and/or complicated (skin abscesses, skin and ear prurulent exudates). Rates of methicilin resistance in order identified Staphylococcus species was null.*

Keywords*: MRSA, dermathological disorders, Staphylococcus*

Introduction

In veterinary dermatological pathology, the bacterial over-infection represents a commonly met complication. Skin ecology is represented by the resident microbial flora (permanently present on skin, and multiplies in niches) and the transitional flora (occasionally found, in limited quantities and periods of time) (1).

In dogs, the bacterial flora found uniformly and constantly on the skin surface, is represented by *Micrococcus, Staphylococcus epidermidis, S.xylosus, Streptococcus group D, Corynebacterium spp., Clostridium sp., Acinetobacter.*

In the same species, the main bacteria responsible for skin infections are (in the decreasing order of frequency): *S. intermedius, S. aureus, S. hycus, S. hominis, S. epidermidis, S. xylosus, Streptococcus sp., Proteus sp., Pseudomonas sp., Echerichia coli,* etc.(1). In cases of primary and secondary piodermatitis, a rapid, appropriate treatment allows to avoid serious consequences like: lesions' extension, dissemination of germs (with the possibility to transfer them to human beings) and/ or the emergence of reactivations. The presence of *Staphylococcus spp.* represents the major cause for the emergence of piodermititis; recent studies conclude that identified and studied Staphylococcus Delta – toxin (DTS), in case of *S. aureus*, induce the allergic skin disease through the activation of mast cells (3).

The study of presence and implications of *Staphylococcus spp.* in veterinary dermatological pathology, with a focus on methycilino - resistant *S. aureus* (MRSA) is

important from the perspective of its transmission from animal to the human being (4, 6, 7, 9).

The present study, regarding the incidence of MRSA at the skin level in company animals (pets), represents a premiere for the North-East region of Romania.

Materials and methods

The study cases were a total of 60 animals: 52 dogs, 7 cats and one horse from the Faculty of Veterinary Medicine and veterinary practices in Iasi. The samples taken into study were represented by crusts, exudate expressed after the crusts removal, scales, purulent collections, ear secretion (in the study there were also included 5 cases of external otitis in context with the dermatological conditions) that presented cytological signs of bacterial infection (fig. 1, 2).

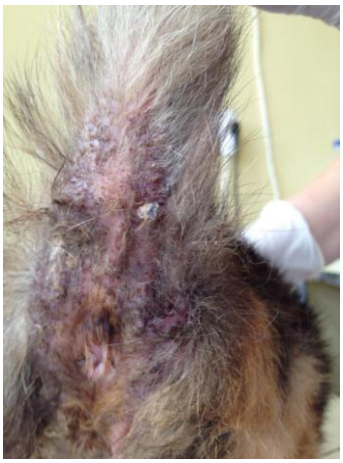

Fig. 1 Cat, perianal area, erythematosus areas, general alopecia, crusts

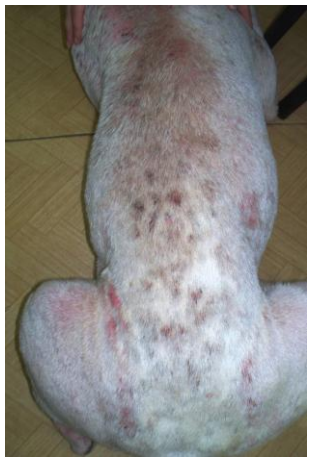

Fig. 2 Dog, pustules with purulent exudates, scratching plagues

The initial identification consisted in samples inseminations on horse blood agar (Oxoid) and mannitol salt agar (Oxoid) - selective medium for Gram positive halophilic bacteria - watching for mannitol fermentation. The incubation was realised at 37ºC for 18-24 hours.

Isolates were identified based on cultural characteristics, their morphology in Gram positive smears and on their ability to grow on mannitol salt agar. The plates in which the colour of the medium was intensely and persistently modified in yellow were presumptively marked as *Staphylococcus aureus.*

For each of the isolated colonies the catalase test was performed (3% hydroxide peroxide). The strains that gave an intense mannitol positive result were tested with STAPHYTECT PLUS, a latex agglutination test for the identification of *Staphylococcus aureus - by* detecting the aggregation factor, protein A and some polysaccharides that are found in methicillin-resistant strains (fig. 3).

The rest of *Staphylococcus spp.* strains isolated using the described methods, were identified with the help of RapiD Staph Plus Panel – Remel(SUA), based on 18 biochemical tests (fig. 4).

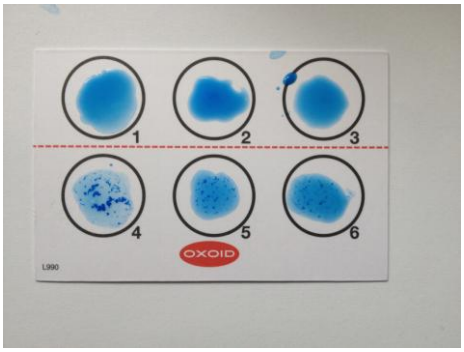

Fig. 3 Latex agglutination test.
Positive control strain, *S. aureus* ATCC 25923 on the left

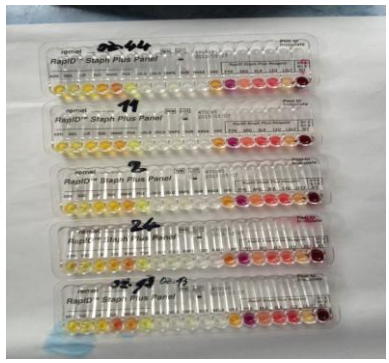

Fig. 4 RapiD Staph Plus Panel strips

After the identification of *Staphylococcus* species, each of those 40 isolated strains has undergone a test for rabbit citrate plasma coagulation (BioRad) (fig. 5). Isolated and identified *Staphylococcus* strains were tested regarding methicillin resistance by performing the antibiogram (fig. 6) using the BSAC methodology. Cefoxitin (10 µg) micro tablets (Oxoid) were used by incubating at 35º for 18-20 h and also oxacillin (1 µg) was used by incubating at 30º for 24 h. Culture suspensions were used at a dilution of 0,5 McFarland (automatic densitometer MacFarland-DEN1). According to BSAC, EUCAST, CLSI recommendations, the detection of methicillin resistance is compulsory in all *S. aureus* strains obtained from clinical cases (8).

For all the identification methods - cultural characteristics, morphological, hemolytic, biochemical, the *Staphylococcus aureus ATCC 25923 was used as a positive control*

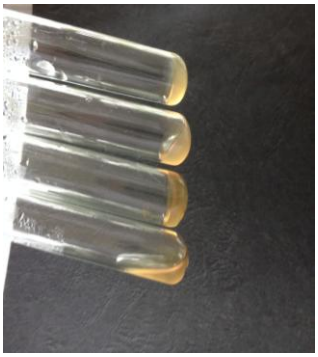

Fig. 5 Rabbit citrate plasma coagulation test

Fig. 6 Cefoxitin susceptibility test

Results and discussions

Correlation of all the obtained data after performing the presented tests led to the results described in fig. 7. 40 *Staphylococcus spp.* strains were isolated from 1 horse, 5 cats and 34 dogs.

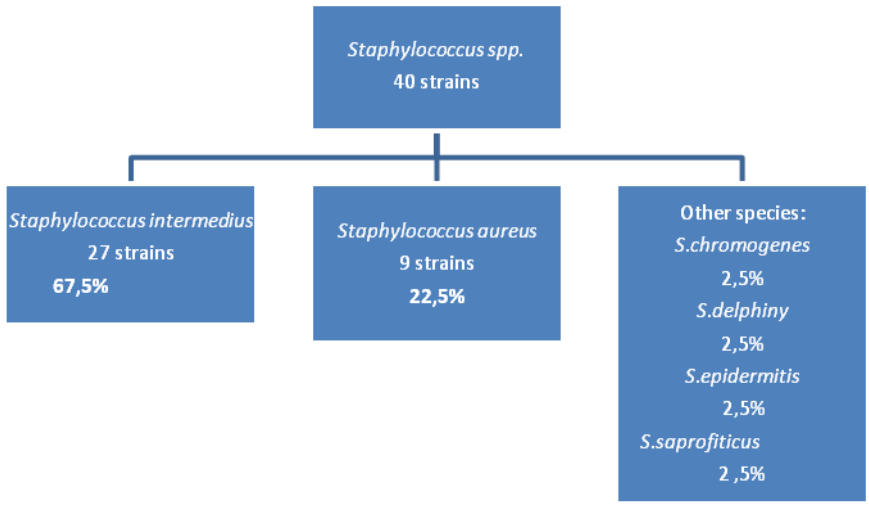

Fig.7. Ditribution of identified *Staphylococcus species*

Of the 9 total *Staphylococcus aureus identified strains*, 6 were methicillin resistant, representing 66,66%. All 6 MRSA strains came from purulent samples.

On a global scale, reports regarding MRSA incidence at cutaneous level in pets are different. In the USA, REES (2008) reports a MRSA incidence of 10% in healthy dogs and 15% in healthy cats (approximately 10% of veterinary staff are S. aureus methicillin resistant carriers, being a professional category considered to present higher risk of MRSA transport)(10) Also in the USA, a study on 25 methicillin resistant strains (isolated from plagues and cutaneous lesions in dogs) reports that 23 strains posses the mecA gene, 9 isolates were MRSA, one was S. intermedius and 15 were coagulase-negative species.

In the results from the present study, the fact that more than half the number of *S. aureus* were identified as methicillin resistant is an alarm signal for both the selection of antibiotic resistant strains as well as for the possibility of transmitting them to humans(6).

A possible explanation for this high percentage of MRSA out of the total identified strains of *S. aureus*, may be that more than half of the samples came from pets treated repeatedly with antibiotics for long periods of time. Relapses or the lack of results in the therapeutic protocol convinced the owners to accept the microbiological laboratory analyses. In the North-Eastern part of Romania, in veterinary medicine, this study presents for the first time a state of MRSA incidence level at cutaneous level in pets - dogs, cats and horses.

Knowing the incidence of MRSA at cutaneous level in pets is important also regarding the transmission of these strains from animals to humans. A study published in Germany concludes that MRSA ST398 can colonise and induce infections in humans and some animal species such as dogs, horses and pigs. The MRSA ST398 isolated strains from that particular study came from a cutaneous infection in a dog and from the veterinary staff from where it was treated, the strain having the same characteristics(7).

The differences of *spa* - types between the isolates contain the same PCR results for SCC *mec* which can be explained by one single genetic event, results that suggest that the MRSA transmission is possible between species(7).

A high rate of MRSA presence is reported among veterinarians and staff that works in veterinary medicine, very close to animals.

Conclusions

1. In the studied group, it was observed a high frequency of MRSA (15% of Staphylococcus strains and 66.66% of S. aureus strains). The results of the study justify further molecular tests in order to confirm the presence of specific genes and to identify the source.
2. The 9 strains of Staphylococcus aureus were isolated from animals with recurrent and/or complicated dermatitis (skin abscesses, skin and ear purulent exudates).
3. No meticillin-resistance in other Staphylococcus species was detected.

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CLASSIFYING *PSEUDOMONAS AERUGINOSA* STRAINS OF ANIMAL ORIGIN IN MDR, XDR AND PDR BY DETERMINING THE RESISTANCE TO ANTIBIOTICS

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Abstract
We used the recent recommendations (Magiorakos et al., 2012) to define MDR, XDR and PDR. By diffusion antibiogram were tested 20 strains of Pseudomonas aeruginosa of animals origin from Clinics of Veterinary Faculty in Iasi according to the results strains were employed in MDR, XDR and MDR.

- *11 of the 20 strains that were tested were proven MDR*
- *9 strains were included in the XDR (extensively drug-resistant) group*
- *No PDR strains were found in this study*

*The quality control of the study was carried out with Pseudomonas aeruginosa strain **ATCC 27853**.*
***Keywords:** antibiotics, Pseudomonas aeruginosa, MDR, PDR, XDR Antimicrobial agents, definitions*

Introduction

MDR has been defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories, XDR has been defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e. bacterial isolates remain susceptible to only one or two categories) and PDR has been defined as non-susceptibility to all agents in all antimicrobial categories. In order to ensure adequate application of these definitions, it is recommended that bacterial isolates are tested against all or nearly all of the antimicrobial agents within the antimicrobial categories and selective reporting and suppression of results should be avoided (Magiorakos AP et al., 2012).

Materials and methods

Antibiotic susceptibility testing

Antibiotic sensitivity of strains included in the study was tested by disc diffusion method. Interpret area results we made based on the rules CLSI (Clinical and Laboratory Standard Institute).

DST diffusion

Principle: the surface of Mueller-Hinton environment seeded with stem test cloth shall be submitted disks with different antibiotics; antibiotic diffuses circular environment at concentrations decreasing; bacterial growth is inhibited in the area where higher concentrations than MIC.

Materials used:

- Strain tested in pure culture;
- Mueller-Hinton agar plates (Oxoid, UK);
- Discs impregnated with antibiotics (Oxoid, UK);
- Sterile saline;
- Sterile pads;
- 0.5 McFarland standard.

Working protocol:

Mueller-Hinton medium I agar prepared according to the manufacturer's instructions and poured into 25 ml petri dishes with a diameter of 90 mm, so that the thickness of the medium is 4 mm;

We prepared in 2 ml of saline, a 0.5 McFarland turbidity of the bacterial suspension with 24 of the culture of the test strain ha, which corresponds to an inoculum of 1-2 x 108 / ml; suspension should be used within 15 minutes of mixing;

We seeded Mueller-Hinton agar plates (pre-dried in the thermostat at 5 to 10 minutes) with a sterile sampling swab soaked in the bacterial suspension in three directions so as to spread out over the entire surface to obtain a confluent growth;

I applied antibiotic discs with automatically dispensaries Oxoid, UK;

We incubated the plates at 37 ℃, aerobically overnight (20-24h);

I measured in mm diameter of inhibition zones around the discs and I played category sensitivity (sensitive resistant, intermediate) according to CLSI standards.

Antimicrobial agents and points of rupture zones of inhibition for *Ps. aeruginosa* (Performance Standards for Antimicrobial amended Susceptibility Testing; Twentieth Informational Supplement. CLSI document M 100 - S20, Wayne, PA, USA, 2010).

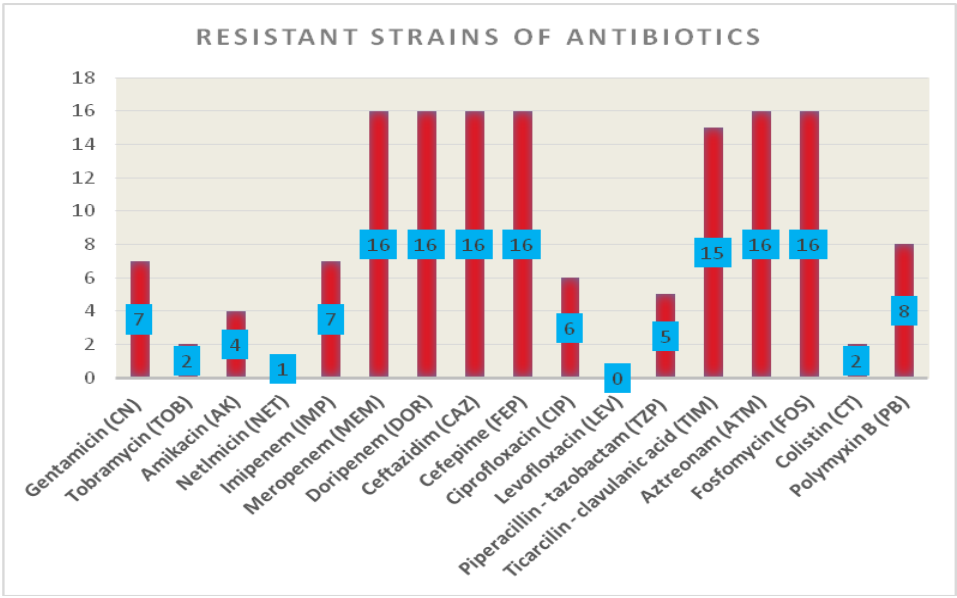
Results

Antimicrobial category	**Antimicrobial agent**	***Isolates***									
		1	2	3	4	5	6	7	8	9	10
Aminoglycosides	***Gentamicin (CN)***	S	S	R	R	S	R	S	R	S	R
	Tobramycin (TOB)	S	S	S	S	S	R	S	S	S	S
	Amikacin (AK)	S	S	R	R	S	S	S	R	S	S
	Netlmicin (NET)	S	S	S	S	S	S	S	R	S	S
Antipseudomonal carbepenems	***Imipenem (IMP)***	S	S	S	R	R	S	S	S	R	R
	Meropenem (MEM)	R	R	R	R	R	R	R	R	R	R
	Doripenem (DOR)	R	R	R	R	R	R	R	R	R	R
Antipseudomonal cephalosporins	***Ceftazidim (CAZ)***	R	R	R	R	R	R	R	R	R	R
	Cefepime (FEP)	R	R	R	R	R	R	R	R	R	R
Antipseudomonal fluoroquinolones	***Ciprofloxacin (CIP)***	S	S	S	S	S	R	R	S	S	R
	Levofloxacin (LEV)	S	S	S	S	S	S	S	S	S	S
Antipseudomonal penicillins+β-lactamase inhibitors	***Piperacillin – tazobactam (TZP)***	S	S	S	S	S	S	S	R	R	R
	Ticarcillin – clavulanic acid (TIM)	S	R	R	R	R	R	R	R	R	R
Monobactams	***Aztreonam (ATM)***	R	R	R	R	R	R	R	R	R	R
Phosphonic acids	***Fosfomycin (FOS)***	R	R	R	R	R	R	R	R	R	R
Polymyxins	***Colistin (CT)***	S	S	S	S	S	R	S	S	S	S
	Polymyxin B (PB)	S	S	R	S	S	R	R	R	S	R

Tab.1a. *Pseudomonas aeruginosa* antimicrobial susceptibility profiles that fit MDR, XDR and PDR

Antimicrobial category	***Antimicrobial agent***	***Isolates***									
		11	12	13	14	15	16	17	18	19	20
Aminoglycosides	***Gentamicin (CN)***	S	S	R	S	S	S	R	R	S	S
	Tobramycin (TOB)	S	S	S	S	S	S	R	S	S	S
	Amikacin (AK)	S	S	S	S	S	S	S	R	S	S
	Netlmicin (NET)	S	S	S	S	S	S	S	S	S	S
Antipseudomonal carbepenems	***Imipenem (IMP)***	S	S	S	S	R	R	S	R	S	S
	Meropenem (MEM)	R	R	R	R	R	R	R	R	R	R
	Doripenem (DOR)	S	R	R	R	R	R	R	R	R	R
Antipseudomonal cephalosporins	***Ceftazidim (CAZ)***	R	R	R	R	R	R	R	R	R	R
	Cefepime (FEP)	R	R	R	R	R	R	R	R	R	R
Antipseudomonal fluoroquinolones	***Ciprofloxacin (CIP)***	S	S	S	S	S	S	R	S	R	R
	Levofloxacin (LEV)	S	S	S	S	S	S	S	S	S	S
Antipseudomonal penicillins+β-lactamase inhibitors	***Piperacillin – tazobactam (TZP)***	S	S	R	S	R	R	S	S	S	S
	Ticarcillin – clavulanic acid (TIM)	R	R	R	R	R	R	R	R	R	R
Monobactams	***Aztreonam (ATM)***	R	R	R	R	R	R	R	R	R	R
Phosphonic acids	***Fosfomycin (FOS)***	R	R	S	R	R	R	R	R	R	R
Polymyxins	***Colistin (CT)***	S	S	R	R	S	S	R	S	S	S
	Polymyxin B (PB)	R	R	R	S	S	S	R	S	R	R

Tab.1b. *Pseudomonas aeruginosa* antimicrobial susceptibility profiles that fit MDR, XDR and PDR



By diffusion antibiogram were tested 20 strains of *Pseudomonas aeruginosa* of animals origin from Clinics of Veterinary Faculty in Iasi according to the results strains were employed in MDR, XDR and MDR.

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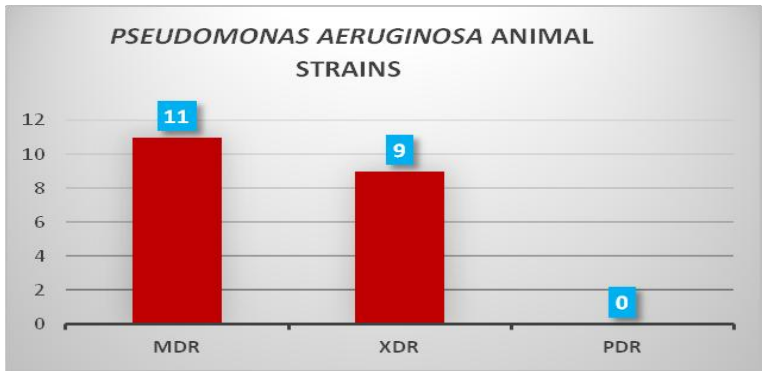
- No PDR strains were found in this study



Conclusion

No PDR strains were found in this study.

Bacterial isolates that are MDR will have many different resistance profiles because by definition, non-susceptible results for even a single agent in only three antimicrobial categories defines an organism as MDR.

'Possible XDR' and 'possible PDR', however, should still be regarded as markers of extensive resistance and their use should be encouraged despite limitations in their interpretation.

When performing routine antimicrobial susceptibility test- ing on bacterial isolates in clinical microbiology laboratories, the limited number of agents generally tested will result in many MDR bacteria being categorized as 'possible XDR' or 'possible PDR'. This practical limitation underscores the necessity of testing an adequate number of antimicrobial agents.

It is important to note that overall a bacterial isolate will be considered non-susceptible to an antimicrobial agent or antimicrobial category, when it is found to be non-susceptible by using any of the available interpretative criteria established by EUCAST, CLSI or the FDA. Furthermore, for results to be compared between surveillance systems or facilities, it will be important to report details about the methods and interpretive criteria used for antimicrobial susceptibility testing along with the results from applying the definitions for MDR, XDR and PDR.

The quality control of the study was carried out with Pseudomonas aeruginosa strain ATCC 27853.

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INVESTIGATION OF DISTRIBUTION AND ECOLOGY OF TICK (ACARI: IXODIDAE) SPECIES IN SOUTHERN ROMANIA FOR SURVEILLANCE OF EMERGING ZOONOTIC DISEASES

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Abstract

Ticks are the most important vectors for zoonotic diseases with widespread distribution that transmit a variety of pathogens causing emerging and remerging diseases. Our aim was to investigate the distribution and ecology of ticks collected in southern Romania, by analyzing the community of ticks in relationship with their hosts. A total number of 732 feeding ticks were collected in different areas in Southern Romania (Olt, Giurgiu, Prahova, Ilfov, Tulcea, Constanta counties, and Bucharest). Ticks were collected in April – June, 2014 from different animal hosts (dogs, cattle, sheep, goats or horses). Of them, 262 (35.8%) were identified as Rhipicephalus sanguineus sensu lato, 112 (15.3%) as Rhipicephalus bursa, 34 (4.6%) as Rhipicephalus annulatus, 161 (21.9%) as Dermacentor reticulatus, 98 (13.3%) as Dermacentor marginatus, 48 (6.5%) as Ixodes ricinus, 11 (1.5%) as Hyalomma marginatum and 6 (1.2%) as Haemaphysalis punctata. The findings emphasize a wide diversity of tick fauna infesting animals in southern Romania, most of them with a great vectorial potential for tick-borne disease of zoontic concern. In conclusion uncovering the ecology and distribution of ticks in relationship with the host could greatly improve the knowledge of tick-borne diseases.

Key words: *ticks, Ixodidae, vector-borne pathogens, zoonoses, emerging diseases*

Introduction

According to the World Health Organization, every year more than 1 million people die from vector-borne diseases worldwide; the most affected are those in developing countries. Costs related to the treatment of vector-borne diseases reach to millions of Euros annually. In recent years the incidence of these zoonoses has increased as a result of climate changes, modifications in the ecology of vectors and massive movement of people and goods but also as a result of the acquisition of resistance to insecticides (2, 3).

Ixodidae ticks (Acari: Ixodidae) are the most important vectors in Europe, spreading a wide variety of pathogens such as: viruses (Tick Borne Encephalitis-Virus (*Flaviviridae*), CBC (*Reoviridae*), Crimea Congo Hemorrhagic Fever virus (*Bunyaviridae*)) bacteria *(Borrelia burgdorferi* sensu lato, *Rickettsia* spp., *Anaplasma phagocytophilum, Francisella tularensis*), protozoa (*Babesia microti, B. divergens, Theileria* spp.) (5, 7, 8). In Romania the number of cases of disease transmitted by ticks gradually increased every year becomes imperative to investigate and surveillance the ticks and tick-borne diseases (1, 4). Data concerning the prevalence of ticks and tick borne diseases present in animals and humans in Romania are limited (9, 10). Therefore, the aim of this paper was to investigated the distribution and ecology of tick (Acari: Ixodidae) species in southern Romania in order to control and prevent tick-borne diseases.

Materials and Methods

A total of 732 feeding ticks were collected in April – June, 2014 from different domestic hosts (dogs, cattle, sheep, goats, and horses) from urban and rural areas in southern Romania (Olt, Giurgiu, Prahova, Ilfov, Tulcea, Constanta counties, and Bucharest). The distribution map of areas where ticks were collected is represented in Figure 1. Ticks were separate by stages and sex, and identified to the species level based on morphological characters using the specific identification keys (5, 6).

Fig. 1. Collection points of ticks from southern Romania areas

Results and Discussions

During of April – June, 2014, a total number of 732 feeding ticks were collected from different animal hosts (dogs, cattle, sheep, goats, and horses). Of them, 434 were females and 298 males. The areas with the highest number of collected ticks were: Ilfov (45.2%) followed by Bucharest (18.7%), Olt (13.3%), Giurgiu (9.2%), Prahova (6.9%), Tulcea (5.0%) and Constanta (1.3%) (Fig. 2)

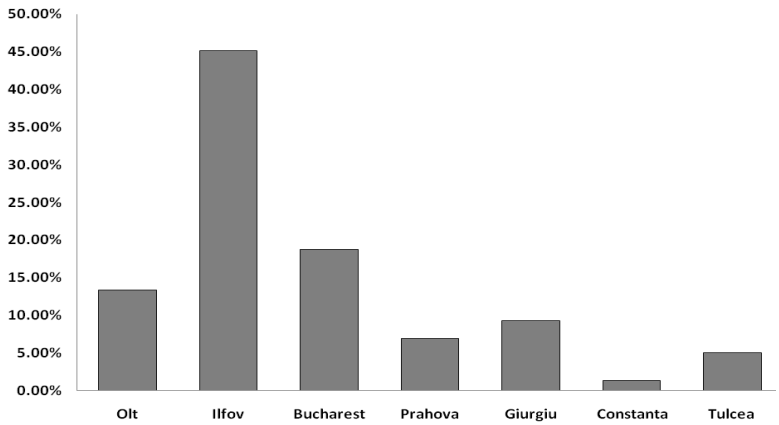

Fig. 2. Proportion of ticks collected from different areas in southern Romania

The main bulk of ticks were collected from dogs (47.6%), followed by sheep (28.6%), cattle (19.2%), horses (3.2%) and goats (1.0%) (Fig. 3).

Tick species were identified as: *Ixodes ricinus* (n = 48/732; 6.5%), *Rhipicephalus sanguineus* (n =262/732; 35.7%), *Rhipicephalus bursa (n = 112/732; 15.3%), Rhipicephalus annulatus (n = 34/732; 4.6%), Dermacentor marginatus* (n = 98/732; 13.3%), *Dermacentor reticulatus, (n=161/732; 22%), Haemaphysalis punctata (n = 6/732; 0.81%), and Hyalomma marginatum* (n = 11/732; 1.5%) (details are shown in Fig. 4 and Table 1).

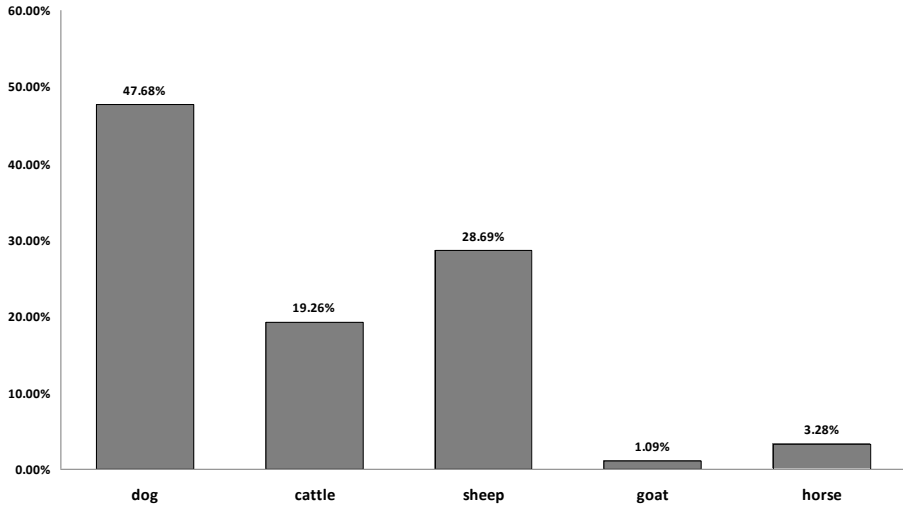

Fig. 3. Ticks distribution stratified by domestic host

Among ticks collected from dogs, *R. sanguineus* was the predominant species (n = 262/349; 75.1 %), followed by *D. reticulatus* (n= 78/349; 22.3%) and *I. ricinus* (n= 9/349; 2.6%).

In cattle, the tick fauna was the most diverse, including six tick species, such as: *D. marginatus* (n= 45/141; 31.9%), followed by *R. annulatus* (n= 34/141; 24.1%), *I. ricinus* (33/141; 23.5%), *R. bursa* (14/141; 9.9%), *H. marginatum* (n= 11/141; 7.8%) and *H. punctata* (n= 4/141; 2.8%).

In sheep the predominat tick species was *R. bursa* (n= 98/210; 46.7%), followed by *D. reticulatus* (n= 62/210; 29.5%), *D. marginatus* (n = 47/210; 22.4%), *I. ricinus* (2/210; 0.95%), and *H. punctata* (n= 1/210; 0.5%). In goats, three tick species were identified, namely *I. ricinus* (4/8; 50.0%), *D. marginatus* (3/8; 37.5%) and *H. punctata* (n= 1/8; 12.5%), respectively.

In horses *D. reticulatus* (n =21/23; 91.3%) and *D. marginatus* (n=3/24; 12.5%) tick species were found.

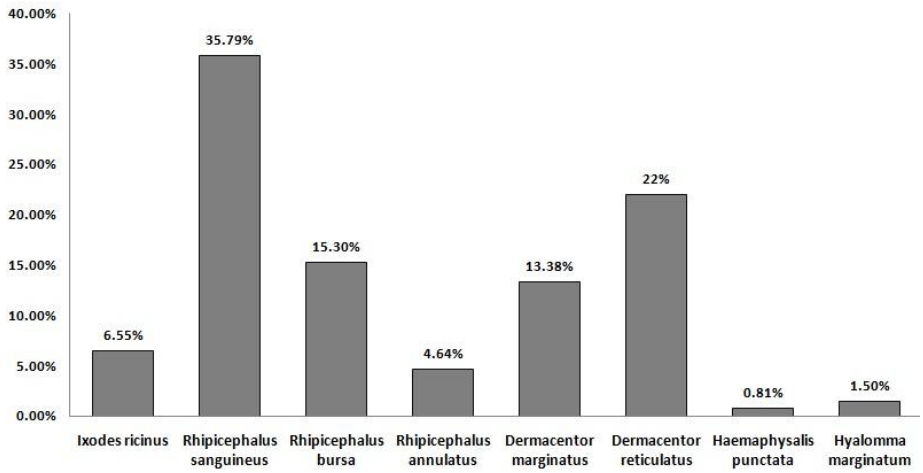

Fig. 4. Distribution of tick species collected from southern Romania

Our data shows that southern Romania is an area with a wide diversity of ticks fauna. Among them, *Rhipicephalus* spp., *Dermacentor* spp. and *Ixodes ricinus*, are the most common and widespread species. Romanian fauna comprise 25 species of ticks with a zoogeographical range restricted by host and climatic factors (6, 9).

In recent years the changes in climate, modifications of ticks habitat and massive movement of people and goods but also acquisition of resistance to insecticides determine modifications in the ecology of ticks (2, 11, 12). Many tick species are important vectors for different pathogens of both medical and veterinary importance. *I ricinus* and *I. scapularis* are responsible for anaplasmosis, babesiosis, and Lyme disease; *Dermacentor* spp. is the most commonly identified species responsible for transmitting *Rickettsia rickettsii* and also tularemia while *R. sanguineus* is the major vector for the Rocky Mountain spotted fever (10).

Other previously study in Romania showed that zoonotic pathogens such as *Rickettsia* ssp., *A. phagocytophilum, Borrelia afzelii*, and *Babesia microti* were found in *I. ricinus* suggesting that this ticks are a major reservoir for transmitting bacterial and protozoan pathogens with the potential of causing both animal and human diseases (8).

Kalmar et al., (2013) analysed more than 12.000 *Ixodes ricinus* ticks from all country of Romania and found that 1.4% are infected with *Borrelia burgdorferi* s.l. Furthermore, by reverse line blot hybridization and RFLP they identified three *Borrelia* genospecies: *B. afzelii*, followed by *B. garinii* and *B. burgdorferi sensu s.s* (10).

However, data concerning the prevalence of ticks and tick borne diseases present in animals and humans in Romania are still limited, not only *Ixodes ricinus* is vector for different pathogens, but also other genera could causes emerging diseases. Therefore our study will continue by collecting more ticks and further analyses to detect the pathogens transmitted by all these vectors.

Table 1. Details on the tick species collected from different areas in Southern Romania, stratified by stage, host, and geographic location.

	Species								
	Ixodes ricinus	*Hyalomma marginatum*	*Dermacentor marginatus*	*Dermacentor reticulatus*	*Haemaphysalis punctata*	*Rhipicephalus annulatus*	*Rhipicephalus bursa*	*Rhipicephalus sanguineus*	Total number of ticks
Stage									
Adult male	13	6	30	99	1	3	46	100	298
Adult female	35	5	68	62	5	31	66	162	434
Host									
Dogs	9	-	-	78	-	-	-	262	349
Cattle	33	11	45	-	4	34	14	-	141
Sheep	2	-	47	62	1	-	98	-	210
Goats	4	-	3	-	1	-	-	-	8
Horses	-	-	3	21	-	-	-	-	24
[a]Location									
B	9	-	-	2	-	-	-	126	137
IF	30	-	57	90	4	-	14	136	331
GR	-	-	41	27	-	-	-	-	68
PH	9	-	-	42	-	-	-	-	51
TL	-	11	-	-	2	24	-	-	37
CT	-	-	-	-	-	10	-	-	10
OT	-	-	-	-	-	-	98	-	98

[a] The letters correspond to the counties: B- Bucharest; IF-Ilfov; GR-Giurgiu; Ph-Prahova; TL-Tulcea; CT-Constanta, OT-Olt

Conclusions

In conclusion, our data suggest that the southern Romania is an area with a wide diversity of tick fauna with a great vectorial potential for transmitting tick-borne disease, some of them of zoontic concern. Uncovering the ecology and distribution of ticks in relationship with the host could greatly improve the knowledge of tick-borne diseases.

Acknowledgement

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PREVALENCE OF NONTUBERCULOUS MYCOBACTERIA ISOLATED FROM CLINICAL SPECIMENS IN IASI COUNTY

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Abstract

Mycobacteria species other than members of Mycobacterium tuberculosis complex are called non-tuberculous mycobacteria (NTM), "atypical" mycobacteria or mycobacteria other than tuberculosis (MOTT). They are usually opportunistic pathogens with variable degrees of virulence. There is a increasing awareness that nontuberculous mycobacteria (NTM) are becoming more prevalent. Nontuberculous mycobacterial infections could be seen as an emerging public-health threat. The aim of the present study was to emphasize the need to isolate the NTM strains in various clinical specimens, and to provide a general overview of infections situation in Iasi county. The situation of cases were analysed according to the isolation rate of NTM isolated at the Clinical Hospital of Pulmonology in Iaşi during the period 2010-2014. All mycobacterial isolates were identified as Nontuberculous mycobacteria (NTM) or Mycobacterium tuberculosis using the conventional methods as the gold standard and by immunochromatographic assay. The isolation rate of NTM was determined as 13,93% in 2010, 20.49% in 2011, 21.31% in 2012, 25.40% in 2013 and 18.85% in 2014 among the mycobacteria culture positive of samples. These results indicated that the prevalence rate has a tendency to increase over the course of the study. The importance of nontuberculous mycobacteria (NTM) infections in Romania must present a high attention because the species spectrum of NTM remains unknown.

Keywords*: Nontuberculous mycobacteria, prevalence, diagnosis*

Introduction

Although it is known that the members of the *Mycobacterium tuberculosis* complex (MTC) are responsible for the majority of mycobacterial infections worldwide, environmental opportunistic infections due to non-tuberculous mycobacteria (NTM) are increasing in recent years and becoming more of public health challenging (Clovice Kankya *et al*., 2011; van Ingen J. *et al*., 2009).

Non-tuberculous mycobacteria (NTM) or so-called atypical mycobacteria or mycobacteria other than tuberculosis (MOTT) have been recognized since Koch's time (Brown-Elliott BA *et al*., 2005; Rosa Maria Carvalho Ferreira *et al*., 2002), but historically they were overshadowed by tuberculosis and dismissed as contaminants (Ting-Shu Wu *et al*., 2009).

NTM include both slow growing mycobacteria (SGM) when colony formation are needed in seven days and the rapid growing mycobacteria (RGM), term which was defined by Runyon (Runyon EH, 1959, 1970) that forming colonies in less than seven days (Clovice Kankya *et al*., 2011; Schroder KH. *et al*., 1997; van Ingen J. *et al*., 2009).

At present more than 130 species of NTM have been identified (E. Tortoli, 2009) approximately 60 of which are suspected or known to be pathogenic (Brown-Elliott BA *et al*., 2005; Jarzembowski J. and Young M., 2008) they have been frequently isolated from water, but also from soil, dust and plants (Falkinham JO, 1996).

The NTM most frequently potentially pathogenic mycobacteria are *M. avium*, *M. intracellulare*, *M. kansasii* (Echevarria şi colab., 1994), *M. xenopi*, and *M. abscessus* (Clovice Kankya *et al*., 2011; Dailloux M. *et al*., 2010; Falkinham JO., 1997).

Furthermore, an increasing number of NTM previously considered non-pathogenic, have proved to cause infections in animals and humans (Clovice Kankya *et al*., 2011; Falkinham JO, 2009; von Reyn CF *et al*., 1993).

NTM are soil and water organisms, and infection is from the environment rather than transmitted from person-to-person, with very rare exceptions. Due to their nearly ubiquitous presence in municipal water supplies, exposure to NTM is common (Brian A. Kendall *et al*., 2013). Further, NTM may affect the respiratory tract without causing disease (Brian A. Kendall *et al*., 2013).

Although it is considered that NTM were not traditionally considered a threat to public health, as person to person transmission occurs rarely if at all, yet these organisms can produce serious morbidity. In addition, are becoming more difficult to diagnose or treat, especially when are drug-resistant strains (Somoskovi *et al*., 2002; WHO, 2000; Wallace *et al*., 1997).

The purpose of the present study was to determine the prevalence of the NTM isolates at the Clinical Hospital of Pulmonology in Iaşi, Romania, during the period 2010 to 2014.

Materials and methods

Samples collection. The isolates from the Bacteriological Laboratory of the Clinic of Pulmonary Disease in Iasi during the 5-year period from January 2010 to September 2014 were collected and analyzed.

In this study all identified NTM isolates were considered to be significant. Thus, were included the isolates of NTM from all clinical specimens from pulmonary and extra pulmonary received between 2010 and 2014.

The specimens were represented from sputum, broncho alveolar lavage, bronchial wash and gastric juice were received from patients with results suggestive of tuberculosis.

From children was collected biopsy of axillary lymph nodes with lymphadenitis and abscesses.

Sterile body fluids such as pleural fluid and others were collected from patients suspected of disseminated mycobacterial infections.

Isolation of NTM. All specimens were collected with aseptic precautions in sterile containers and transported to the laboratory.

Clinical specimens were stained with auramine-rhodamine (AR) and Ziehl-Neelsen (ZN) stain. The results of the smear microscopy were reported semiquantitatively.

For culture examination samples were concentrated and decontaminated using 4% sodium hydroxide (Petroff), 15-20 min, before cultivation. For neutralisation was used HCl 8%, and blue-bromtimol like pH marker.

After neutralization the processed specimens were centrifuged and the sediment were plated onto Lowenstein Jensen medium, and/or on Middlebrook liquid medium. For shorter incubation times and for confirmation the results was used cultivation on MB/BacT bottles (bioMérieux, France), incubated at automated MB/BacT system (Organon Teknika).

The inoculated tubes were incubated at 37°C and then inspected weekly for 6 weeks.

The culture-positive tubes were examined by ZN staining for a possible contamination and to investigate the presence of acid-fast bacilli (AFB).

Interpretation and expression of the results of Lowenstein Jensen culture growth was performed as for microscopic examination, semiquantitatively (Daniela Homorodean *et al*.,

2005; European Centre for Disease Prevention and Control, 2011), taking into account the bacterial growth medium.

Identification of isolates. All AFB isolates were assessed to distinguish between *M. tuberculosis* and NTM, according to growth rates, colonial morphologies, and pigmentation, and by MPT64 antigen assays with immunochromatographic test capable to discriminate between Mycobacterium tuberculosis complex (MTBC) from NTM (SD BIOLINE TB Ag MPT 64 Rapid®", manufactured by Standard Diagnostics, Seoul, South Korea).

Results and discussions

A total of 122 clinical NTM isolates were included in the study. Of these, 101 NTM (82.78%) were isolated from pulmonary specimens (sputum, bronchial wash, bronchoalveolar lavage fluid and gastric fluid) and 21 NTM (12.21%) from extrapulmonary specimens (pus, tissue biopsies, axillary lymph nodes). Distribution of NTM isolated from all specimens between 2010 and 2014 is shown in the Table 1.

Table 1.

Annual distribution of NTM from different clinical specimens

Specimens	**2010**	**2011**	**2012**	**2013**	**2014**
Sputum	10	14	16	19	9
BAL		1	1	3	1
Bronchial wash	5	9	2	3	8
Biopsy			3	3	2
Gastric juice	2	1	1	-	3
Pus			3	3	-
Total	**17**	**25**	**26**	**31**	**23**

The pulmonary and extrapulmonary cases was 82.79% and 17.21% with NTM infections which have increased significantly (Figure 1.A). About 90% of cases involved the pulmonary system; the rest involve lymph nodes, soft tissues and pus.

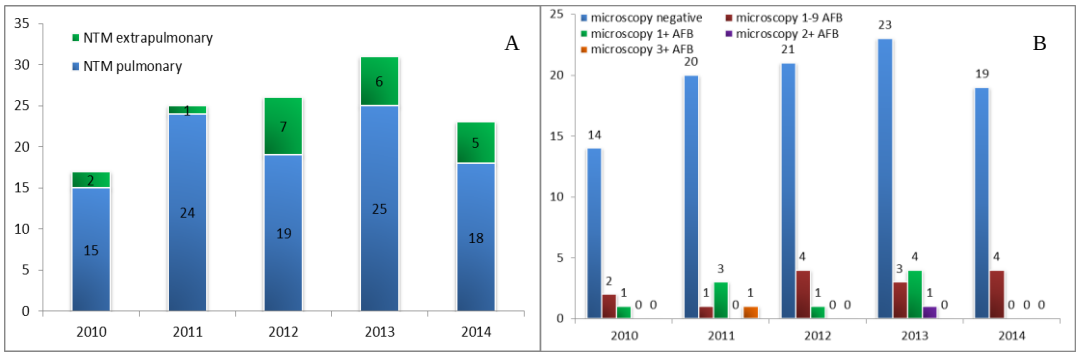

Fig. 1.A - Annual number of pulmonary and extrapulmonary NTM infection;
B - Semi-quantitative examination after ZN at NTM.

After the semi-quantitative evaluation of the results of microscopy 21.32% the acid fast bacilli were positive and 78.68% smears were negative, as presumptive diagnosis of

mycobacterial infection (Figure 1.B). Instead, the number of isolates of NTM were different, after the semi-quantitative evaluation of the results of culture. Thus, were cultures positive 88.52% and 11.48% were negative (Figure 2.A), but positive after incubation in the automated system MB/BacT.

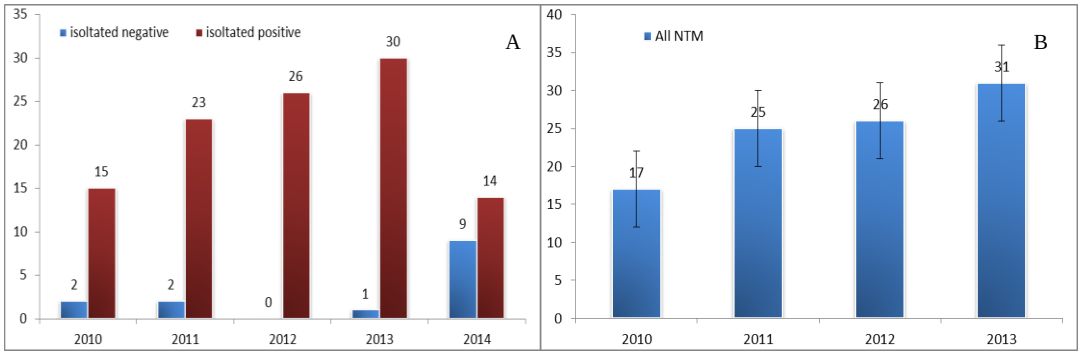

Fig. 2.A - Annual number of isolates at NTM;
B - Isolation rate of NTM from clinical specimens during the period 2010-2013

The median age of the study with positive NTM cultures was 60.5 years, most patients with disease were > 60 years of age (Figure 3.A); 54.10% specimens were isolated from males and 45.90% specimens were isolated from females (Figure 3.B).

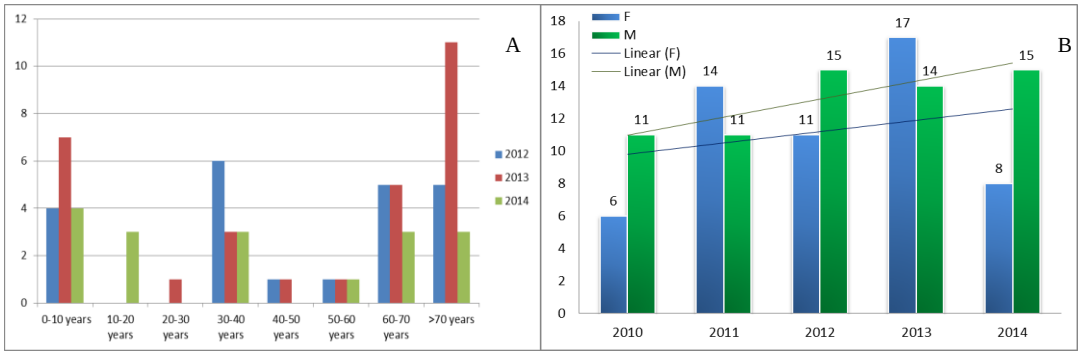

Fig. 3.A - The median age of the patients with positive NTM
B - Isolation rate of NTM from males and female

The isolation rate of NTM was determined as 13,93% in 2010, 20.49% in 2011, 21.31% in 2012, 25.40% in 2013 (Figure 2.B) and 18.85% in 2014 among the non-tuberculous mycobacteria culture positive of samples.

Data from NTM isolates among mycobacteria positive cultures done in 2010 and in 2013 showed that the isolate rate had increased with 11.47%. Frequency of cases with nontuberculous strains are low in urban and in rural areas is increased.

During the 5 year period, has increased the prevalence of NTM strains significantly. In concordance with the increased of NTM isolations, incidence of NTM diseases also increased considerable.

A reason for the increase can be by improving of cultivation methods using the broth medium in systems automat.

There is findings wich showing that liquid medium based by system are more sensitive than solid media based systems for recovering NTM (Rishi S., *et al*., 2007). Thus, to a certain extent, the increase in detected cases of disease due to NTM may reflect an improvement in methodology for diagnosis with a isolation and more rapid identification of NTM.

In population of this study, the older patients were with a significant number of NTM cases. This is explained by the fact that immune function, and in particular cellular immune function, declines with age, and there is every reason to think that older people are more susceptible to developing NTM than younger people (Sugihara E., *et al*.. 2007).

Infections with NTM are not reportable to public health authorities, thus, epidemiological and surveillance data are not readily available. . Epidemiologic factors of NTM diseases are particularly important in diagnosis and management when compared to other diseases. Nonetheless, the prevalence of pulmonary NTM disease has increased dramatically in the globally over the past 3 decades. (Brian A. Kendall *et al*., 2013).

Conclusion

The study reveals a clear increase in the prevalence of NTM infection in Clinical Hospital of Pulmonology in Iaşi during the past five years.

The studies should focus on the the presence and of species diversity of nontuberculous mycobacteria (NTM) for prevention of infections.

The importance of nontuberculous mycobacteria (NTM) infections in Romania must present a high attention because the species spectrum of NTM remains unknown.

In conclusion, regular documentation and reporting of these NTM is essential to be aware of the possible spectrum of diseases associated.

Acknowledgments

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STRUCTURAL ANALYSIS OF NEW MUTATIONS IN KatG SELECTED ON ISONIAZID-RESISTANT STRAINS OF *MYCOBACTERIUM TUBERCULOSIS IN VITRO*

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Abstract

Isoniazid (INH) or hydrazide of isonicotinic acid, is a highly effective drug of first-line treatment of tuberculosis. Resistance to INH is due to acquisition of mutations in the KatG protein (the catalase-peroxidase), particularly in position 315 (Ser315Thr, representing 70% of strains), and also in the InhA protein (the enoyl-acyl carrier protein reductase) implicated in biosynthesis of mycobacterial cell wall or his promoter. The structural bases for INH-resistant mutations in the katG gene are still poorly understood. Considering this appearance, the aim of this study was to analyze at the structural level the new mutations in KatG protein, selected from strains resistant to isoniazid. The molecular role of these mutations have never been characterized before. For the new mutations, the consequences and the position of these in the katG were modeled from the crystal structure of the Mycobacterium tuberculosis KatG protein. The crystallography analyses will hopefully shed better light the molecular modeling of new katG mutations selected in vitro, which can be extrapolated to the clinical setting. The mutations showed a reduction in catalase activity or even the loss of all enzymatic activities of KatG.

Keywords: *Isoniazid, Mycobacterium tuberculosis, katG*

Introduction

Isoniazid (isonicotinic acid hydrazide, INH) is a prodrug, the first line antibiotic used against *Mycobacterium tuberculosis* infections (Robitzek *et al*., 1952). Isoniazid is the most widely used antituberculosis drug since the recognition of its clinical activity in the early 1950s. INH has a powerful bactericidal activity against *M. tuberculosis* (Brossier, 2011).

The mode of action of isoniazid is not completely understood, but evidence that certain isoniazid resistant strains of mycobacteria have reduced catalase peroxidase (KatG) suggested that the pro-drug is acted upon by this enzyme (Chouchane *et al*., 2000).

The molecular basis of the mechanism INH-resistance is less well characterized, and mutations in several genes have been associated with it (Ramaswamy *et al*., 1998).

Mutation in *katG* gene coding for catalase-peroxidase-KatG is a major mechanism of INH resistance (Heym *et al*., 1995; Zhang *et al*., 1992) KatG presents a key role in activating the prodrug INH, an important drug for tuberculosis (Unissa *et al*., 2014).

It is considered that approximately 80% of INH-resistant clinical isolates, presents mutations missense, partial or complete deletions of *katG* gene, with the mutation Ser315Thr representing 70% of the strains, associated with a high level of isoniazid resistance (Brossier, 2011).

It has been demonstrated that the mutations in *inhA* gene or its promoter region can cause also isoniazid resistance, with promoter mutations that are more frequent than mutations in the structural gene (Musser et al., 1996).

Thus, the mutations in the promoter region of the *inhA* gene are 15 to 35%, and mostly in -15c→t and a percentage of 0-5% resistant clincal strains INH with mutation in the *inhA* gene, associated with a low level of isoniazid resistance (Brossier, 2011).

It is considered that a number of isolates clinical resistant to INH namely the less than 5% of strains have no mutations in the genes known to be involved in resistance to INH (Brossier *et al*., 2009; Hazbón *et al*., 2006).

The KatG is a homodimer with the 81 kDa the subunits (740 amino acids), each subunit being linked to a heme b (Johnsson *et al*., 1997). The site active which contain proteins of the heme to the proteins of KatG has a proximal portion and a distal portion on both sides of the heme with the almost identical amino acid positions (Smulevich *et al*., 2006). Thus, the triad of of His-Trp-Asp in the part proximal (His270, Trp321, Asp381) and the triad of His-Arg-Trp in the part distal (His108, Arg104, Trp107) are conserved (Bertrand *et al*., 2004).

The aim of this study was to perform a crystallographic analysis of the mutations in KatG selected on isoniazid-resistant strains of *Mycobacterium tuberculosis* obtained *in vitro*. For these mutations were performed structural and biochemical studies to determine their resistance profile.

Materials and methods

For the molecular modeling of mutations identified in vitro were included the following amino acid substitutions in katG: P232L, T394M, T275I, R484H and D137Y.

In order to model the position and the consequences of new mutations detected in KatG were studied using the crystallographic structure of the Mycobacterium tuberculosis KatG protein (Zhao et al., 2006; PDB, 2CCA) with software Pymol.

Results and discussions

The KatG protein presents a covalently bound heme is surrounded by proximal and distal pockets.

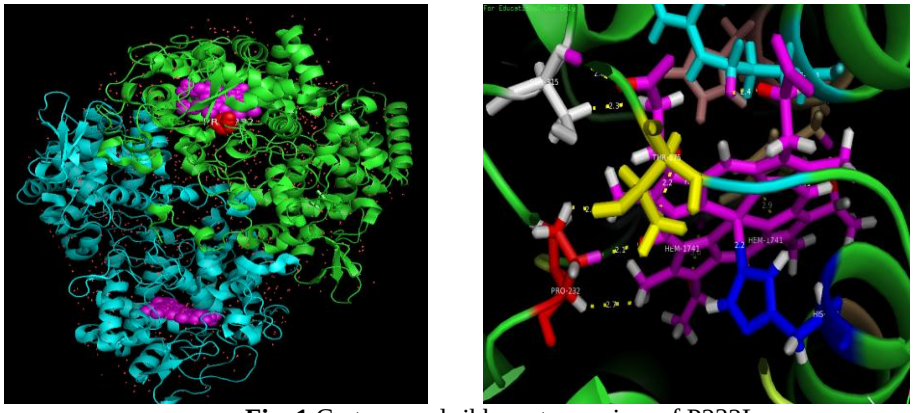

Fig. 1 Cartoon and ribbon stereo view of P232L,
including the heme with the almost identical amino acid positions

The missense mutation P232L presents a great interest, because P232 is part of the proximal pocket and is almost of the heme (Figure 1). Therefore, produce a possible loss to

the covalent bond due to the proximity of the heme and obligatory the catalytic activity of KatG is lost.

The T394M mutation associated with the high levels of INH-R is located in an alpha-helix located near the proximal pocket of the KatG, fails to produce a steric hindrance in this region (Figure 2).

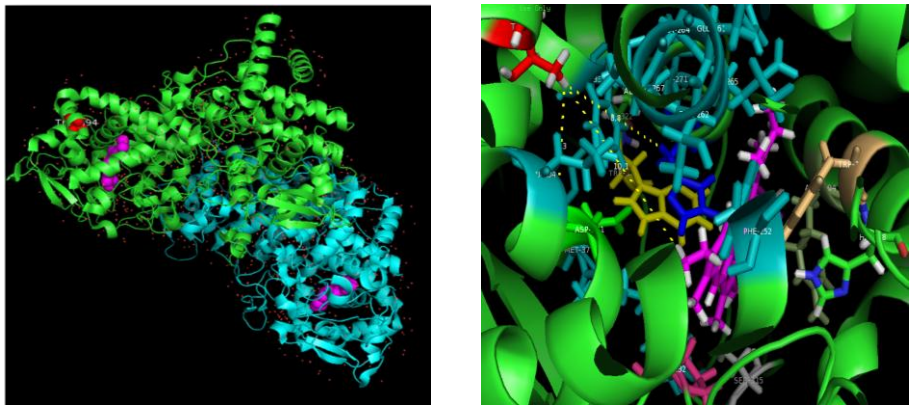

Fig. 2 Cartoon and ribbon stereo view of T394M
including the heme with the almost identical amino acid positions

Another mutation T275I protein located in the the proximal pocket of the protein, near the heme, resulted in a possible loss of catalytic activity (Figure 3).

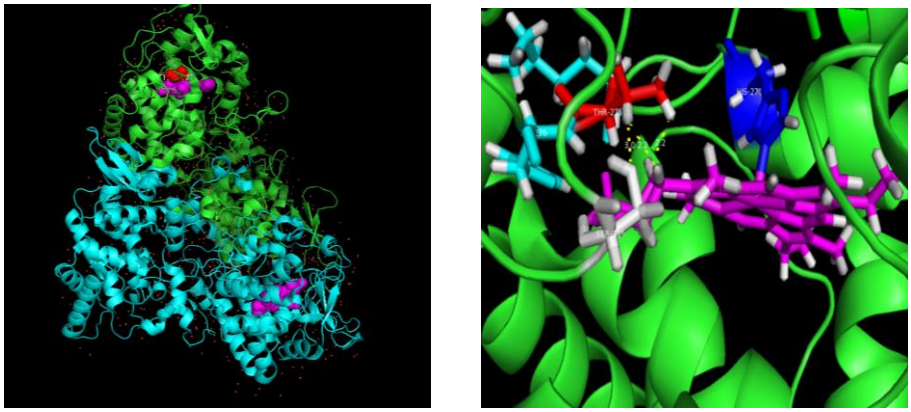

Fig. 3 Cartoon and ribbon stereo view of T275I,
including the heme with the almost identical amino acid positions

The other mutation (R484H) was located in the alpha-helix positioned away from the heme probably contributes to the destabilization of alpha-helix of the secondary structure (Figure 4).

Finally, D137Y has formed together with Ser315 a short and narrow channel (Figure 5) which extends from the surface of the enzyme near to pyrrole IV propionate of the heme distal side, thus succeeding to penetrate the heme.

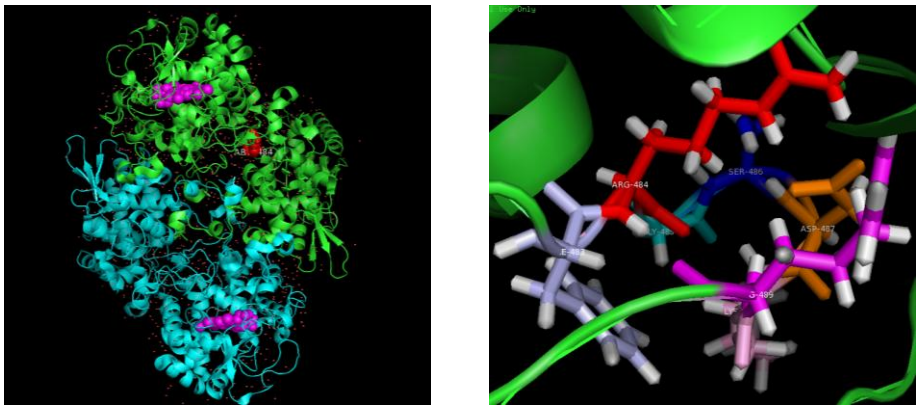

Fig. 4 Cartoon and ribbon stereo view of R484H,
including the heme with the almost identical amino acid positions

The peroxidatic activation is regulated by separations of residues Ser 316 and Asp137 (Figure 4) by an heme access channel. Thus, the steric restriction is blocked by this channel, but the residues attenuates this bottleneck (Zhao *et al*., 2013).

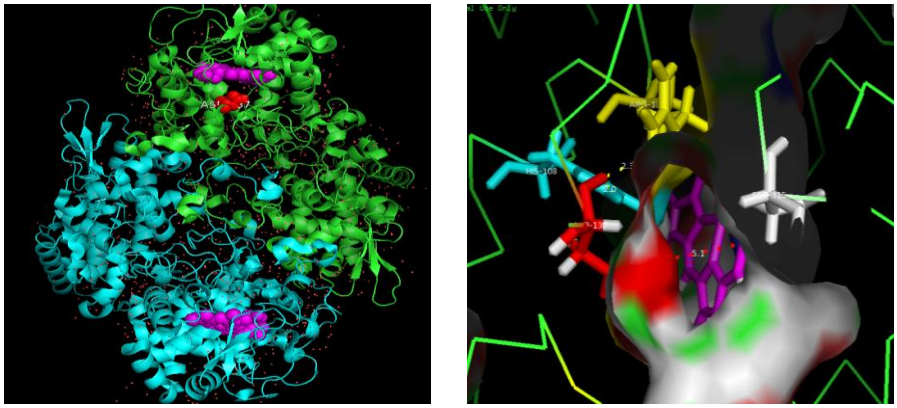

Fig. 5 Cartoon and ribbon stereo view of D137Y with S315,
including the heme and acces channel

From the results described the construction of the crystal structure of *M. tuberculosis* KatG (Bertrand *et al*., 2004) represents a major breakthrough in the explanation of the molecular mechanism of INH activation with the structural similarity both in the overall structures and at the active sites of KatG (Unissa *et al*., 2014).

The resistance conferring mutations further away from the heme active site also are exhibited interesting reactivity patterns (Cade *et al*., 2009).

The residue location is a critical role in determining INH resistance mechanisms associated with INH activation. The different mutations at the same location can produce vastly different reactivities that are oxidant-specific (Cade *et al*., 2009).

The study of these mutations allow the establishment of a relationship between the level of INH resistance and the catalytic effects observed in the protein structure of KatG through this comparative study between the mutations.

Conclusion

P232L and T275I showed reduction of enzymatic activities in agreement with the molecular modeling.

D137Y, residue of a heme access channel, attenuated the enzymatic activity (peroxidase) of KatG.

For the remaining mutations, the enzyme activities were not significantly reduced with a role that is difficult to specify.

More studies of the molecular modeling must exist in order to improve the understanding of the mechanisms of resistance to INH.

***Acknowledgments**:* The authors are thankful to the Pitié-Salpêtrière Laboratory of Bacteriology of Paris for conducting the research work. This work was supported from the Project: "Improvement and Development of Human Resources for Research and Innovation by Doctoral School'', Contract: POSDRU – CPP107-DMI1/5/S/77222; University of Agricultural Sciences and Veterinary Medicine, Iaşi, Romania.

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BOVINE TUBERCULOSIS. CASE STUDY

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Abstract
Bovine tuberculosis is a infecto-contageous disease caused by Mycobacterium bovis ,usually evolving chronic and endemic.The clinical picture is polymorph ,and the anatomopathological picture is characterized by granulomatous lesions or exudative caseous. The reasearches were conducted in a herd of 12 cattle (8 adults and 4 youth 10-16 months) existing in a non- professional holding in which for two years have not been conducted tuberculin vaccination.For bacterioscopic exam fingerprint smears were made from lymph nodes stained by Ziehl-Neelsen and the histological sections were made from lymph nodes and lungs stained by the method HEA. In the young animals and adult animals the lymph nodes mentioned were increased in volume, and on the section the lesions found were hyperplastic macrocelular and caseous. The caseous lymphadenitis considered the most serious injury, lymph nodes were enlarged, and on the sectional area was observed in some animals a radial cazeous appearance or total lymph nodes caseification. In limphoreticulitis hyperplastic were highlighted giant cells with multiple nuclei arranged at the periphery (Langhans cells).
Key words: *tuberculosis, bovine, Mycobacterium*

Bovine tuberculosis is a infecto-contageous disease caused by Mycobacterium bovis ,usually evolving chronic and endemic.The clinical picture is polymorph ,and the anatomopathological picture is characterized by granulomatous lesions or exudative caseous (3).

In cattle the disease occurrence and evolution are influenced by many factors, grouped into two categories, namely intrinsic andextrinsic factors.As a result of control measures,including tuberculin vaccination ,the disease evolves as primary infection,with the formation of the primary localized frequently in the lungs(primary emotion) and bronchialand mediastinal lymph nodes complex (2,4).

Materials and Methods

The reasearches were conducted in a herd of 12 cattle (8 adults and 4 youth 10-16 months) existing in a non- professional holding in which for two years have not been conducted tuberculin vaccination.

The following year, at the single test conducted on the entire population, all animals reacted positively and on the comparison test were framed simultaneously in the positive category.

Slaughter inspection was carried out legally, the animals were sacrificed and the lungs and lymph nodes sampled for laboratory examination (bacterioscopic and histology).

For bacterioscopic exam fingerprint smears were made from lymph nodes stained by Ziehl-Neelsen (1) and the histological sections were made from lymph nodes and lungs stained by the method HEA (3).

The results

Animals were slaughtered legally being considered the pathological organs affected frequently by the disease.

Macroscopic lesions characteristic of tuberculosis were absent in lungs and other organs, are present only in bronchial and mediastinal lymph nodes.

In the young animals and adult animals the lymph nodes mentioned were increased in volume, and on the section the lesions found were hyperplastic macrocelular and caseous.

In hyperplastic lymphadenitis the nodes were enlarged with increased consistency with section surface look glossy and fat (Fig. 1).

The caseous lymphadenitis considered the most serious injury, lymph nodes were enlarged, and on the sectional area was observed in some animals a radial cazeous appearance or total lymph nodes caseification (Fig. 2, 3).

Nodular lymphadenitis (granulomatous) or areas of calcification was absent.

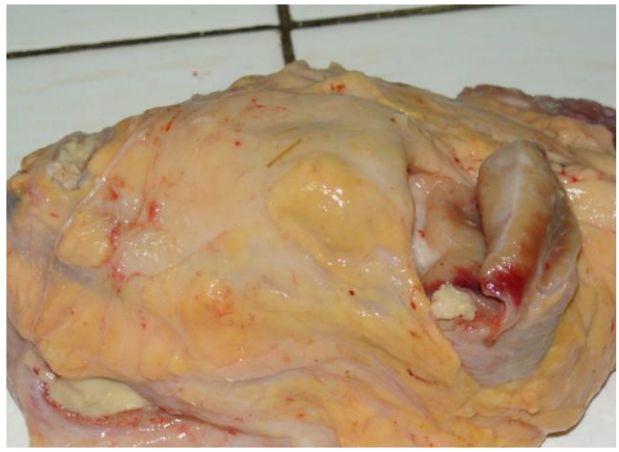

Fig. 1 Hyperplastic lymphadenitis, the lymph node

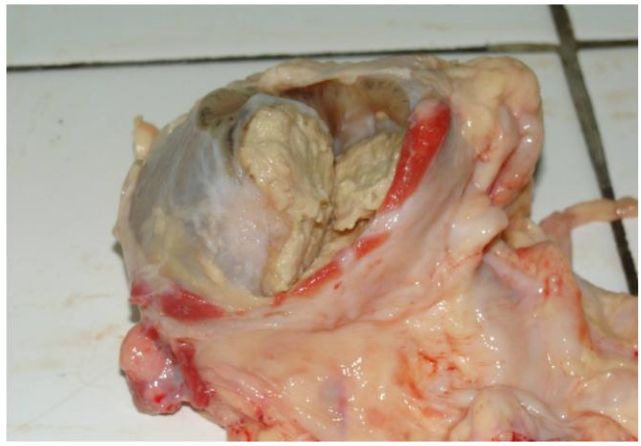

Fig. 2 The caseous lymphadenitis

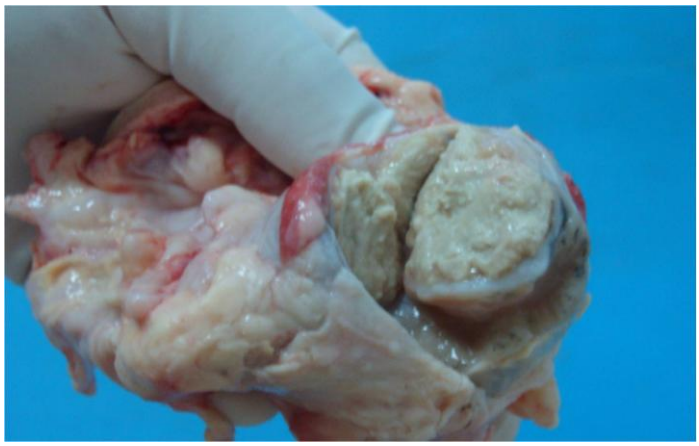

Fig. 3 The caseous lymphadenitis

Histological examination revealed microscopic lesions characteristic of tuberculosis, only in the lymph nodes, in other organs they were absent.

In limphoreticulitis hyperplastic were highlighted giant cells with multiple nuclei arranged at the periphery (Langhans cells). It was also identified areas of fibrosis conjunctiva (activated fibroblasts) well defined and infiltration of neutrophils, lymphocytes and epithelioid cells. Because in the histological sections examined was observed only Langhans type giant cell presence and did not reveal areas of necrosis or foci of calcification,the aspects characteristic for tuberculous granuloma in chronic form, we can say only that they were snapped early forms of TB characteristic for hyperplastic lymphoreticulitis.

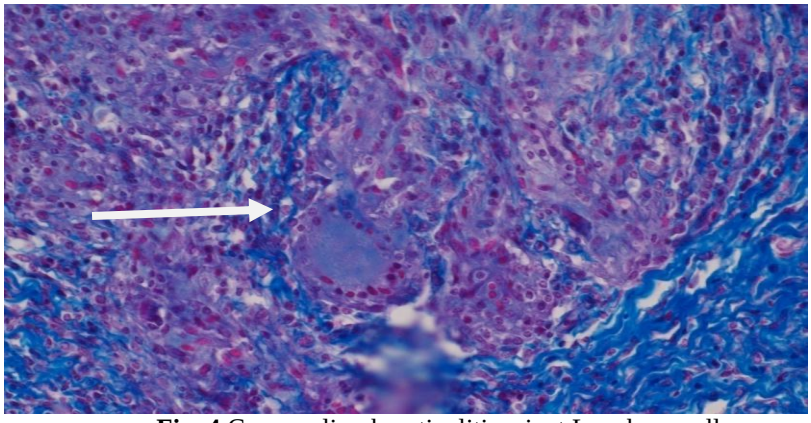

Fig. 4 Caseous limphoreticulitis, giant Langhans cells

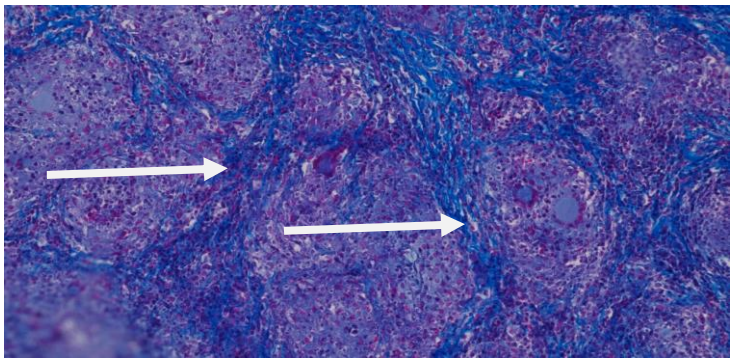

Fig. 5 Caseous limphoreticulitis, giant Langhans cells

In the lymph nodes with caseous lesions were highlighted only caseous histological lesions without differentiating the tuberculous granuloma structure .

These lesions argues for the existence of a chronic evolution of tuberculosis in these animals. In the lungs and other organs were not present histological lesions characteristic.

By bacterioscopic examination rare mycobacteria were found only in smears made from the lymph nodes with hyperplastic lesions.

The results confirm the development of tuberculosis in a herd of cattle (youth and adult) characterized by hyperplastic lymphoreticulitis specific to an acute primary infection and a caseous lymphoreticulitis specific to a chronic developments. Also it can be said that in this outbreak of tuberculosis it had a particular evolution because the lesions were present only in bronchial and mediastinal lymph nodes without affecting the lungs. This development is incomplete primary complex type (Fig. 4, 5).

Conclusions

In young animals aged 10-16 months were found tuberculous lesions only in lymph nodes both macroscopic and microscopic as being characteristic for the hyperplastic macrocelulare lymphoreticulitis.

In adult animals the tuberculous lesions were present only in the lymph nodes characteristic for caseous llymphoreticulitis type.

In lymph nodes and in the lungs and other organs were not identified microscopic lesions of granulomatous type.

Bacterioscopic examination revealed only small number of mycobacteria in smears made from the lymph nodes with hyperplastic lymphoreticulitis lesions.

In the investigated outbreake the tuberculosis evolved with specific injuries located in lymph nodes characteristic of acute infection and chronic infection, depending on age.

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ASSESSMENT OF THE ANTIMICROBIAL RESISTANCE FOR THE *E. COLI* STRAINS ISOLATED FROM CHICKEN CARCASSES FROM HYPERMARKETS

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Abstract

The research is an endeavor to assess the E. coli contamination burden on chicken carcasses and to determine the antimicrobial resistance. The research was made on 50 chicken carcasses purchased from hypermarkets, from different producers and stored in the same conditions. The microbial burden ranged from 1.6 x 10^3 to 3.76 x 10^4. Noteworthy, the E. coli strains isolated from the surface of the carcasses were resistant in different percentages to the tested antibiotics: 100% to enrofloxacin and ciprofloxacin, 60% to ampicillin and ticarcillin, 60% to sulfamethoxazole and trimethoprim, 40% to chloramphenicol, 20% to gentamicin and amikacin. The disk diffusion method (CLSI M 100 S-18) identified 60% from the tested E. coli strains as multidrug resistant (MDR), a fact with a high importance for human health.

Key words: *E. coli, chicken carcasses, antimicrobial resistance.*

Introduction

Is a global well known fact that bacterial infections became almost impossible to treat and to eradicate due to the effect that antimicrobial resistance is indirectly proportional with the antimicrobial discovery (Jansen et al., 2014). While the discovery and release on the market of new drugs is heavily challenged and takes place at a small pace, on the other hand, there are recent scientific reports on the emergence of multi (MDR) and extensively drug resistant (XDR) bacteria (WHO, 2012).

The emergence and the spread of resistant pathogenic bacteria are considered to be the effect of extended injudicious use of antibiotics in agriculture, in veterinary and human medicine (Aarestrup, 2005; Rodriguez-Rojas et al., 2013).

The appearance of the multi-resistant food borne pathogens represents a great risk for public health determining difficult to treat infections to humans. Faye K., 2007, describes the risk for resistant microorganisms to contaminate the food.

Material and Methods

For the experimental design there were purchased a total of 50 fresh chicken carcasses, 10 carcasses at 5 different intervals of time (n = 50), within 3 months time period, from April to June, from various producers, commercialised in hypermarkets and stored in same conditions (temperature of 4° C).

The antimicrobials (BioRad, France) used were at a standard concentration, belonging to groups A, B and C as recommended by the CLSI, M100 S18, 2008.

The determination of the bacterial burden from chicken carcasses was made by weighting 10g of skin harvested from carcasses (figure 1.), in sterile conditions and mixed with 90 mL 0.9% saline solution, for an initial tenfold dilution. Three subsequent decimal dilutions to 10^{-4} were made in order to better appreciate the contamination degree. The isolation was made by inoculation of 1 mL of the first three decimal dilutions, in duplicates, in VRBG agar medium (Oxoid LTD, Hampshire, England), double layer technique. The

inoculated plates were incubated at 37° C for 24 hours. After expiration of the incubation time, the determination of the bacterial burden was performed, by calculating the CFU from the three decimal dilutions, in duplicates.

Figure 1. The fresh chicken carcasses prepared for determination of the bacterial burden

From each VRBG agar medium plates, there were isolated between five and ten colonies in order to identify them and to perform susceptibility tests. The strains were inoculated on non selective media, incubated for 24 h at 37° C, then the strains were streaked on TBX agar (Oxoid LTD, Hampshire, England) and incubated at 44,5° C for 4 hours and agar Levine (Oxoid LTD, Hampshire, England) and incubated for 24 h at 37° C. The indole test was also performed in order to indentify the indole positive *E. coli* strains. There were selected and conserved on BHI broth (Oxoid LTD, Hampshire, England) with 20% glycerol at -80° C only the strains β-glucuronidase and indole positive, for susceptibility tests.

The susceptibility of the bacterial strains was assessed through Kirby-Bauer method (or disk diffusion antibiotic sensitivity testing) (Schwalbe *et al.*, 2007) as recommended by CLSI standard M100 S18, 2008.

Results and discussions

From all 50 fresh chicken carcasses was assessed the bacterial contamination burden (table 1.).

The contamination burden identified was from 1.3 x 10^3 to 1.7 x 10^4. The identified bacteria were coliforms belonging to *Enterobacteriaceae* family and identified as being *E. coli* and bacteria from different genera that were identified with usual biochemical tests API 20E (BioMerieux, France).

Table 1. The bacterial contamination burden on fresh chicken carcasses.

Samples n=50	Bacterial burden
Sampling I	1.7 x 10^4
Sampling II	2.4x10^3
Sampling III	1.8x10^3
Sampling IV	1.3x10^3
Sampling V	2.3x10^3

After the isolation of pure bacterial strains, there were selected only the beta-glucuronidase and indole positive bacteria identified as *E. coli* strains. The *E. coli* can be distinguished from other coliform bacteria by its growth and colour reaction on certain types of culture media. When cultured on EMB (Eosin Methylene Blue Agar – Levine's Formukation) plate, a positive result for *E. coli* is metallic green shine on a dark purple media, or blue colour on TBX agar.

There were isolated and conserved 80 *E. coli* strains for susceptibility testing. Therefore, each bacterial strain was considered susceptible, intermediary or resistant to a certain antibiotic.

The susceptibility was as follows: the *E. coli* strains were resistant 100% to the tested quinolones (enrofloxacin and ciprofloxacin, respectively); towards the beta lactam antibiotics the *E. coli* presented a different resistance percentage, thus reaching to 60% for ampicillin and ticarcillin, 11.25% for cefazolin and susceptible to piperacillin-tazobactam, amoxicillin-clavulanic acid, cefotaxime and cefoxitin. There were 5 strains (6.25%) which presented resistance towards aztreonam, cefpodoxime and ceftazidime indicating the presence of ESBLs (Sundin, 2009). All the *E. coli* strains were susceptible to imipenem.

The bacterial strains were resistant to sulfamethoxazole-trimethoprim up to 60% and to chloramphenicol to 40%. Only 16 strains of *E. coli* (20%) were resistant to gentamicin and amikacin. It may be said there are bacterial strains MDR in a percentage of 60% (Sundin, 2009).

These data are similar to the ones described by Garcia-Migura et al., 2014, for *E. coli* strains isolated from broiler chickens from different European countries, with a resistance percentage for ciprofloxacin from 50.8% to 56.2% between 2005 and 2011 in Netherlands. According to the data presented in their mini-review, the levels of resistance to third generation of cephalosporins in *E. coli* isolated from healthy animals appeared to have increased over the last years, especially in the poultry sector.

Conclusions

1. The contamination bacterial burden identified on fresh chicken carcasses was from 1.3×10^3 to 1.7×10^4.
2. There were 5 strains (6.25%) which presented resistance towards aztreonam, cefpodoxime and ceftazidime indicating the presence of ESBLs.
3. The *E. coli* strains were resistant 100% to the tested quinolones (enrofloxacin and ciprofloxacin, respectively).
4. The bacterial strains MDR were identified in percentage of 60%.

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MYCOPLASMOSIS IN QUAIL: ISOLATION AND IDENTIFICATION OF *MYCOPLASMA GALLISEPTICUM* AND *MYCOPLASMA SYNOVIAE*

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Abstract

The mycoplasmas are important pathogens responsable for causing diseases, not only in chickens and turkeys, but in quails as well. The objectives of this study were to isolate and identify Mycoplasma gallisepticum (MG) and Mycoplasma synoviae (MS) in quails with clinical and pathological signs of chronic respiratory disease in Northern Romania. 12 organ samples (brain, sinus, trachea, lungs, cloaca, testis and ovary) and blood sera were collected from two quail. Following ELISA test, both serums samples were positive for MG. After cultivation on specific medium was obtained nine primary cultures from sinuses, trachea, cloaca and brain and eight strains were isolated only from the sinuses and conjunctiva. Identification was made using molecular biology tests (PCR) using specific primers for MG, MS and Mycoplasma genus. All eight isolated strains were positive for MG, none of them being positive for MS. The results are in agreement with other similar research data, MG being the main species responsible for the occurrence of chronic respiratory disease in quail. Mycoplasmosis is a important disease in quail bred and it's control can prevents the spread of mycoplasmosis to other birds directly targeted such as chickens and turkeys and reduce economic losses.

Key words*: Mycoplasma gallisepticum, Mycoplasma synoviae, micoplasmas cultivation, PCR, quail.*

Introduction

Mycoplasma gallisepticum (MG) infections can cause significant economic losses on poultry farms from chronic respiratory disease, reduced feed efficiency, decreased growth and egg production. The carcasses of birds sent to slaughter may also be downgraded. MG causes disease in chickens, turkeys, and it also causes disease in other avian species including pheasants, partridges, bobwhite quail, and Japanese quail (Jordan, F.T.W et all, 1980)

First *Mycoplasma gallisepticum* infection in quail was described by Madden et al. (1967). They isolated the organism from a commercial flock of Bobwhite quail (Colinus virginianus) that had been affected by chronic respiratory disease. Thereafter have been several subsequent reports of cases of mycoplasmosis in quail.

Mycoplasma synoviae most frequently occurs as a subclinical upper respiratory infection but may result in airsacculitis and synovitis in chickens and turkeys (Kleven, 2003).

Culture combined with identification by immunological methods or PCR is still considered to be „the gold standard„ according to OIE **(OIE, 2008)**

The objectives of this study were to isolate and identify *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in quails (*Coturnix coturnix japonica*) with clinical and pathological signs of chronic respiratory disease (CRD),

Materials and methods

In this study, samples of organs and sera were used. Tow diseased quails (Figure 1) were picked from about two hundred household quails flock with different ages (5-24 months) in Northern Romania. In the same household were chickens and turkeys of different

ages and origins. Serum samples were collected from 2 diseased quails, 26 weeks old. Blood samples were aseptically collected from the wing veins using 5 mL sterile disposable syringes and needles. Blood was allowed to clot in the syringe at room temperature. After this, serum of each sample was transferred to sterile microtubes and kept at -20◦C until the moment of use.

For mycoplasma isolation, the tow diseased quails (Figure 1) were picked from the flock. Samples of organs were taken aseptically from conjunctivae, infraorbital sinuses, tracheae, air sacs, lunge, testicle, ovar and brain. The organs were conservate imediately in sterile microtubes at -70 C until cultivation.

Sera were analyzed for antibodies against MG and MS using a commercially Enzyme-Linked Immunosorbent Assay (ELISA) antibody test kit (*Mycoplasma gallisepticum* antibody Test Kit and *Mycoplasma synoviae* antibody Test Kit (Idexx Laboratories, USA) according to the manufacturer's instructions. Samples were diluted five-hundred fold (1 : 500) with the diluent, and 100 µl of each sample was dispensed in a well of a plate previously coated with MG/MS antigen. The plate was incubated for 30 minutes at room temperature. After that, plate was washed with deionized water, and 100 µl of the conjugate was placed in each well. Plate was incubated for 30 minutes and washed again. Finally, 100 µl of the substrate solution (tetramethylbenzidine) was dispensed into each well and incubated for 15 minutes at room temperature. The reaction was blocked with 100 µl of stop solution. Absorbance was measured in an Sunrise Absorbance Reader (TECAN) at 405-410 nm. Results were expressed as serum-to-positive ratios (S/P ratios) relative to a standard positive control. Serum samples, with S/P ratios greater than 0.5 (titers greater than 1,076) were considered positive.

Before cultivation, all tissue samples samples were suspended in 2 ml peptone water and a volume of 1/10 were used to prepare serial dilutions up to 10^{-3} in Frey's broth (Frey *et al.*, 1968). Broths were incubated at 37°C and subcultured onto mycoplasma agar when there was a colour change for cloning step and incubated at 37°C and 5% CO_2. Three colonies for each positive culture were taken and recultivated on broth media.

All positive cultures were centrifuged for 10 min at 13400 x g and the pellets resuspended in 200 µl of sterile PBS. DNA was extracted using the protocol QIAamp DNA Mini Kit (Qiagen).

Figure 1. Quail with sinusitis and swelling of the eyelids accompanying increased lacrimation.

The extracted DNA was analized using *Mycoplasma spp*., *Mycoplasma gallisepticum* and *Mycoplasma synoviae* specific PCR.

One pair of primers was selected for the detection of *Mycoplasma gallisepticum* (330 bp) (Kempf *et al*., 1993). The primer 1 sequence was: 5'-TAA CTA TCG CAT GAG AAT AAC-3'. The primer 2 sequence was: 5'- GTT ACT TAT TCA AAT GGT ACA G-3'. The PCR reaction mixture (total volume of 50 µl) was: 5 µl (1X) of 10 X reaction buffer (Roche), 1 µl of 50 mM MgCl2, 2,5 µl of 10 mM of dNTP (Eurobio), 1 µl of primer (containing 400 ng of each forward and reverse primer), 5 µl of DNA template, 0.2 µl (5 units) of taq DNA polymerase (Roche) and distilled water to complete the mixture to 50 µl . PCR was performed on a MJ Mini^{TM} Gradient Thermal Cycler (Bio-Rad) programmable thermal controller. The amplification was performed following an initial 1 min incubation at 90°C, thermal cycling (40 cycles) proceeded with each segment of one cycle being: 95°C for 15 sec, 60°C for 20 s, and 75°C for 15 s. An additional cycle (95°C for 15 s; 60°C for 45 s and 75°C for 5 min) was included as a final step. The analysis of PCR products was performed by using 10 µl of the amplified PCR product, mixed with 2 µl loading buffer and electrophoresed through 2% agarose gel and DNA was visualized by UV flourescence after ethidium bromide staining, and then photographed in Gel Doc XR System (Bio-Rad).

Mycoplasma synoviae (207 bp): MSL1: 5'-GAG AAG CAA AAT AGT GAT ATC A-3', MSL2: 5'-CAG TCG TCT CCG AAG TTA ACA A-3' (Lauerman et al, 1993) The PCR reaction mixture (total volume of 50 µl) was: 5 µl (1X) of 10 X reaction buffer (Roche), 2 µl of 50 mM MgCl2, 1 µl of 10 mM of dNTP (Eurobio), 0,5 µl of primer (containing 400 ng of each forward and reverse primer), 5 µl of DNA template, 0.25 µl (5 units) of taq DNA polymerase (Roche) and distilled water to complete the mixture to 50 µl. The amplification was performed following an initial 5 min incubation at 94°C, thermal cycling (35 cycles) proceeded with each segment of one cycle being: 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min. A final step of 5 min at 72°C was included.

Mycoplasma spp. (270 bp): GPO3: 5'-GGG AGC AAA CAG GAT TAG ATA CCC T-3', MGSO: 5'-TGC ACC ATC TGT CAC TCT GTT AAC CTC-3' (Van Kuppeveld et al, 1992). The PCR reaction mixture (total volume of 50 µl) was: 5 µl (1X) of 10 X reaction buffer (Roche), 5 µl of 50 mM MgCl2, 2,5 µl of 10 mM of dNTP (Eurobio), 0,5 µl of primer (containing 400 ng of each forward and reverse primer), 5 µl of DNA template, 0.2 µl (5 units) of taq DNA polymerase (Roche) and distilled water to complete the mixture to 50 µl. The amplification was performed following an initial denaturation step (10 min at 95°C), 45 thermal cycling with each segment of one cycle being: 95°C for 15 sec, 58°C for 20 sec, and 75°C for 20 sec. A final step of 5 min at 75°C was included.

Results and discussions

Performing ELISA test, both of serums were positif for MG. For MS, one serum was suspect and one was negative.

Inoculum made up of organ sample and 2 mL peptone water, was tested by Mycoplasma spp.-PCR, MG-PCR and MS-PCR. All were negative.

By cultivation, 15 primary cultures were obtained and all were tested with *Mycoplasma spp*.-PCR. 9 (60%) out of 15 were negative, the rest of 6 (40%) were positif (Figure 3). The negative cultures were similar cultural characters as the positive, changing the color of the broth without causing turbidity. Subcultured onto mycoplasma agar, no colonies growth was observed even after 21 days of incubation.

The six *Mycoplasma spp*-PCR positive broth were obtained from sinuses, conjunctiva and trachea. Subcultured onto mycoplasma agar and incubation at 37°C, 3 diferent colonies for each culture were transfered in mycoplasma broth for cloning step. Positive agar cultures were obtained after 2 to 7 days of incubation and after clonage step were obtained eight strains only from the sinuses and conjunctiva.

All were tested by *Mycoplasma synoviae* and *Mycoplasma gallisepticum*-PCR for identification and to ensure the purity of obtained strains. All eight were MG-PCR positif and MS-PCR negative (Figure 4, Figure 5)

Cultural characters of the positive cultures were polymorphic, yielding colonies with typical „fried egg„ aspect but also lenticular colonies without central spot and size of 0.1 mm to 1 mm (Figure 2).

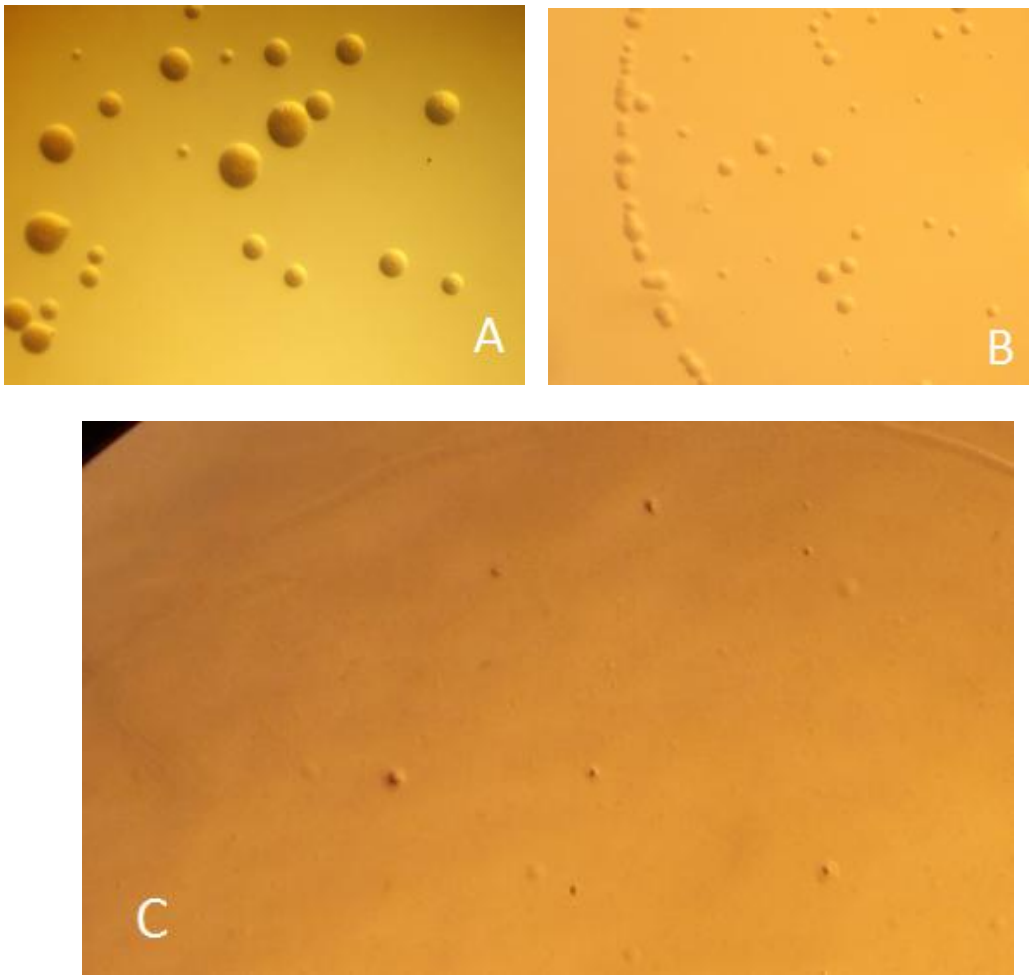

Figure 2. Diferent aspects of the colonies: A-primary culture of sinus; B-clone 3 from sinus; C- clone 1 from conjunctiva.

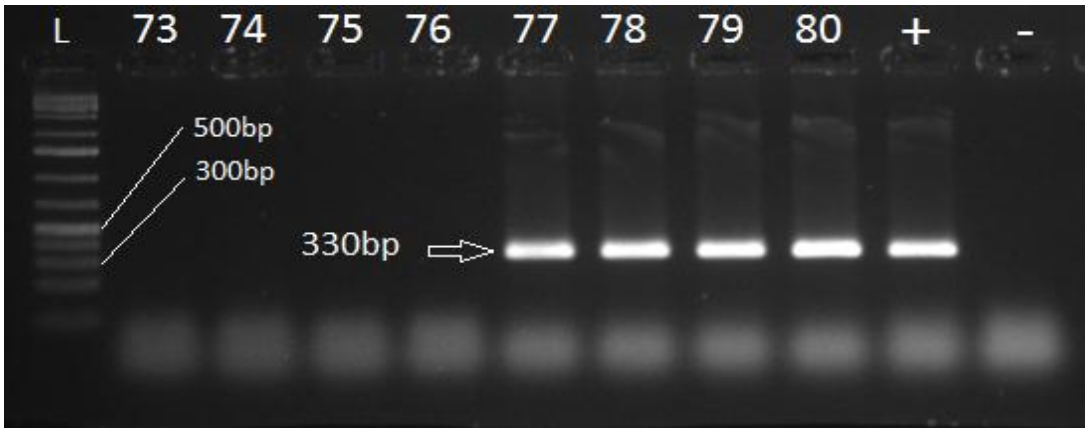

Figure 3. Agarose gel electrophoresis of MG-PCR. Lane L: molecular weight marker, 100 base pair DNA Lanes 73 to 76: negative samples; Lanes 77 to 80: positive samples; Lane +: positive control; Lane -: negative control.

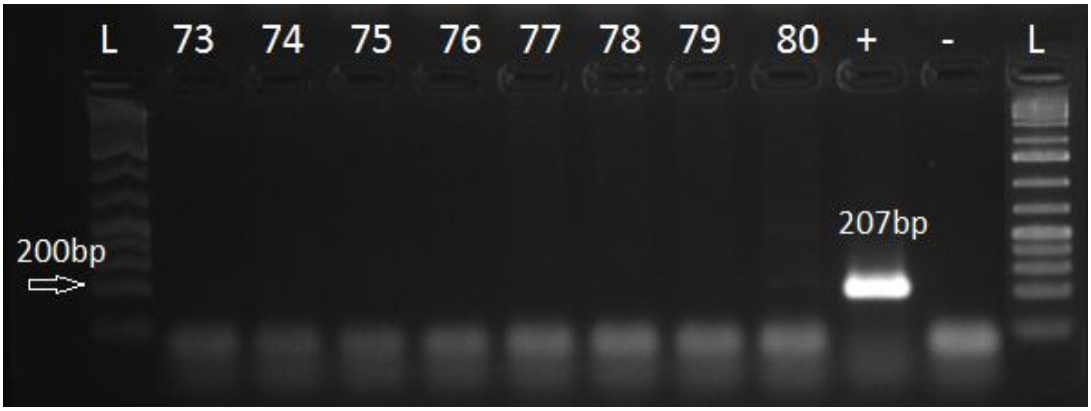

Figure 4. Agarose gel electrophoresis of MS-PCR. Lane L: molecular weight marker, 100 bp DNA; Lanes 73 to 80: negative samples; Lane +: positive control; Lane -: negative control,

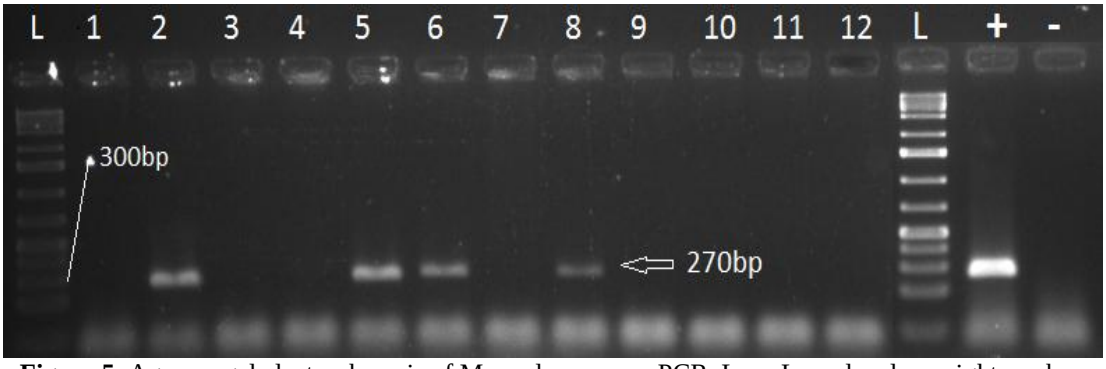

Figure 5. Agarose gel electrophoresis of Mycoplasma spp.-PCR. Lane L: molecular weight marker, 100 base pair DNA Lanes 1,3,4,7,9-12: negative samples; Lanes 2,5,6,8: positive samples; Lane +: positive control; Lane -: negative control,

According to OIE standard protocol for definitive diagnosis, *Mycoplasma* culture has the disadvantage of being very laborious, particularly in cases of mixed infections, and it also depends on the presence of viable organisms. While positive results may be obtained within 4 to 7 days, up to 30 days may be required for a negative result. (Kleven, 1994).

Bacterial or yeast contamination and the presence of non viable mycoplasmas could be one reason for the low number of positive cultures (Eissa, S.I. et al, 2009).

Cultivation techniques require awareness of any recent antibiotic treatment who can inhibit isolation of the organisms and other problems include overgrowth by faster growing mycoplasma species or other bacteria (Garcia et al., 1995). For better results swabs or organ samples should be collected from affected organs, tissues and exudates for mycoplasma culture. The samples can be taken from live birds, recently dead birds or carcasses frozen soon after death. Tissues or swab samples can be transported in mycoplasma broth. Samples should be sent to the laboratory as soon as possible and kept with ice.

With the increased use of vaccination, more powerful tools are required to trace the source of contamination and to differentiate vaccine strains from circulating field isolates to aid better understanding of the epidemiology of the disease (Ferguson et al., 2005).

Conclusion

Mycoplasma positive cultures were obtained only from the sinus, conjunctiva and tracheea samples and *Mycoplasma gallisepticum* was identified only from the sinus and conjunctiva samples to the booth quails. *Mycoplasma synoviae* was not identified in none of the samples.

These study suggested that MG was the likely cause of the outbreak of conjunctivitis and sinovitis in quail flock. This infection can be transmitted naturally to an additional host species.

Mycoplasma gallisepticum infections should be considered in poultry or game birds with upper respiratory disease and conjunctivitis (Spickler, A. R., 2007).

PCR tests are fast diagnostic tools that take only 1 to 2 days for the detection of *Mycoplasmas* and are not dependent upon viability of the organisms, but the result can be negative if the bacterial load is low or there is inhibitory substances in the sample.

Acknowledgments

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SEROLOGICAL INVESTIGATION REGARDING THE INCIDENCE OF *MYCOPLASMA GALLISEPTICUM* AND *MYCOPLASMA SYNOVIAE* IN CHICKENS AND TURKEYS IN DIFFERENT POULTRY FLOCKS

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Abstract

Mycoplasma gallisepticum (MG) together with Mycoplasma synoviae (MS) are the principal causative agents of respiratory disease among Mycoplasma species in chickens and synovitis in turkeys and are responsible for significant economic losses in the poultry industry worldwide. The aim of this study was to determine incidence of the infection caused by these species in chickens and turkeys from different poultry farms. With ELISA quantitative tests for MG and MS antibodies were analyzed 516 serums (90 from turkeys and 426 from chickens) from different age categories in four intensive livestock farms and five households. In farm A, 1 (2.17%) out of 46 samples was found positive for MG, similar result was obtained for MS. In farm B, 11 (7.3%) out of 150 tested serum from broilers were positive for MG and 27 (18%) out of 150 were positive for MS and from 54 serums from young breeding chicks vaccinated with inactivated vaccine, 53 (98%) were positive for MG. In farm C, 78% of serums from unvaccinated laying hens (71/90) were positive for MG and 58.8% samples (53/90) were positive for MS. In farm D, 44.4% of meat turkeys serums (40/90) were positive for MG and 7.7% serums (7/90) were positive MS. In households, all 50 serum samples from chickens were positive for MG and 46 (92%) were positive for MS. The results showed that the infection is more common in multi-age farms. Antibody titer is very high in poultry bred in households, and this is correlated with frequent exposure to infectious agents.

Key words*: Mycoplasma gallisepticum, Mycoplasma synoviae, serology, ELISA*

Introduction

Mycoplasma gallisepticum is the most economically significant mycoplasmal pathogen of poultry. M. gallisepticum infections can cause significant economic losses on poultry farms from chronic respiratory disease, reduced feed efficiency, decreased growth and decreased egg production. The carcasses of birds sent to slaughter may also be downgraded (Spickler, A. R., 2007). It is especially serious in broiler chickens in which it often acts synergistically with other agents, such as respiratory viruses or pathogenic strains of *Escherichia coli* to provoke chronic respiratory disease. In laying hens, it may also cause loss of egg production (Bradbury, 1998).

Mycoplasma synoviae is considered the second most important mycoplasma affecting chickens (Stipkovits & Kempf, 1996; Kleven, 2003). It causes respiratory disease and subsequent condemnations due to airsacculitis although most respiratory tract infections seem to be subclinical. M. synoviae also causes synovitis in chickens and turkeys and eggshell apex abnormalities (EAA) in laying hens (Landman & Feberwee, 2001, 2004; Kleven, 2003).

Since 2000, eggshell apex abnormalities (EAA), produced by *Mycoplasma synoviae*, have been increasingly seen in layer flocks in Netherlands, and there have been several subsequent reports of cases in many other country. The EAA are characterized by a roughened shell surface, shell thinning, increased translucency, cracks and breaks. The abnormalities are confined to the top cone of the egg, up to approximately 2 cm from the

apex, and almost always have a very clear demarcation zone. The proportion of affected eggs varies between flocks, from a few percent up to 25% (Feberwee A. et al, 2009, Bouchardon A., 2012)

Mycoplasma gallisepticum is transmitted during close contact between birds as well as on fomites. Aerosol spread occurs over short distances and can be responsible for transmission within a flock. M. gallisepticum is also transmitted vertically in eggs. Egg transmission is more frequent in birds infected during laying than in birds infected before they mature. Infected birds carry M. gallisepticum for life, and can remain asymptomatic until they are stressed (Spickler, A. R., 2007).

Experimentally infected poultry develop symptoms after 6 to 21 days. In natural infections, the incubation period is variable; infected birds may be asymptomatic for days or months until stressed.

M. gallisepticum/M synoviae–negative breeding stock can be identified and maintained by serologic testing and is particularly helpful in screening poultry flocks. Serology is less useful in individual birds, as nonspecific reactions are common (Kleven et al, 2003). Commonly used assays include a rapid serum agglutination (RSA) test, enzyme-linked immunosorbent assays (ELISAs) and hemagglutination inhibition.

Control of *M. gallisepticum* has generally been based on eradication of the organism from breeder flocks and maintenance of a mycoplasma-free status in the breeders and their progeny by implementation of biosecurity measures.

Material and methods

Using ELISA quantitative tests for MG and MS antibodies, were analyzed 516 serums (90 from turkeys and 426 from chickens) from different ages and categories in four intensive livestock farms and five households in North-Eastern and Central Romania. The study was carried out in the years 2012 and 2013.

The A farm is a broilers farm only. The B farm is a multi-age farm in which one are raising broilers, breeding hens and laying hens. The blood samples were taken from the broilers with clinical signs of mycoplasmosis or weight loss.

In farm C is raising a small flock (about 2000 chicken) of laying hens from Rhode-Island race. In D farm is raising turkey for meat production.

In household system, chicken are raising in multi-age and multi-species flock and they have contact with wild birds.

For this study blood samples were aseptically collected from the wing veins using 5 mL sterile disposable syringes and needles. Blood was allowed to clot in the syringe at room temperature. After this, serum of each sample was transferred to sterile microtubes and kept at -20◦C until the moment of use.

Two comercial ELISA kit were used: *Mycoplasma gallisepticum Antibody Test Kit* and *Mycoplasma synoviae Antibody Test Kit* (BioChek B.V, Holland)

The MG ELISA kit will measure the amount of antibody to MG in the serum of chickens. Microtitre plates have been pre-coated with inactivated MG antigen. Chicken serum samples are diluted five-hundred fold (1 : 500) with the diluent, and 100 µl of each sample were added to the microtitre wells where any anti-MG antibodies present will bind and form an antigen-antibody complex. The plate was incubated for 30 minutes at room temperature. Non specific antibodies and other serum proteins are then washed away with deionized water. 100 µl of the conjugate (anti-chicken IgG labelled with the enzyme alkaline phosphatase) was

placed in each well and the plate was incubated for 30 min. Anti-chicken IgG binds to any chicken anti-MG antibodies originally bound to the antigen. After another wash to remove unreacted conjugate, 100 µl of the substrate is added in the form of pNPP chromogen and incubated for 15 minutes at room temperature. A yellow colour is developed if anti-MG antibody is present and the intensity is directly related to the amount of anti-MG present in the sample. Reaction was blocked with 100 µl of stop solution. The plate was read in an Sunrise Absorbance Reader (TECAN) at 405-410 nm.

Results were expressed as serum-to-positive ratios (S/P ratios) relative to a standard positive control. Samples with an S/P of .500 or greater contain anti-Mg antibodies and are considered positive. The following equation relates the S/P of a sample at a 1 : 500 dilution to an end point titre: Log10 Titre = 1.1 (log10 S/P) + 3.156.

For MS antibody detection was used the same protocole from MG ELISA kit, the difference was the plate that was pre-coated with inactivated MS antigen.

Results and discussions

For A Farm were colected 46 serum samples from broilers, age 39-43 days. Populating is done with day-old chickens from specialized commercial units. They are housing in 18.000-20.000 chicken per hall and they are not vaccinated with MG or MS vaccine. One (2.17%) sample was found positive for MG, and one sample was positive for MS (Table 1.). The antibody titre was low, and can showing a recent infection.

In B Farm were taken 240 blood samples from broilers and young breeding hens. The breeding hens were vaccinated with attenuated *Mycoplasma gallisepticum* vaccine (strain MG 6/85) at 50 days old and rappel with inactivated MG vaccine at 130 days old. From 150 broilers serum samples, 11 (7.3%) out of 150 tested serum were positive for MG and 27 (18%) out of 150 were positive for MS. The antibodies titre was low for MG and very high for MS. The low antibodies titre for MG can be explained by the fact that broilers come from MG vaccinated parents who transmitting maternal antibodies to progeny. For MS, the very high antibodies titre can be explained by an active MS infection confirmed by the presence of fibrinous airsacculitis and synovitis at necropsied dead birds. From 36 breeding chicks (10 weeks old) vaccinated with attenuated vaccin at 50 days old, three serum samples (8,33%) were positive for MG and none for MS. The MG 6/85 strain gives cellular immunity, without inducing antibody. The antibody titer obtained was low and can be explained by a natural infection. In another case, when the rappel with inactivated vaccine is made, all birds become positive for MG. Therefore, from 54 analyzed serum samples 53 (98,14%) were MG positive. The inactivated vaccine gives humoral immunity and the high antibodies titre that was obtained show a good immunization of the flock (Table 1). In this case, using serology can not differentiate natural infection.

In C Farm were sampled 90 blood samples from laying hens, ages 55-60 weeks that are not vaccinated with any MG or MS vaccine. 71 (78%) out of 90 samples were found positive for MG and 53 (58.8%) out of 90 serums were positive for MS. The level of antibodies titre was high (9370 for MG and 8011 for MS) and that results show a natural infection.

In a farm of meat turkeys (Farm D), day-old chicks are purchased from specialized units from Europe with documents that certifying that they are free from Mycoplasma. All 10 serum samples from day-old turkeys were negative for MG and MS. From 80 turkeys that were aged between 99 and 152 days, 40 samples (50%) were positive for MG and 7 (8,75%)

were positive for MS. The antibodies titre was high for booth mycoplasma species. In this case, chickens brought for populating the halls were free of Mycoplasma but during the breding period, natural infection occurred.

In households, all 50 (100%) serum samples were MG positive and 46 (92%) were MS positive. In households growth system, multi-age/ multi-species flock and permanent exposure to the wild birds contribute to the occurrence of repeated infections with *Mycoplasma*. This is shown by the very high level of antibodies titre (Table 1).

The results show a high incidence of Mycoplasma infection in households flocks and laying hens flocks (Figure 1)

Serological tests are useful for examining flocks, but they sometimes lack specificity or sensitivity (Kleven et al, 2003; Yoder, 1991).

Table 1. Number of positive samples tested by ELISA.

Flock identification	Category	Age	Number of samples	Number of positive/total		Antibody titre***	
				MG	**MS**	**MG**	**MS**
Farm A	broilers	39-43 d	46	1/46	1/46	1312	632
Farm B	broilers	32-47 d	150	11/150	27/150	1154	14622
	breeding hens	10 w*	36	3/36	0/36	1233	-
		20 w**	54	53/54	0/54	5135	-
Farm C	laying hens	55-60 w	90	71/90	53/90	9370	8011
Farm D	Turkey for meat	99-152 d	80	40/80	7/80	5128	1622
		1 d	10	0/10	0/10	-	-
Households	chicken	2 m-4 y	50	50/50	46/50	13593	12259
Total			516	229/516	84/516		

* vaccinated with attenuated vaccine (MG 6/85 strain) at 9 weeks

** vaccinated with attenuated vaccine (MG 6/85 strain) at 9 weeks and rappel with inactivated vaccine at 19 weeks

*** the average value titer of antibody for positive samples

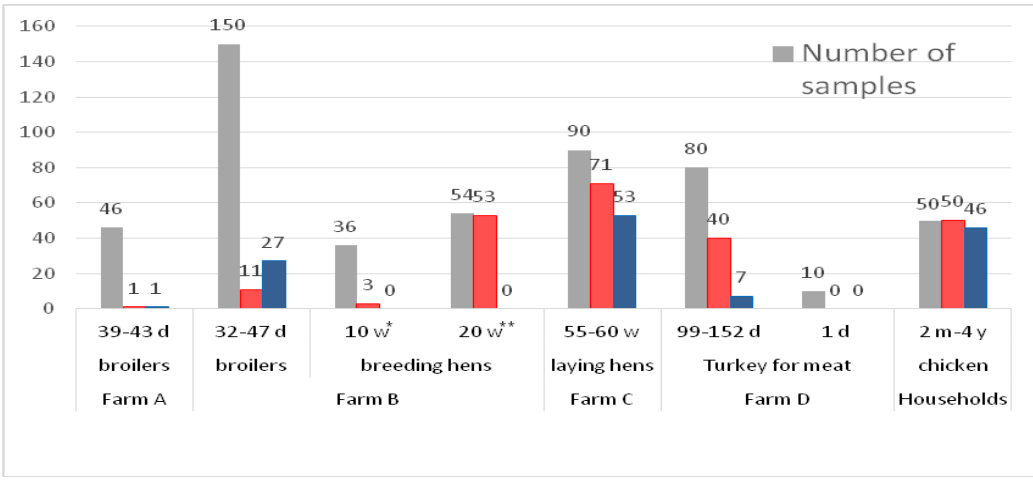

Figure 1. The comparative results of the serological tests

* vaccinated with attenuated vaccine (MG 6/85 strain) at 9 weeks

** vaccinated with attenuated vaccine (MG 6/85 strain) at 9 weeks and rappel with inactivated vaccine at 19 weeks

Conclusions

Moreover, serology is not a valid screening method if any commercial *M. gallisepticum* or *M. synoviae* vaccines have been used, because current serological tests can not differentiate between responses to vaccine or field strains.

Results showed that the infection is present in all analyzed poultry flocks, more common in multi-age flocks and comercial laying flocks.

M. gallisepticum and can be introduced into a flock by live birds or hatching eggs, as well as the movement of people and fomites. Subclinically infected small backyard flocks can be a source of infection for commercial poultry.

Biosecurity measures are important in preventing transmission on fomites. Wild birds can also carry *Mycoplasma spp*. and should be excluded from poultry operations.

Eradication from large, multiple-age commercial egg laying flocks is complicated by persistent infections and periodic shedding under stress.

Acknowledgments

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THE FREQUENCY SEROGROUPS OF APEC STRAINS IN BROILER FLOCKS IN WESTERN COUNTRY

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Abstract
The APEC strains frequently isolated from broilers are framed in serogroups O1, O2, O35, O78. The researches had as objective serological typing for 57 APEC strains isolated from broilers and establishing the frequency of APEC strains serogroups farms monitored. Following the serological typing strains framed into 4 serogroups was as follows: O78 K80, O157, O18a O18c:K77, O55 K59. A number of 11 strains framed in serogroup O157 have been that include strains verotoxigene increased zoonotic risk. The dominant strains of serogroup O78 was isolated from the ages of 7, 14 and 28 days, and strains of serogroup O157 (verotoxigenic) were isolated from the age of one day, seven days and 28 days.
Key words: *APEC, broiler, E.coli, serogroup.*

Introduction

The APEC pathotype (Avian pathogenic E. coli) includes strains of the E. coli isolated from birds from antigenically point of view, belong to of several serogroups.

Frequently isolated from birds isolated strains classified within the serogroups O1, O2 and O78. The E. coli strains included in the APEC pathotype, have genotypic and phenotypic characteristics, they offer a level of special pathogenic for birds, but also presents a zoonotic risk increased

The researches had as objective establishing the frequency of and serogroups serological typing APEC strains of broiler farms monitored.

Material and methods

The serological typing, strains of *E. coli* was performed using the kit Escherichia coli O Antisera produced by Plasmatec Laboratory Products. For inclusion in the group "O" was inactivated bacterial suspensions of *E. coli* and the following commercial antisera: O2, O18a O18c: K77, O20a O20b: K84, O55 K59, O78: K80, O157.

The strains subjected to typing have been isolated from broiler cadavers with colibacillosis lesions as follows: from the farm A were performed 20 bacteriological exams, from the farm B - 14 bacteriological exams, of farm C - 27 bacteriological exams and the farm D 20 bacteriological exams. The preparation of suspensions of 57 strains of E. coli, was performed according to the protocol recommended by the producing company (7).

Results and discussion

From the broilers cadavers with lesions were made a number of:
- 20 bacteriological exams, in farm A, from which were isolated 5 strains of *E. coli*;
- 41 bacteriological exams, in farm B, from which were isolated 30 strains of *E. coli*;
- 27 bacteriological exams, in farm C, from which were isolated 13 strains of *E. coli*;
- 20 bacteriological exams from of farm D were isolated from nine strains of *E. coli*.

The serological typing was performed with the aim of classification the APEC the strains isolated from broilers in different serogroups. Were a total of 57 typed the APEC strains isolated from broilers of different ages.

The antigens obtained from each strain were contacted on a glass slide with the following sera group "O" O2, O18a O18c: K77, O20a O20b: K84, K59 O55, O78: K80, O157, produced by Plasmatec Laboratory.

Using the methodology described by manufacturer and by other authors, we found that the antigens obtained (diluted in 0.5 ml 0.85% saline suspension) are highly concentrated, which influenced the antigen-antibody reaction in the sense that it could not be their correct interpretation

To remove this impediment antigen were diluted to a volume of 1 ml saline solution (0.85%). Using this procedure we found that the agglutination reactions were held normally, positive feedback is visible. To increase the contrast of glass slides were placed on dark substrates. A number of 12 strains could not be typed in the kit sera, suggesting that they belonged to other serogroups.

The serological typing of the 57 the APEC strains, according to the methodology described, allowed classification into several serogroups, results are presented in Table 1 and Figure 1.

Analyzing these results it is observed that most strains were classified into serogroup O78: K80, followed in order by serogroups: O157, O55: K59, O18aO18c: K77, and serogroups O2 and O20aO20b: K84 were not framed none of isolates.

The research followed the distribution of the serogroups in broiler farms, the results are presented in Table 1 and in Figures 1, 2, 3, 4.

Table 1. The frequency of serogroups in monitored farms

Farm	**Serogoups**													
	O2		**O18a O18c:K77**		**O20a O20b:K84**		**O55 K59**		**O78 K80**		**O157**		**E. coli untyped**	
	No	%	No	%	No	%	No	%	No	%	No	%	No	%
A	0	0	0	0	0	0	0	0	4	7,01	0	0	1	1,75
B	0	0	1	1,75	0	0	2	3,5	16	28,07	5	8,77	6	10,52
C	0	0	0	0	0	0	1	1,75	8	14,03	0	0	4	7,01
D	0	0	1	1,75	0	0	0	0	1	1,75	6	10,52	1	1,75

From analysis of these data shows that in farms monitored was the frequency of the variable serogroups.

In the farm A was found a single serogroup, in farm B four serogroup were identified in farm C identified two serogroup end in D farm three serogroups.

These results suggest that referred farms, chicken flocks had different origins, their acquisition was made from multiple providers, serogroups frequency being correlated with broiler origin. In this case the highest frequency of serogroup O78 had it all. The frequency of serogroups according to the age is shown in Table 2.

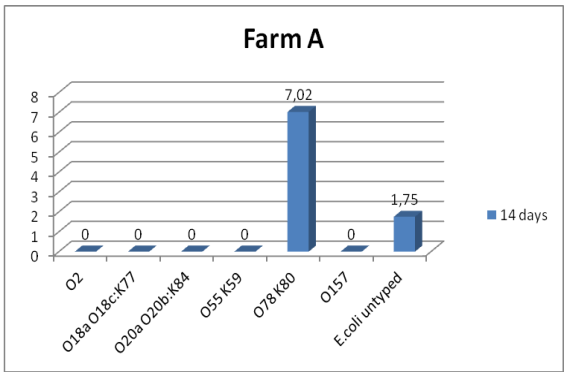

Fig. 1 Farm A

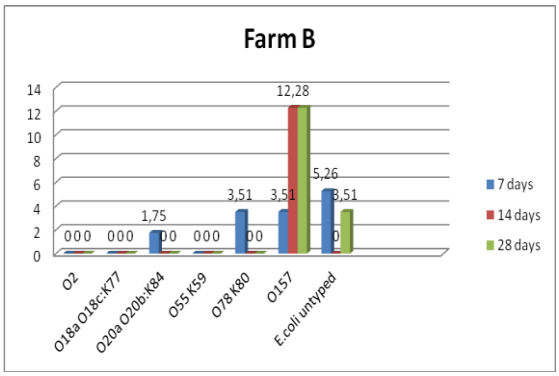

Fig. 2 Farm B

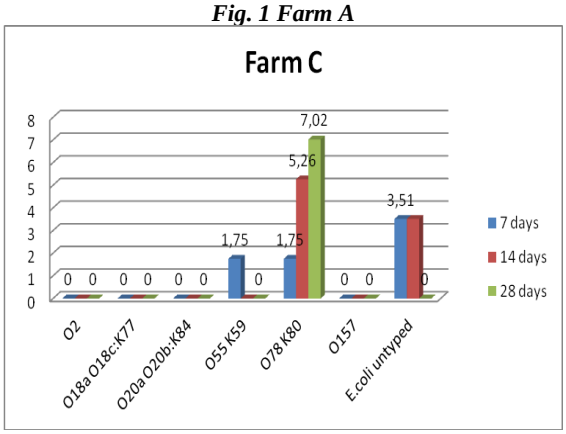

Fig. 3Farm C

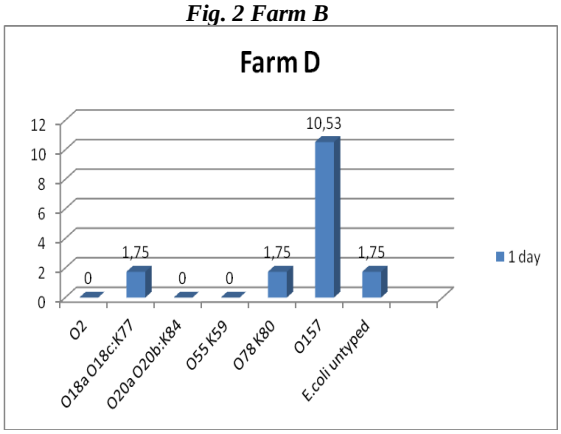

Fig. 4 Farm D

Table 2. Distribution of serogroups by age

Age	Serogroups						
	O2	O18a O18c:K77	O20a O20b:K84	O55 K59	O78 K80	O157	Total
1 day	0	1	0	0	1	6	8
7 days	0	1	0	3	3	3	10
14 days	0	0	0	0	14	0	14
28 days	0	0	0	0	11	2	13

The results show that at age of one day, serogroup O 157 has the highest frequency, and the serogroup O 78 was dominant at the age of 7, 14 and 28 days.

The apec strains that cause avian colibacillosis are classified into several serogroups, some more frequently, and others less frequently.

A definitive serological classification of the apec strains, up to now there is not, there are different from one country to another and from author to author (1, 2).

The frequency of serogroup o 157, in recent years has increased, a phenomenon confirmed in researches.

In according to the research conducted by the ewers christa et. All. (2003) the apec strains fall into serogroups considers that 61% of the apec strains: o1:k1, o2:k2, o78:k80, and serogroups o15, o18, o35, o109, o115, o116 have a lower frequency.

Typify serologically 51 the apec strains using 12 sera of group, kumar a.d et al. In 2003 found that 23.5% of the the strains belonged to serogroup o78 tested, and 14% belonged to serogroup o115 the remaining strains were distributed in varying proportions in other serogroups.

Rodriguez siek kylie et al. In 2005, in a study performed on a total of 451 strains apec, found that 70.5% of strains were classified into a single serogroup respectively o78 serogroup, and the remaining strains were classified in varying proportions in other serogroup.

In 2006 sylvester et al. Typifies serological for the first time in the apec strain serogroup o157, considered verotoxin producing serogroup.

In Korea and Japan on a number of 101 the APEC strains, recent studies performed by YONG WUN J. et al. (2013) with 8 sera of groups found the following distribution of strains: 19.8% by serogroup O78, 3% by O18, 2% by O1, 2% by O115, 1% in O 21, the remaining was untyped strains.

Conclusions

The strains subjected to serological typing were classified into 4 serogroups as follows: O78 K80, O157, O18a O18c:K77, O55 K59.

There were a total of 11 isolates typed belonging to serogroup O157, which includes strains verotoxigene increased zoonotic risk.

The serogroups frequency in the four farms was variable, as follows: farm A was found a single serogroup, B farm four serogroups were identified, in the firm C identified two serogroups and in farm D 3 serogroups.

The serogroups frequency was correlated with the age of the chickens, the dominant strains of serogroup O78 was isolated from the ages of 7, 14 and 28 days, and strains of serogroup O157 (verotoxigenic) were isolated from the age of one day, seven days and 28 days.

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CONTROL OF INDOOR TEMPERATURE IN HOOP SWINE EXPERIMENTAL UNIT

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Abstract

The aim of this study was to observe and measure the indoor environmental factors in relation with temperature at swine's level in hoop swine experimental unit during summer trial. The outdoor environmental factors were collected every 30 minutes using automatic weather station and the indoor measurements were performed collecting the environmental parameters in 9 control points. The hoop structure acted well in West Romania summer climate. No thermic stress was observed in the pigs. The indoor temperature was influenced by the outdoor environmental factors; it is strong and positive correlated (at p <0.000) with outdoor temperature (r =+0.936), wind chill (r =+0.924) and strong negative correlated (at p<0.000) with indoor humidity (r =-0.712) outdoor humidity (r =-0.599), relative press and absolute pressure (both with r =-0.660). By automatic linear modelling procedure for 16 environmental factors, (model significant at p=0.000) three factors can decrease the level of indoor temperature: indoor humidity (coefficient -0.196, importance 0.714 at p<000), total rainfall (coefficient -0.086, importance 0.025 at p<000), and relative pressure (coefficient -0.074, importance 0.011 at p<000 but just one can be technologically controlled and modified in future experiments.

Keywords: *hoops structure, indoor temperature*

In the last two decades, some American universities have been active in developing alternative swine production systems with the Swedish 1980's concept - an intensive swine production system using deep bedded litter which allowed animals to express their natural behaviours *(1,3).* Various Cooperative Extension programs in the United States and Canada have promoted these systems which typically use deep bedded hoop barns as the primary production facility. Nowadays, the USA, Canada, Denmark, Sweden, and lately Ukraine and Poland, had a significant presence of hoop structure management systems.

The hoop structure of Experimental Units is a new and unconventional facility for research purposes. The aims of study were to monitor and analyse the indoor temperature in relation with environmental factors during summer experimental trial with farm pigs.

Materials and methods

The hoop structure of Experimental Units[1] (figure no. 1) are built out of light materials and designed to protect the animals inside from environmental conditions such as rain, wind, solar radiation and extreme temperatures, using just natural ventilation. The orientation of structure is Est – West.

The structure used for experiment has a feeding area located along one sidewall; the floor area is divided into resting and feeding areas.

The feeding area is 2.5 *m* wide in a 9.20 *m* wide barn uses 30% of the available floor area – see fig no1. a) and b). Feed was provided using self-feeders located along the sidewall. Water is provided by nipple waters with cup - see fig. no 1 c).

Flooring in the feeding area should be rough concrete at a 0.4 *m* elevation from resting area with a transverse slope of 1 to 5%. The entire concrete area along the sidewall is bedded and used as resting area. The resting area will cover 70% of the available floor area and it is deep bedded. Round bale of wheat straws were used as deeding material.

During the trial, the structure was populated with 45 piglets; the piglets were a hybrid between Landrace and Large White and had in the beginning 25 kg of body weight. The environmental data was collected automatically with electronic weather station and by operator at piglets' level every 30 minutes interval. The following sixteen environmental parameters were registered by the weather station:

1. *indoor temperature (°C)*
2. *indoor humidity (%)*
3. *outdoor temperature (°C)*
4. *outdoor humidity (%)*
5. *relative pressure (hpa)*
6. *absolute pressure (hpa)*
7. *wind speed (km/h)*
8. *gust (km/h)*
9. *wind direction*
10. *dew point (t°C)*
11. *wind chill (°C)*
12. *hour rainfall (mm)*
13. *24 hour rainfall (mm)*
14. *week rainfall (mm)*
15. *month rainfall (mm)*
16. *total rainfall (mm)*

On a regularly basis, during the day period and in 24 hours period during the weekend, every 30 minutes, the operator registers other 27 measurements in nine control points at the pig's level (see figure no 2); the measurements collected ware wind speed, temperature and relative humidity in P1-P9 checking points.

The measurements were included in data bases using Excel and statistically analysed using MINITAB and SPSS; the Pearson correlations, ANOVA and Automatic Linear Modelling procedures were considered.

Results and Discussion

The indoor temperature measured by weather station all day long, at every 30 minutes together with other environmental factors measured during 2nd to 22th of August and all the values and the variability are in table no 1.

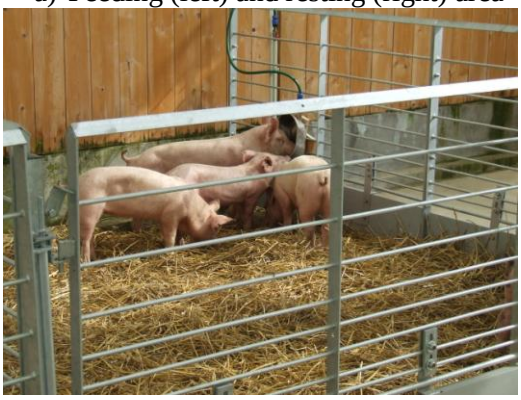
Table no. 1 Descriptive Statistics of environmental factor *(n = 959)*

Variable	***Mean ± Std. Error***	***Std. Dev.***	***Minimum***	***Maximum***
Indoor temperature *(°C)*	24,37 ±0,17	5,20	13,80	36,80
Indoor humidity (%)	67,60 ±0,43	13,20	39,00	90,00
Outdoor temperature *(°C)*	24,32 ±0,19	6,01	12,20	39,10
Outdoor humidity (%)	69,90 ±0,64	19,86	29,00	99,00
Relative press (hpa)	1020,29 ±0,07	2,21	1012,70	1025,40
Absolute pressure (hpa)	1003,39 ±0,07	2,21	995,80	1008,50
Wind speed (km/h)	4,07 ±0,12	3,79	0,00	27,00
Gust *(km/h)*	6,71 ±0,17	5,39	0,00	39,20
Dewpoint *(°C)*	17,69 ±0,10	3,00	10,30	23,20
Wind chill *(°C)*	24,21 ±0,19	6,02	12,20	39,10

Hour rainfalls (mm)	0,05 ±0,01	0,40	0,00	6,30
Day rainfalls (mm)	1,16 ±0,07	2,08	0,00	7,20
Week rainfalls (mm)	7,82 ±0,09	2,82	1,50	14,40
Monthly rainfalls (mm)	20,52 ±0,11	3,39	15,00	27,90
Total rainfalls (mm)	28,02 ±0,22	6,93	17,40	36,60

a) Feeding (left) and resting (right) area

b) Self-feeders



c) Drinking and dirty area

d) Level difference between functional areas

e) Unstressed interaction with operator

f) Exploratory and welfare behaviour

Figure no 1. The Swine Experimental Unit - hoop structure

The mean of all-day indoor temperature is strong and positive correlated (at $p < 0.000$) with outdoor temperature ($r = +0.936$), wind chill ($r = +0.924$) and strong negative correlated (at $p < 0.000$) with indoor humidity ($r = -0.712$) outdoor humidity ($r = -0.599$), relative press and absolute pressure (both with $r = -0.660$). Between indoor temperature and the indoor temperature measured at pigs level the correlation was strong and positive - ($r = +0.954$ at $p < 0.000$).

The warmest period was between 10:00 am to 17:00 pm; the difference between outdoor temperature (29.86±0.28 °C) and indoor temperature measured in P4 – resting area (29.67±0.24 °C), the difference is significant (t =2.007 at p =0.048) but by practical point of view do not differ too much (the difference between them was just 0,19 °C). If the comparison is done between all day measurements the experiment cannot sustain the hypothesis of differences between outdoor and indoor temperature (t=-0.976 at p = 0.329). During trial no effect of thermic stress was noticed in pigs either in clinical observations or biochemical analyses – cortizol analyses.

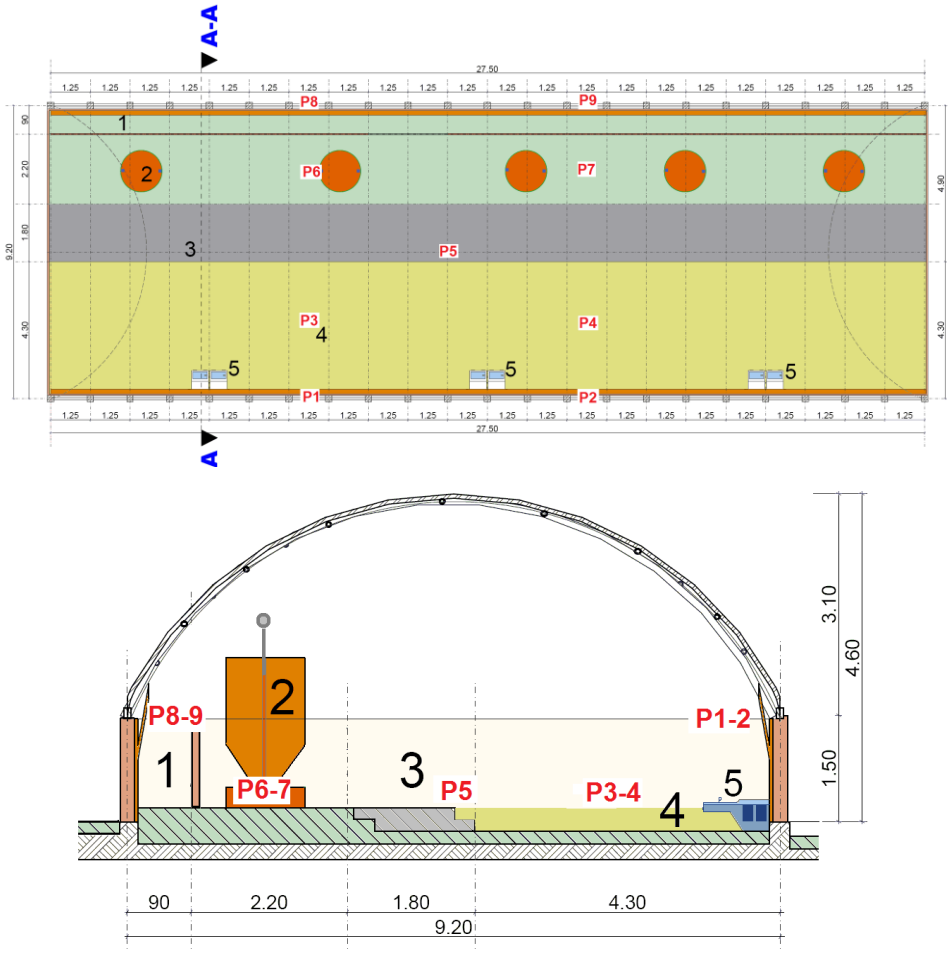


Figure no 2. Floor plan *(upper)* and cross-section *(lower)* of a grow/finish unit with feeding area on one side P1 – P9 measurements check points. 1. service alley; 2. feeder and waterer; 3. concrete feeding area; 4. deep bedded resting area; 5. heated frost proof water fountain

Practically, points have to be oriented in order to reduce the effects of heatstroke by resistance at UV, white colour of tarpaulin, trees and any vegetation around the hoop structure. Hoop barns are covered by a UV resistant white tarpaulin; practically the outdoor temperature is more or less similar to indoor temperature. In this context the thermal comfort of pigs must be supported by increasing the speed of air. Hoop barns require no artificial ventilation; natural flow through the structure serves to refresh the air and remove moisture accumulation. These air flows are of two types: longitudinal and ascending transverse. The tunnel effect created by the long narrow shape of the building establishes the longitudinal flow pattern *(5).*

During the experiment, the doors and the end-walls were 1/3 opened to increase the air's movement through the structure. Sidewall slots were kept entirely open and deflection panels removed as *Honeyman et. al. (1998)* recommended. During measurement of the indoor air speed (n=215 measures) and comparing the Nord-South sides and resting *vs.* feeding area, the following results were found. The air speed in Nord side was 0.16±0.014 m/s and in South part 0.98±0.030m/s - the difference are significant (*t = - 26.69*) at p<0.000. The air speed in resting area was 0.17±0.015 m/s and in feeding area 1.14±0.043m/s - the difference is significant statistic (*t = - 22.65*) at p<0.000. Probably, the end-walls must be removed ½ or totally – future hypothesis for testing

By automatic linear modelling procedure (model significant at *p*=0.000), the indoor temperature (*indoor*$_{temp}$) seems to be influenced by: indoor humidity (*in*$_{HR\%}$ - coefficient -0.196, importance 0.714 at *p*<000), outdoor temperature (*out*$_{temp}$ coefficient +0.558, importance 0.150 at *p*<000), gust (coefficient +0.051, importance 0.028 at *p*<000), total rainfall (coefficient -0.086, importance 0.025 at *p*<000), dewpoint (coefficient +0.235, importance 0.022 at *p*<000), outdoor humidity (coefficient +0.049, importance 0.017 at *p*<000), week rainfall (coefficient +0.128, importance 0.016 at *p*<000), relative pressure (coefficient -0.074, importance 0.011 at *p*<000), temperature in resting area (coefficient +0.191, importance 0.008 at *p*<000).

$$Indoor_{temp} = 85.727 - 0.196 \times in_{HR\%} + 0.558 \times out_{temp} + 0.051 \times gust + 0.086 \times rain_{total} + 0.235 \times dew_{point} + 0.235 \times dew_{point} + 0.049 \times out_{HR\%} + 0.128 \times rain_{week} + 0.074 \times pres_{relative} + 0.191 \times P3_{temp} \quad (1)$$

From above paragraphs, becomes clear that those three variables decreased the level of indoor temperature (indoor humidity, total rainfall and relative pressure), but only one can be technologically modified - indoor humidity. So, future research and testing the technology must concentrate on indoor humidity's control, which technically can be relative easier applicable by water nebulization.

Such a swine management system offers significant advantages for Romania and other Eastern European countries as well. The most prominent of these is the low initial investment in structures as compared to that of conventional systems. In addition, this system requires significantly less energy use for heating, cooling and ventilation. It is also more environmentally friendly with low impact on water quality and reduced odour and other air quality concerns *(1,4).*

The base unit for the model would be a medium sized family farm which uses its own labour. These operations are not typically very intensive, but would nevertheless have to meet all environmental standards for its peculiar location, including factors affected by topography,

soil type, hydrology - both surface and underground, and air. Such farms will require the experts' technical assistance for swine production practices as well as environmental practices. These types of farmers are in general active as community members. They are also likely to form production cooperatives and be proactive with responsible production practices and marketing.

In order for such a model to succeed, it is imperative that these farmers have access to free current technological information regarding genetics, nutrition, management, and marketing of swine products. Young producers and beginners are a critical part of this model and they in particular will oftentimes require extensive initial training in production practices as well as environmental and community issues. In addition, ongoing continuing education needs to be available to keep all producers abreast of current developments. Such education programs should encourage innovation for alternative management systems. Innovation is a prime mover of sustainable development.

Conclusions

- In the hoop barns used under Romanian temperate climate peculiarities, the indoor temperature follows the variations of outdoor temperature.
- The hoop barns can be used for pigs and hogs without mechanical ventilation – no effect of thermic stress were seen during trial.
- Three environmental factors can decrease the level of indoor hoop barns temperature: indoor humidity, total rainfall and relative pressure.
- The decreasing of indoor temperature in hoop structure naturally ventilated can be generated through by technological actions focused on indoor humidity.

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RESEARCH REGARDING THE UTERINE INVOLUTION DYNAMICS IN THE PUERPERAL PERIOD OF COWS

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Abstract

For cows, the post-partum uterine involution means anatomical, cytological and biochemical modifications of the uterus. These phenomena are progressive following a dynamics that regards the anatomical and physiological rehabilitation of the genital system. It is remarked that, after parturition, the uterus has a length of approximately 100 cm, a thickness of 40 cm and a weigh of 10-12 kg. The first 5 days post-partum are characterized by an accelerated uterine involution, followed by a decreased intensity of involution (days 5-10 post-partum) and again, a more accentuated dynamics in days 10-15 post-partum, in such a manner that in 2 weeks the uterus has involved with a proportion of approximately 50%. The uterine liquids eliminated through the genitalia are represented by a mixture of annex leaking, fetal annexes, blood, cellular detritus and mucus. Monitorizing the dynamics in uterine involution includes the rhythm of the eliminated uterine liquids, which was achieved by investigating several aspects: the usage of ultrasound in uterine involution, as well as the diagnosis of some morphological modifications in the genital tractus. The observations followed several aspects by monitorizing 20 cows. These investigations referred to determining the length of the uterus, its diameter and its weight during 21 one days post-partum. From this point of view, the research shows a diminishing in the uterus length from 97 cm in day 1 post-partum to 22 cm in day 21 post-partum. Similar aspects were registered in the case of uterus involution which diminishes its diameter from 43 cm in day 1 post-partum to 4 cm in day 21 post-partum. The weight of the uterus is presented with a reduction from 8 kg in day 1 post-partum to 1 kg in day 21 post-partum. In correlation with the uterine involution is also the elimination of liquids after parturition. Research shows that a normal elimination has been observed until days 14-15 post partum, characteristic to the 3 periods of the involution process: days 1-7, 7-14, 14-21. The clinical expression was given by the ultrasound periodic exam in days 1, 7, 14, 21, post-partum.

Key words: *cow, puerperal, uterus.*

Methods and materials

Using ultrasound on milk cows reproductive system begins to have a great practical importance regarding the state of uterine health in different stages of physiological solicitation.

The main methods of investigating the characteristics of uterine involution are the transrectal palpation and the ultrasound exam. There have been monitored after being slaughtered, 4 females in different stages of their puerperal period, being determined the length, weight and diameter of the uterus.

The main parameters monitored in this exam are referred to morphological modifications of the genital tractus and at the first estrus post-partum or different pathological states. The transrectal exam refers especially at the:

- topography of the fallopian tubes;
- aspect of the liquid eliminated after birth;
- shape of the fallopian tubes;
- degree of involution of uterus and cervix;
- ovary exam.

It has been used the ultrasound Aquila Vet, with a transrectal probe with a double frecuency of 6-8 MHz.

Results and Discussion

The research has been conducted in a bovine raising unit from the NE of Romania on a number of 154 cows from the breed Baltata with Negru Romaneasca. The gynecological investigation was made in the period 2011-2013 on 20 cows, monitored in their puerperal period.

In the research it has been monitored: the topography of fallopian tubes, their shape, the aspect of the post-partum eliminated liquid, the degree of uterus involution.

Table 1. The results of the morphological examination after slaughter between the days 1-21 post-partum (medium results)

Nr. crt.	ziua post-partum	lungimea (cm)	diametru (cm)	Greutatea (kg)
1	1	97	43	8
2	7	49	35	3
3	14	35	5	1,5
4	21	22	4	1

To assess the rate of uterine involution there were determined after slaughtering several elements: length, diameter and weight of the uterus during the first 21 days post-partum.

Monitoring the 20 cows taken into observation revealed the following: transrectal gynecological examination was performed on days 1,7,14 and 21 post-partum and referred to the determination of uterine length, diameter and weight.

It is found that the length of the uterus varies in the postpartum period between 97 and 22 cm, and when is close to in day 21 to the preconception value (table 1).

Findings indicate that the diameter of the uterus at the same time reduces its size from 43 cm (day 1 post-partum), to 4 cm on day 21 post-partum.

In the uterine weight is a decrease from 8 kg on day 1 post-partum, to 1 kg on day 21 after parturition.

Our research has been conducted over four periods of time (1,7,14,21 days post-partum).

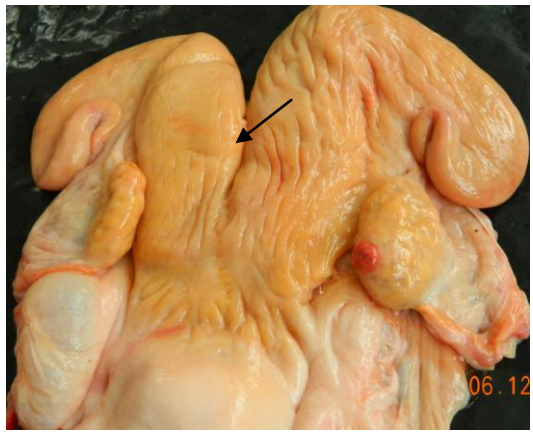

Fig. 1 The macroscopic aspect of the cow's genitalia 7 days post-partum (non-involuted luteal corpus)

In this image it can be observed the aspect of the uterine horn at the beginning of involution (fig. 1).

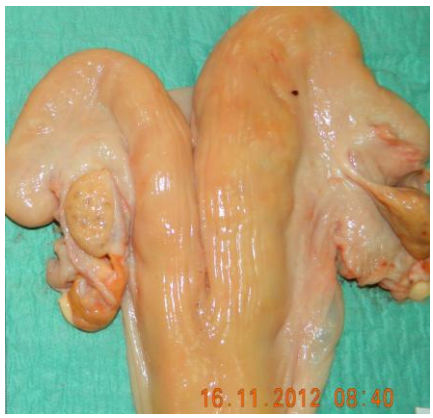

Fig. 2 The macroscopic aspect, cow genitalia 14 days post-partum

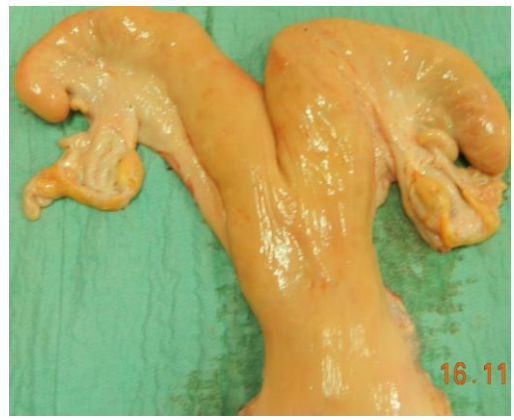

Fig. 3 The macroscopic aspect, cow genitalia 21 days post-partum

At 14 days post-partum is highlighted the longitudinal folds of the uterine wall due to myometrial contractions that occur in the involution of it (fig. 2).

At the end of morphological involution (21 days post-partum), genital takes a before-gestation appearance, both in shape and in size (fig. 3).

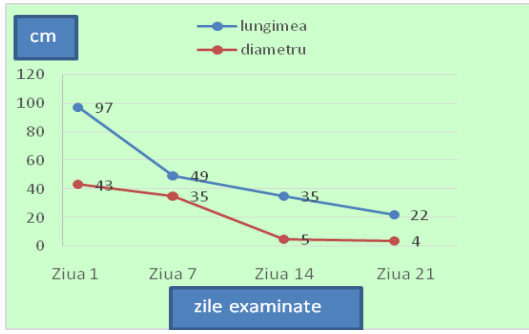

Fig.4 Dynamics of uterine involution (length and diameter) in the first 21 days post-partum

Closely related to uterine involution and regenerative processes is the elimination of liquid after birth, which contains alanto-amniotic fluid, fetal roofing debris, blood resulting from the breakage the ombilical chord, caruncular epithelium, degenerated caruncules, mucus secreted by the uterine epithelium, bacteria etc. As the uterus regresses, its size is reduced and the liquid after birth is eliminated, its amount is gradually reduced (table 2, fig. 5).

The post-partum eliminated liquid quantity and quality were determined by measuring the volume in a graduated beaker consecutive to the transrectal pelvic exam.

Their appearance has changed, first being blood-red, then,in the 7-8 days post-partum, to have a chocolate brown color, and towards the end it had a whitish mucus looking at 20-21 days after parturition. It is sometimes noted the presence in the first days after parturition of a creamy yellow liquid.

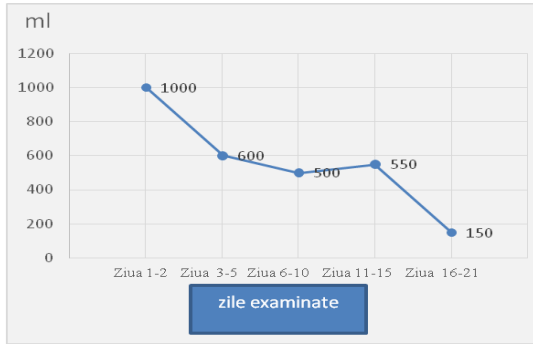

Fig. 5 The quantitative evolution of the liquid eliminated after giving birth in the puerperal period

Elimination of liquid after parturition is a normal physiological process during the first 14-15 days post-partum; all secretions, apart from estrus mucus secretions, that continues to appear after 21 days is within the pathology zone. The prolonged elimination is the main symptom for diagnosis of endometritis or various types of uterine involution.

Another aspect of our research on the monitoring of uterine involution was the study of the dynamics of post-partum changes during 1-21 days. In this regard included observations during puerperal changes produced in 3 periods: 1-7 days, 7-14 days, 14-21 days post-partum.

The research was conducted using transrectal ultrasound, appreciating the involution rate by considering the thickness of the uterine wall.

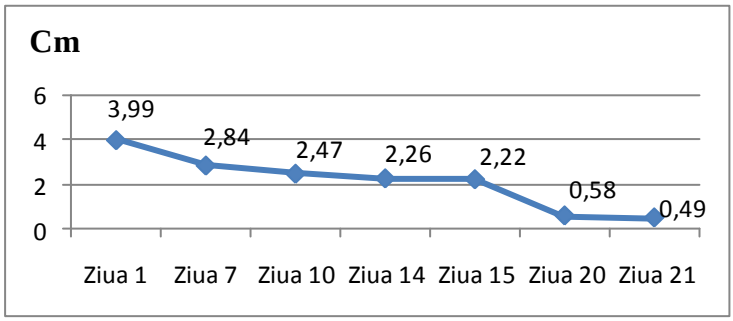

Fig. 6 The thickness of the uterus wall in the 1 to 21 days period post-partum at cows

Thus we find that the first phase of involution has a faster pace from 3.99 to 2.84 cm with a difference of 1.15 cm.

In the second step, up to 14 days post-partum, uterine involution has a slower rate with a decrease in uterine wall thickness of 0.58 cm.

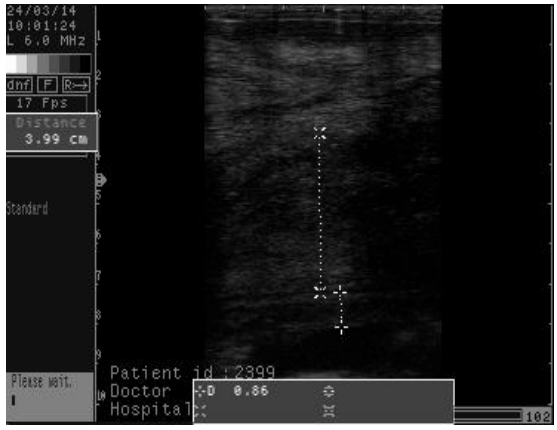

Fig. 7 The ultrasound aspect of the cow`s uterus and uterine mucus in day 1 post-partum. Linear probe, frequency 6 MHz.

There is a uterine wall thickness of 3.99 cm and 0.86 cm mucosa on day 1 post-partum (fig. 7).

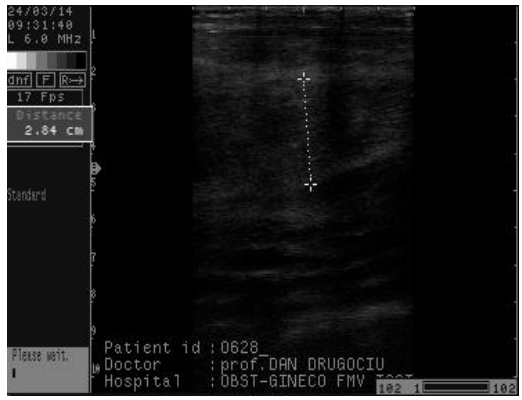

Fig. 8 The ultrasound aspect of the cow`s uterine wall thickness 7 days post-partum. Linear probe, frequency 6 MHz.

Ultrasound appearance confirms the increased pace of involution in the period 1-7 days post-partum (fig. 8).

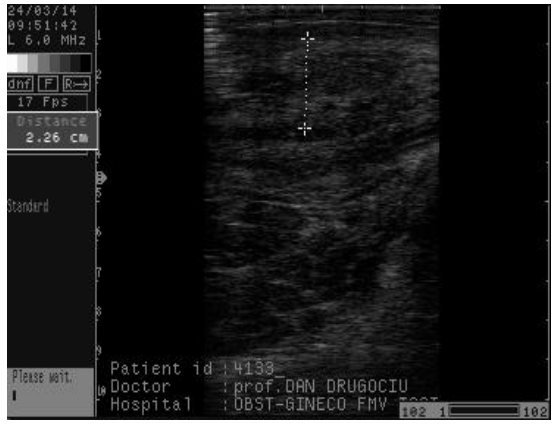

Fig. 9 The ultrasound aspect of the cow`s uterine wall thickness 14 days post-partum. Linear probe, frequency 6 MHz.

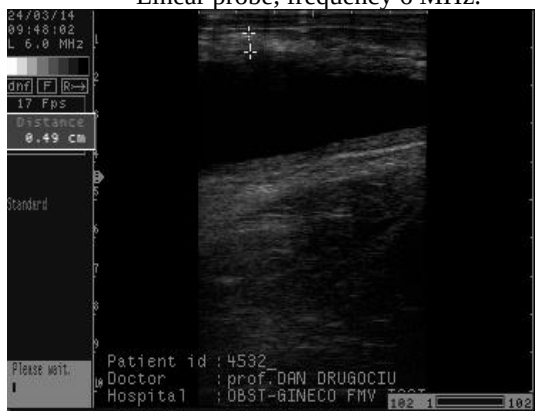

Fig. 10 The ultrasound aspect of the cow`s uterine wall thickness 21 days post-partum. Linear probe, frequency 6 MHz.

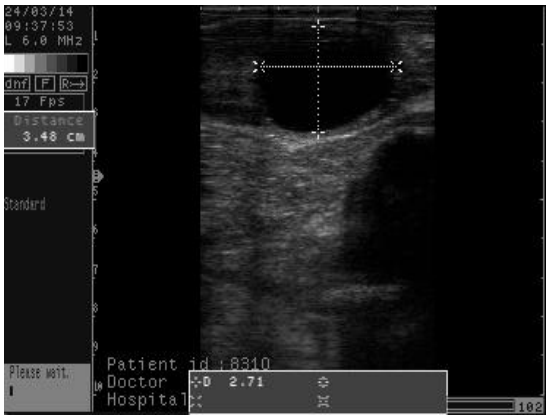

Fig. 11 The ultrasound aspect of a luteinic cyst post-partum.
Linear probe, frequency 6 MHz.

In some cases it has been noticed an extension of uterine involution morphological exceeding 21 days post-partum, delaying the complete restoration of the uterus in order to reinitiate the breeding ciclograma and for the first post-partum estrus to occur.

Our observations recorded during this period the presence of a corpus luteum cyst on the right ovary, which through its secretion of progesterone inhibits and delays the uterine involution. The dimensions of this corpus were of 3.48 / 2.71 cm (fig. 11)

Conclusions

1. The research conducted on a group of 20 cows monitored the uterine involution dynamics in terms of three parameters: length, diameter and weight of the uterus.

2. The length of the uterus will vary in the postpartum period (1-21 days), it varied from 97-22 cm. The diameter of the uterus during this period reduces its size from 43 cm (day 1) to 4 inches (day 21) post-partum. The uterine weight shows a decrease from 8 kg on day 1 post-partum, to about 1 kg on day 21.

3. The after birth liquid elimination rate during the 1-21 days post-partum is characterized by a progressive decrease from approximately 1000 ml in 1-2 days, to 500 ml in days 6-10 and 150 ml in 16-21 days post-partum. A synchronization was found between the rate of uterine involution and the liquid elimination rate.

4. The confirmation of clinical gynecological examination is shown in ultrasound aspects of uterine involution during the 21 days post-partum. It can be noticed a involution in three steps: an accelerated one during 1-7 days, a weaker pace in 10-15 days and again an increase in the rate of uterine involution within 20 to 21 days post-partum.

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OBSERVATIONS REGARDING UTERINE CONDITIONS AS A FACTOR OF INFERTILITY FOR COWS

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Abstract

Bovine fertility can be maintained high during the entire period of sexual life, if a normal parturition and a puerperal period are assured, without any complications.A pathological puerperal period causes important economical damages by the great losses in production that it involves, firstly by the high percentage of infecundity and infertility generated. The data in specialized literature shows that the percentage of infertility varies in high limits from an author to another, between 10-40 % of the number of cows. From the numerous cases of bovine infertility, the uterine conditions have a high incidence. The most frequent uterine conditions evolve from a pathological puerperal period. An important role in affecting the uterus in the puerperal period is played by environmental factors, prolonged stabling, lack of corporal hygiene, low or high temperatures etc. With this desire, we aim to study the influence that different puerperal conditions have on the development of bovine reproduction process, the causes that led for some conditions to appear and the mechanism of different factors that act against the animal organism and against the genital system, in particular. The study has been performed on a period of 2 years, on 154 females in 2012 and 156 cows in 2013 and it shows that the biggest share is occupied by gynecological conditions, 33.7 %. Being analyzed based on the types of conditions, the research shows a percentage of 46,4 of severe endometritis and 18% cases of chronic endometritis inside the farm. Observations from the annual dynamics show that the highest level of severe endometritis is registered during two seasons, spring 29,6% and summer 25,8% , while in autumn the minimum level is 20%. One aspect of the research has referred to the correlations between the productive level of cows and the puerperal conditions. It has been observed that the uterine infections raise incidence directly proportional to the raise of milk production: minimum value (11,1%) of a production of 3000-4000 l milk/cow/year and maximum value (19,5%) of a production of 7000 l milk/cow/year.

Key words: *cow, gynecological conditions, infertility.*

Methods and materials

In order to obtain exact results in the research, there have been used data from inside the farm, information regarding the milk production, gynecological records as well as the existing consultation and treatment register.

The research has been conducted between 2012 and 2013, in a bovine raising unit from the N-E of Romania.

The research material was represented by cows from the breed Baltata with Negru Romaneasca, the number consisting of 154 cows in 2012 and 156 females in 2013.

The animals have benefited from good feeding, maintenance and exploitation conditions.

The clinical diagnosis of the gynecological conditions was effectuated by: general clinical examination and gynecological examination.

The general clinical exam consisted of prevailing the anamnesis data referring to the health state and of the conditions, the manner of manifestation of the estrus, their intensity and the number of artificial inseminations for one fecundation. The data concerning the development of anterior parturitions were analyzed.

The general physiological constants were taken into consideration, especially in cases where the general state of the animals was modified.

In order to track down pathological conditions in the puerperal period the gynecological investigation was done on the entire number of cows. This investigation consisted in:

a) Anamnesis;
b) General clinical exam;
c) Gynecological clinical exam.

The main objectives of the gynecological exam referred to:

- establishing or excluding a state of gestation;
- determining the state of estrus for females that have a non-expressed estrus;
- the diagnosis of the estrus disorders.

a) *Anamnesis.* It represents the first step taken to determine the estrus disorders diagnosis or the state of gestation. For this, the investigations refer to:

- the reproduction program, the manner of artificial insemination;
- feeding with estimation of the fodder quality, the quantity and the administration mode;
- the exploitation technology, movement, rest, microclimate, the animal density and hygiene conditions;
- monitor of the establishment of the estrus activities, the parturition and the puerperal period;
- data that regard lactation and the health state of the mammary gland;
- the frequency of reproduction disorders and the abortions;
- examination of the vaginal secretion.

All these data were written on a monthly basis in the reproduction registers and in the gynecological registers of the females.

b) *The external clinical exam* – done using near and distant investigations.

In the abdominal examination it has been studied the depth or abdominal retraction, the abdominal symmetry, the development of the pelvis depending on the age and the physical development. There have been also studied the state of the sacrum-ischion ligaments, which appear, if relaxed or infiltrated, as two wholes near the base of the tail. In the examination of the vulva, there can be observed modifications of the labia, of the genital secretions, signs of a genital infection.

c) *The internal clinical exam* was done using two methods:

- trans-rectal exam;
- colposcopic exam.

In the colposcopic exam we have obtained data to emphasize the permeability of the uterine cervix and the modifications of the aspect, the color of the vaginal mucosa and eventual secretions.

Results and Discussion

Within the studied number of cows, in 2012 there have been registered several conditions: medical 10,38 %, surgical 5,19 %, the biggest share being occupied by gynecological conditions, 33.7 % (fig. 1).

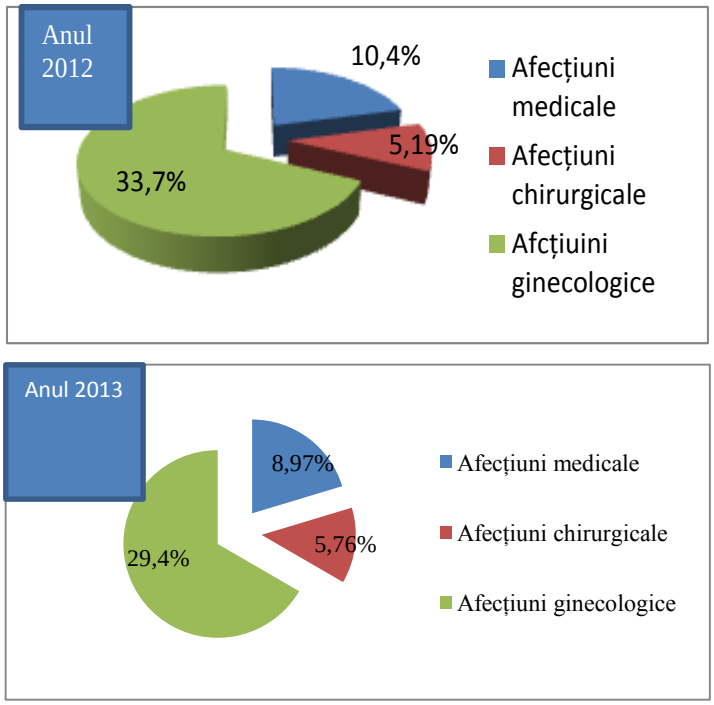

Fig. 1 The distribution of the medical conditions in 2012-2013.

In the following researches between 2013-2014, there have been found 31 cows with placenta retentions (20%), 72 cases of severe endometritis (46,4%), 28 cases of chronic endometritis (18%) inside the farm.

Among the cases of severe endometritis, in 2012, there have been registered an incidence of 39 cases (25,3%), and in 2013, 33 cases (21,1%) (fig. 2).

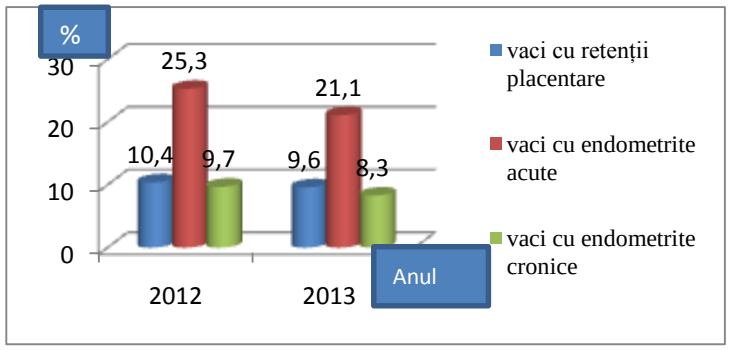

Fig. 2 The incidence of the gynecological conditions in 2012-2013.

The number and quality of the bacterial colonies inside the puerperal uterus depend on the normal or pathological evolution of this period. Therefore, the number of germs in the liquid eliminated after birth is maximum in the first days (4-8) post-partum, the frequency of

the isolated bacteria and their number in the next days, depending on the puerperal development.

All of these, together with the improvement of corporal hygienic conditions and of the shelters, a better assistance to parturition, equilibrating and completion of the food ratio, led to the minimization of the frequency of uterine conditions.

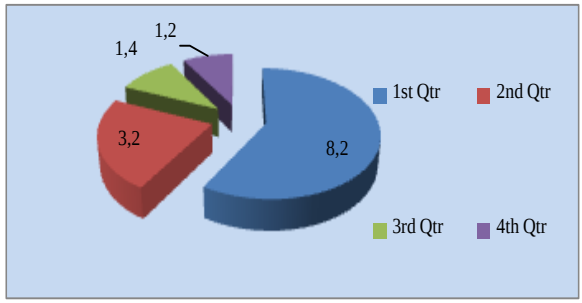

Fig. 3 The frequency of the puerperal conditions in 2012

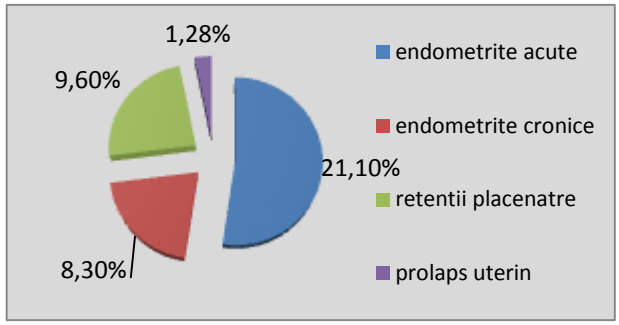

Fig. 4 The frequency of the puerperal conditions in 2013

In the figures 3, 4 we observe that a lower percentage is registered in chronic endometritis than in severe ones, but it is obvious that in 2013, due to preventive treatments and improvement of the assistance to parturition in the post-partum period, the rate of severe endometritis minimized from 25,3% to 21,1% and of the chronicle endometritis from 9,7% to 8,3%.

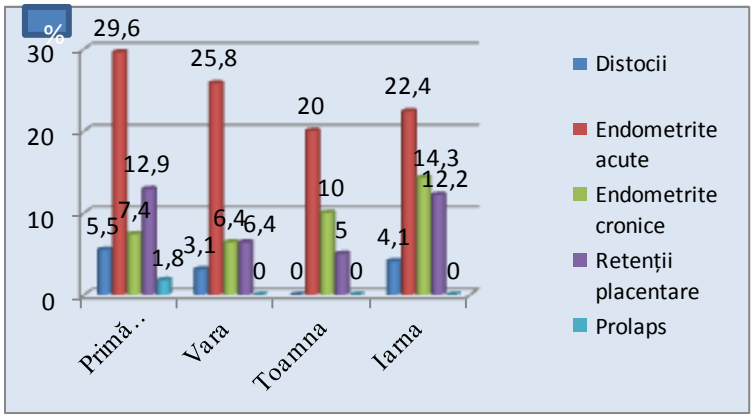

Fig. 5 The incidence of the puerperal conditions in 2012 according to the season

Analyzing figure 5, we can observe that the distocics have a higher level during two of the seasons: spring (5,5%) and winter (4,1%), in the summer being a decrease down to 3,1%.

Following the same pattern, the severe endometritis are more frequent in the spring (29,6%) and in the summer (25,8%), and in the autumn being lowered down to 20%.

The highest percentage of chronic endometritis is of 14,3% in winter, while in the summer the percentage is low, of 6,4%.

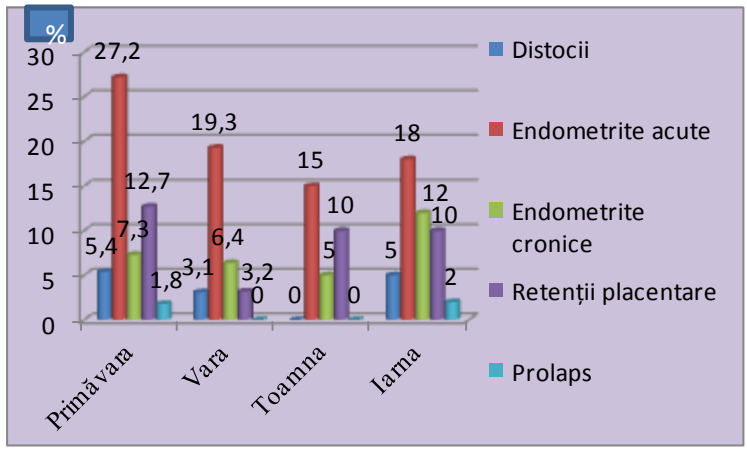

Fig. 6 The prevalence of the puerperal conditions in 2013 according to the season

Fetal retentions reach a peak in the spring season of12.9%, in winter of 12.2% and the lowest was found in autumn, 5%.

In 2013 the frequency of puerperal disorders is lower than in 2012. Comparing the same period, during the spring of 2012 were respectively reported 39 cases (25.3%) of acute endometritis, and in the spring of 2013 were reported 33 acute cases of endometriosis (21.1%).

The milk production is one of the main factors on which depends the evolution of puerperal period and the recovery of females in case of uterine conditions.

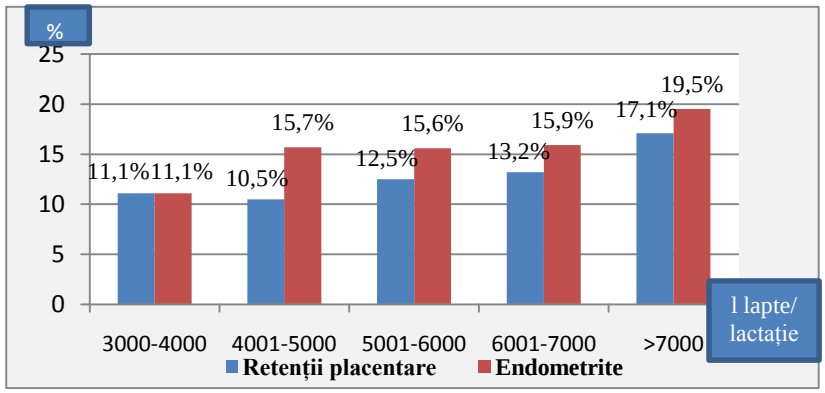

Fig. 7 Correlations between the productive level and the puerperal conditions

Analyzing figure 7 it can be observed that: uterine infections increase their incidence proportion to the increase in milk production, with a minimum of 11.1% at an output of 3000-4000 liters of milk / cow / year and a maximum of 19.5% at an output of over 7000 liters of milk / lactation / year.

Conclusions

1. In the case of gynecological diseases it is observed that a lower percentage of chronic endometritis to the acute (in 2013) due to preventive treatments and improving care at parturition in the post-partum period. Acute endometritis rate decreased from 25.3% to 21.1%, and chronic endometritis from 9.7% to 8.3%.

2. Cows with different post-partum disorders generally have lower milk yields than clinically healthy.

3. Uterine infections increase their incidence proportion to the increase in milk production, with a minimum of 11.1% at an output of 3000-4000 liters of milk / cow / year and a maximum of 19.5% at an output of over 7000 liters of milk / lactation / year.

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BREED DEPENDENT CLINICAL ASPECTS IN CANINE ATOPIC DERMATITIS

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Abstract
Canine atopic dermatitis is an allergic condition with genetic background and primarily dermatological manifestations represented by pruritus, eritema, papulae and secondary lesions due to an abnormally increased IgE production consequent to sensibilization towards airborne allergens. The aim of this study was to find correlations between the literature data and the clinical cases of canine atopic dermatitis registered at the Faculty of Veterinary Medicine Iaşi, between 1 october 2012 – 1 may 2014, regarding breed site predispositions of the skin lesions, with special interest in West Highland White Terriers, Labrador Retrievers and German Shepherds. Generalized pruritus was found in all dogs, erythema and alopecia in the typical sites: axillae and abdomena but in GS and LR there was a preference for the inguinal region and a diffuse alopecia was found on the distal parts of the paws. In LR periorbital alopecia and erythema of the pinnae was more often than in other breeds. In GS, a constant finding were the numerous papulae diffusely localized on the dorso-lumbar area and the involvement of the ventral side of the neck. In WHWT a special aspect was represented by the intense hyperpigmentation and parakeratosis on all ventral parts of the body, along with bacterial and fungal infection. Otitis externa was also found especially in GS and WHWT. The specific site predispositions of different dog breeds in atopic dermatitis can facilitate the clinical examination and treatment approach in order to assure a favorable evolution of the disease, without further unconsidered complications.
Keywords: *canine atopic dermatitis, symptoms, skin, predisposition*

Introduction

Canine atopic dermatitis is an allergic, inflammatory and pruritic disease that appears after the summation of different factors: genetic, individual, environment. The affected dogs belong or are crossbreeds of breeds known to be predisposed (Jaeger, 2010). Atopy is a hypersensitivity reaction towards common air allergens: pollens, storage mites, cockroach feces, scales. Typical signs include pruritus (mostly generalized), erythema, alopecia and papules. The secondary lesions are excoriations and erosions that follow scratching (they are frequently associated with superficial pyoderma), hyperpigmentation and parakeratosis.

Besides air allergens, responsible for triggering the clinical manifestation of the disease are also food allergens and flea saliva leading to the summation effect and crossing the threshold value (Gauguere, 2004; Solcan, 2011).

Genetic predisposition for atopic dermatitis involves both breed and individual, leading to certain lesions on specific body sites.

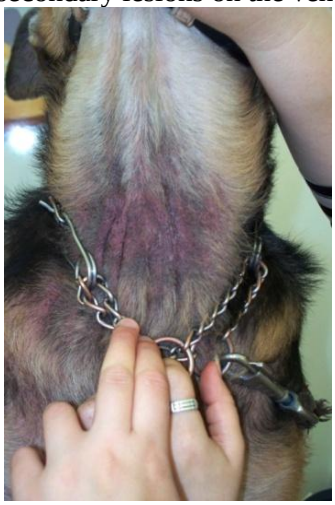
Materials and methods

Between 1 october 2012 – 1 may 2014, 41 dogs were registered with atopic dermatitis. Out of these 28 were crossbreeds and 13 were pure breed dogs: 5 West Highland West Terriers, 4 German Shepherds şi 4 Labrador Retrievers that were diagnosed with atopy not associated with flea or food allergy. Differential diagnostic was carried out based on anamnesis, clinical signs, microscopical examination of skin scapings and response to an elimination diet.

Results

All the dogs considered for this study had clinical signs typical for an allergic dermatitis. After supplementary investigations the definitive diagnostic was set for atopic dermatitis (DeBoer, 2001). The lesions and their location on the body were characteristic (Favrot, 2010; Griffin, 2001) for this disease but we observed a series of particularities regarding their severity, distribution and evolution linked with the breed of the patient.

In German Shepherds we observed an increased incidence of papules located on the dorso-lumbar area (Fig.3, 4) followed by alopecia, dry skin and emergence of primary and secondary lesions on the ventral cervical area (Fig. 1, 2).



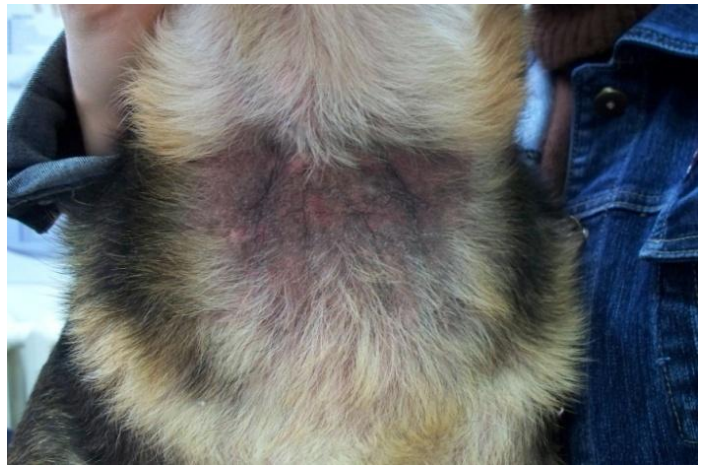
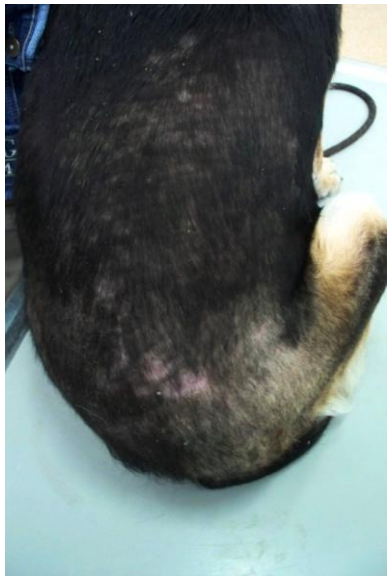


Fig. 1, Fig. 2 – Alopecia, erythema and hyperpigmentation in the cervical region in two German Shepherds



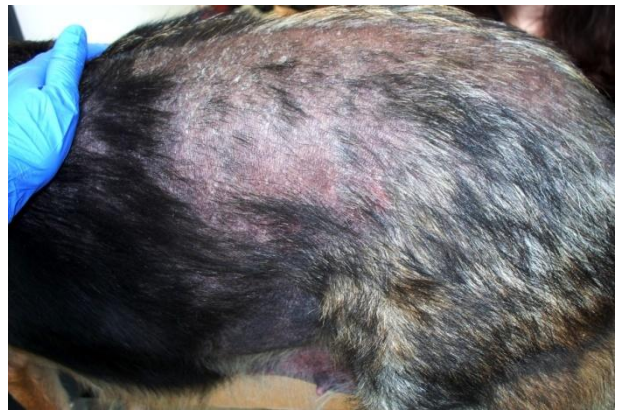


Fig. 3, Fig. 4 – Diffuse alopecia, papules and erythema localized in the dorso-lumbar area in two German Shepherds

In this same breed (Fig. 5, 6) but also in Labrador Retrievers (Fig. 7) the distal parts of the legs were affected with a diffuse alopecia and severe pruritus that lead to self inflicted erosions.

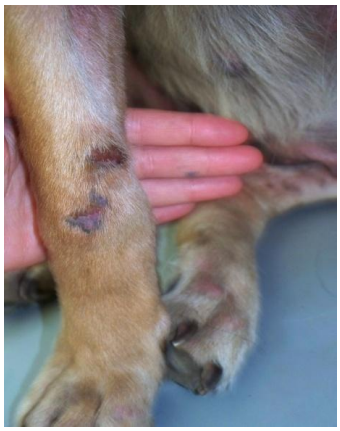
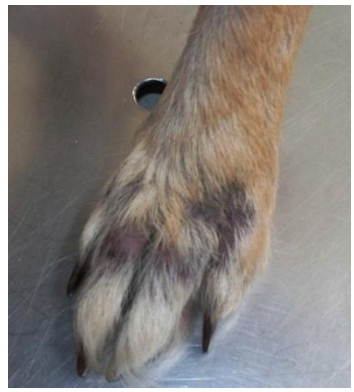
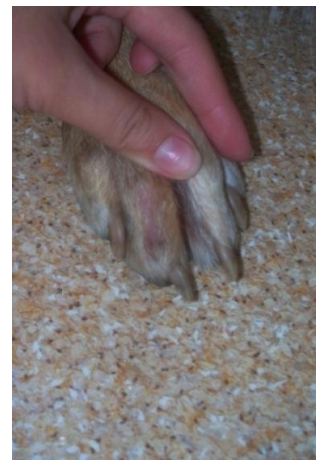




Fig. 5, Fig. 6, Fig. 7 – Affected distal parts of the front and hind limbs in dogs with atopic dermatitis (German Shepherds and Labrador Retriever).

Periorbital alopecia (Fig. 8) and erythema followed by papules on the pinna (Fig. 9) were seen especially in Labradors and external otitis was found in all examined breeds, but more severe in WHWT (Fig. 10, 11), where the ear canal was completely blocked with secretions and hypertrophic masses.

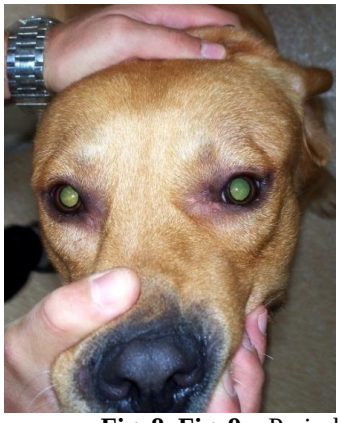
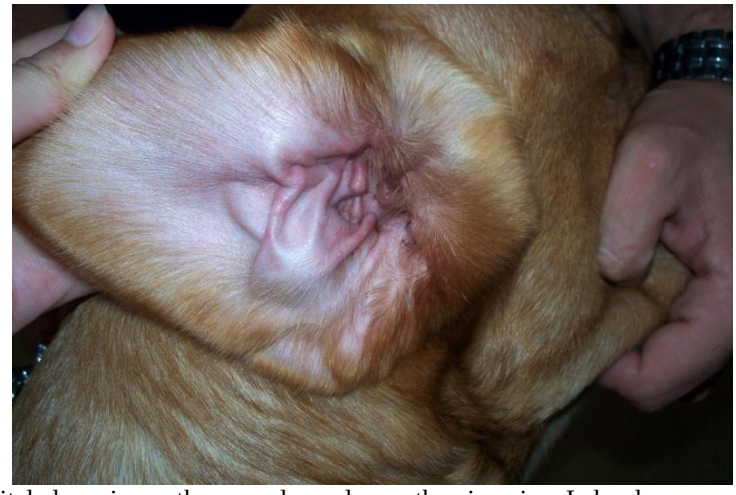



Fig. 8, Fig. 9 – Periorbital alopecia, erythema and papules on the pinna in a Labrador

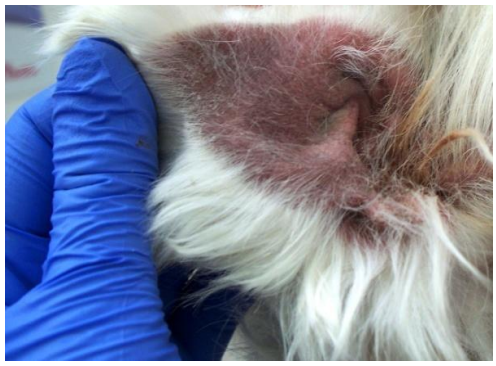
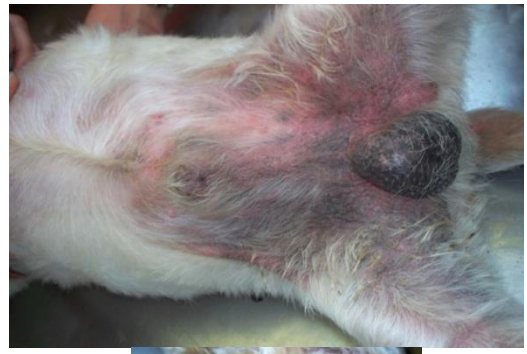



Fig. 10, Fig. 11 – External otitis, hyperpigmentation and parakeratosis in two West Highland White Terriers

In WHWT the most important aspect we observed was the involvement of all the ventral parts of the body and a rapid evolution from erythema and alopecia (Fig. 12) to hyperpigmentation, parakeratosis and bacterial (*Pseudomonas spp.)* and fungal (*Malassezia spp.)* infections (Fig. 13, 14, 15).

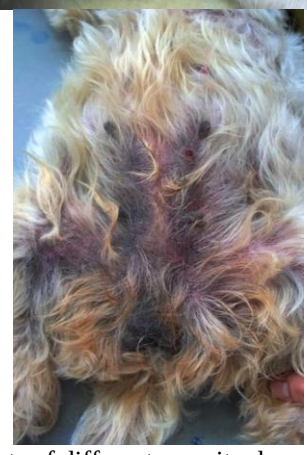


Fig. 12, Fig. 13, Fig. 14, Fig. 15 – Lesional aspects of different severity degrees in 4 West Highland White Terriers with atopic dermatitis.

Discusions

Atopic dermatitis is a disease with a strong genetic background shown both by the breed and the individual predisposition. The breeds considered in this study are among those with a high incidence for this disease. In 2010 (Jaeger, 2010; Wilhem, 2010), in a study done on 843 atopic dogs (both food induced and strictly aeroallergenic), Sylvia Wilhem (9) identified phenotypic patterns regarding the lesions and their distribution in this type of dermatitis in 9 breeds: Boxer, German Shepherd, Golden Retriever, Shar-pei, Dalmatian, Labrador Retriever, French Bulldog, West Highland White Terrier, Jack Russell Terrier. We chose only three of these breeds: German Shepherd, Labrador Retriever and West Highland White Terrier in whom we did observe constant locations of the typical lesions and a particular evolution pattern. The obtained results don't match exactly with those of Wilhem's, possibly because of the smaller number of subjects. In GS we found in all individuals the involvement of the ventral neck region, with lichenification and hyperpigmentation and the dorso-lumbar area, usually associated with flea allergy.

The lesions we found were the ones typical for atopic dermatitis, mentioned in literature (Nuttal, 2009; Scott, 2001; Solcan, 2011). The evolution pattern and associated complications were surprising in WHWT. The extension degree on all ventral body areas (cervical, armpits, pectoral, abdominal, inguinal) and also on the medial sides of the front and back legs signifies an increase in the severity of the clinical manifestation, the same as the rapid crossing from pruritus to hyperpigmentation, parakeratosis and associated infections in approximately 14 days. Also, although the fungal infection with *Malassezia spp.* is frequently found in this breed (Griffin, 2001; Solcan, 2011), we found it in only one patient, from the rest we isolated *Pseudomonas spp.* In this same breed we observed a predisposition for the inflammation and infection of the supracaudal organ.

External otitis represented a form of evolution of atopy in all breeds included in this study, associated or not with dermatological lesions. The most severe form was seen in WHWT with the total closure of the external ear canal and warty masses. The most superficial form included erythema and papules on the pinna and at the entry of the external ear canal in Labradors.

Conclusions

1. Atopic dermatitis is a disease with nonspecific clinical manifestations that require a carefully performed differential diagnosis.
2. Atopy may manifest itself differently from breed to breed but also individually.
3. Labradors manifest mainly the primary lesions of this disease.
4. West Highland White Terriers show the most severe forms of atopic dermatitis with rapid evolution.

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NESTED-MULTIPLEX PCR DETECTION OF PARAPOXVIRUS (ORF VIRUS) AND PAPILLOMAVIRUS DIRECTLY FROM SAMPLES COLECTED FROM GOATS AND CATTLE FROM ROMANIA

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Abstract

Differents viral species cause skin lesions in domestic ruminants, among all notifiable FMDV, BTV and poxviruses but also parapoxviruses (PPVs), Herpesvirus and Papillomavirus. The clinical aspect of skin disorders lead to frequent cases of misdiagnosis with possible severe consequences in terms of economic impact and zoonotic transmition. The possibility to identify the viral agents responsable of skin lesions with rapid direct method could help the definitive diagnosis. In the last years, in Romania the most common skin diseases affecting the domestic ruminananants are mainly those caused by PPVs ., Orf virus (OV), the prototype of PPV genus, affected the adult goats and the kids, with lesions localised in the mouth area, legs and on in the hudder. Cattle are frequently found to be affected with viruses causing multiple skin lesions spread all over the body traditionally defined as papilomatosis. To identify the etiological agents causing cutaneos diseases in domestic ruminants from Romania, skin and scab samples have been collected from affected cattle and goats respectively. The animals were farmed in two different locations Iasi and Botosani. PCR has been performed on DNA extracted from either skin or scabs, in particular for the identification of PPVs a fragment of the conserved gene B2L has been amplified using primers PPPI and IV while for the detection of bovine papillomavirus we used FAP59 and FAP64 primers targeted to the conserved L1 gene of Human papillomavirus (HPV) but able to amplify a broad spectrum of papillomavirus species. Our results showed that the skin lesions found in goats were caused by PPV and subsequent sequencing demonstrated orf virus as the causative agent. The PCR performed on bovine lesions confirmed the presence of Bovine papillomavirus DNA. This study demonstrated that PCR can be effective for a rapid identification of viral agents responsible of skin diseases of ruminants. This technique is also useful as a starting point for genomic characterization of virus strains circulating in Romania.

Key words:*PPV, HPVs,multiplex PCR*

Materials and methods

In order to detect the two taxonomic groups of the virus, viral DNA extractions was used from scabs and tissues collected from six ill animals (three cattle with BPV infection and 3 goats with PPV infection). The extracted DNA was subjected to the PCR multiplex reaction. For this purpose, various combinations were made between the two DNA's extracted from the isolated viruses in order to see if there is a possibility of gene recombination and the development of new re-combined strains. To identify a portion of the B2L gene (592bp), which is specific to Parapox viruses, PPP1 (GTCGTCCACGATGAGCAG) and PPP4 (TACGTGGGAAGCGCCTCGCT) primers were used; for Papiloma viruses FAP 59 (TAACWGTIGGICAYCCWTATT) and FAP64 (CCWATATCWVHCATITCICCATC3) primers were used – all of previous primes being used to identify theL1 gene with a length of 400bp, isolated from the human HPV type (Table.1) .

Table 1. Reaction mix used for multiplex PCR

Buffer 10X	**5µl**	**Primeri**
Sol Q	**10µl**	
dNTPs 10mM	**8µl**	
PPP1 (primer foward)	**1µl**	**5' GTCGTCCACGATGAGCAGCT 3'**
PPP4 (primer revers)	**1µl**	**5' TACGTGGGAAGCGCCTCGCT 3'**
FAP 59 (primer foward)	**1µl**	**5' TAACWGTIGGICAYCCWTATT 3'**
FAP 64 (primer revers)	**1µl**	**5' CCWATATCWVHCATITCICCATC3'**
Taq	**1µl**	
H2O	**17µl**	
DNA	**5µl**	

Extraction of viral DNA was performed using NucleoSpin®Tissue kit (Macherey - Nagel, USA). The mix was prepared under the initial distortion at a temperature of 94ºC for 10 min, followed by 45 cycles of amplification, each cycle being followed by a denaturation step at a temperature of 94ºC for 1 minute and 30 secondseach than an alignment phase at 50 ° C for 1 minute and 30 seconds each and after a elongation phase at 72ºC for 1 minute. At the end of the 45 cycles, the mix was exposed to a final extension for a period of 6 min at 72ºC temperature. For a final PRC multiplex process, the amplifier was subject to a 2% agarose gel electrophoresis (100mA, 400V). The gel was loaded with 5µl of amplifier and 1µl of bromine blue - phenol. DNA amplifier was revealed using ethidium bromide gel while viewing the transluminal (UV). For each electrophoretic migration a quantitative marker of known molecular weight was used (100bp DNA Ladder BioLabs, New England) as well as a negative control. The images were viewed using Vision software Works®LS attached to GelDoc-It310 image analyzer (UVP, UK). Purification amplifier obtained by PCR was done using NucleoSpin®Extract II kit (Macherey-Nagel, USA), which is intended to eliminate the amplified primers, nucleotides and other substances that were used to prepare the PCR mix.

Results and discussion

The PRC multiplex results that were obtained after the viral DNA extraction from tissue samples taken from animals with ectima lesions (Figure) and also from animals with papilloma (Figure:) confirmed the diagnosis, showing that this technique is useful in differentiating the two types of DNA (Figure.1).

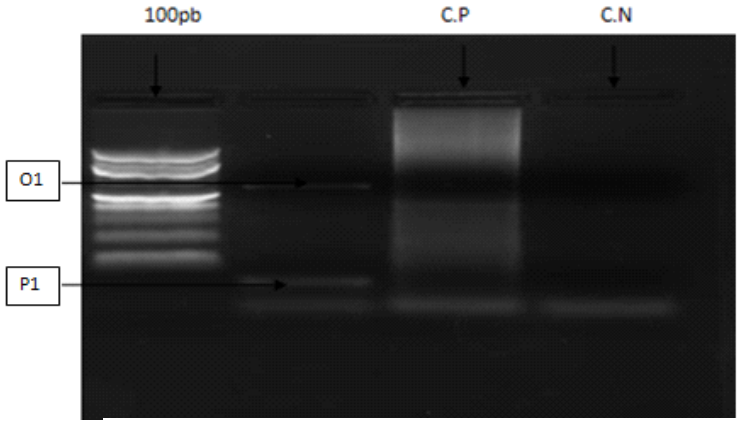

Figure 1. Detection of viral DNA of BPV/PPV by multiplex PCR technique
(100pb marker, CP-positive control, C.N- negative control, O1- ORF virus positive sample, P1- papillomavirus positive sample)

B2L gene sequences that encode the protein found in the PPV tire and which is similar to p37K protein from the major envelope of vaccinia virus was used as a control for molecular diagnostics and viral species identification. The GenBank database is a large number of sequences which are used as a reference for the phylogenetic and epidemiological analyzes. As a result of the obtained sequences it was possible to see the isolated strains of (for example) the Orf virus within the goat population from Romania. This fact indicates that the outbreaks infections occurring in these species are associated with Orf virus and not other species that belong to the parapoxvirus type. Phylogenetic analysis performed on the amino acid sequence showed that both proteins, the formation of two major groups are not linked to any geographic origin or animal species or type of virus strain. Regarding the identity that exists between the amino acid sequences encoded by ORF 109, we can say that it can vary from one strain to another, the similarity degree between them being about 50% (viral strains 613 and 987 also 987, 613 and 8L strain, 613 and C2). Regarding the identity percentage of the amino acid sequences encoded by gene Orf 110, this can vary from a maximum of 100% between viral strains 613 and 987 and a minimum of 43.6% between strains 613 and NZ2. The analysis of the phylogenetic and sequence alignment with a length of 498 bp it was shown that the strain which affects mostly goats is NZ2, sheeps beeing more resistant, having a milder form of the disease unlike goats.

Conclusions

1. The research results obtained provide us relevant information regarding the evolution of the Poxviruses within 2 different geographical areas characterized by household system grown animal population – in farms or herds.
2. The Poxviruses, due to large genome and double-stranded DNA, are able to encode a large number of genes. The presence of multiple genes allows the virus to easily tolerate mutations related to selective pressure.

3. Methods used to extract the virus from the crust were PCR methods of molecular biology. Viral DNA was extracted using NucleoSpin®Tissue kit (Macherey - Nagel, USA), following the protocol.
4. The PRC multiplex, is a fast method to confirm the laboratory diagnosis even if the appearance is characteristic to anatomo-clinical BPV infection and PPV. The method itself is recommended in order to properly identify the gene pathogen, the amplification being carried out by a simultaneous co-infection of DNA extracted from samples of animals infected with the two viruses.
5. As a result of phylogenetic analysis of sequences with a length of 498 bp, based on the B2L gene,it was showen a phylogenetic affinity between ORF4-f-PPP1 and ORF 5-f-PPP1 also a phylogenetic similarity between ORF1-f-PPP1 and ORF6-f-PPP1 .

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RESEARCHES REGARDING THE CONTAGIOUS ECTHYMA IMMUNOPROPHYLAXIS IN SHEEP AND GOATS

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Abstract

Contagious ecthyma is one of the contagious infection entities responsible for the high death number among young animals, especially those with the age between 2- 3 weeks to 3 months. Parapoxviruses are viruses that have DNA in their structure and belong to the family Poxviridae, subfamily Chordopoxvirinae. These viruses cause eruptive skin disease affecting domestic mammals, wild mammals and man. Viruses of this type cause: bovine papular stomatitis, bovine pseudocowpox, contagious ORF virus which leads to ecthyma in sheep and goats, All the species of this type are transmissible to humans. Current knowledge about molecular biology of parapoxviruses and particularly virulent genes that infects hosts are limited and generally based on studies done on poxvirusures.To prevent the contagious ecthymma illness in youth, it was used the Scabivax vaccine, administered in the first days after lambs and kids birth, as well in adults before and after calving. By molecular biology methods (PCR), it was shown that the vaccine contains NZ2 strain, which is circulating in animals in Eastern Romania and provides immunity for ORF virus infection, being recommended in combating the contagious ecthyma.

Key words: *PPV, PCR*

The only vaccine that we were able to purchase is SCABIVAX ™ Forte, which is manufactured in England and throughout PRC procedure we have also revealed that it contains within it's composition the NZ2 strain which circulated throughout Europe. The Scabivax vaccine (Figure above) can be used 3or 4 weeks in advance before the appearance of typical contagious ectima symptoms which means that the proper administration for lambs and goat kids is done during Spring and Autumn period, especially birth season. Lambs and goat kids can be vaccinated immediately, either 2 days after birth or after the first sign of the ectima symptom. The vaccine for adults can be administered during or after pregnacy. The extraction of the viral DNA was performed using NucleoSpin®Tissue kit (Macherey - Nagel, USA). Thus the viral DNA was extracted from a volume of 25 µl of the vaccine over which a 180 µl Buffer T1 solution and a 25 µl of Proteinase K was added; the final sample incubated for 1-3 hours at 56 º C. After incubation time, the sample is being mixed with the aid of the vortex after adding 200 µl of Buffer B3 and then incubated at 70 ° C; 10 minutes later the sample is being removed from the incubator and added 210 µl of ethanol 100%. The amount of material obtained, is loaded into a tube that can be found within the kit and which is fitted with a storing filter that allows the centrifuging of the sample at 11000 RPM(rotation per minute). This type of tube contains a silicon gel membrane that allows the DNA selection within the sample. The contaminating samples, including the inhibition enzymes, are neutralized after washing with a quantity of 600 µl of Buffer B5, the buffer itself being neutralized while spinning at 11000 RPM. The final step in this process is obtained by adding the Buffer BE which was heated and maintained at a temperature of 70 ° C throughout the whole process. After centrifugation the necessary DNA quantity was obtained in order to prepare the PCR mix.

1.B2L gene amplification.

The B2L gene with a length of 594 bp, coding for the protein p37K was amplified using universal primers ppp1 forward (5 'GTCGTCCACGATGAGCAGCT 3') and reverse PPP4 (5 'TACGTGGGAAGCGCCTCGCT 3'). For preparing the mix, the kit Taq DNA polymerase was used (Qiagen, Germany) and the mix was prepared using the amounts specified in the table below (Table 1).

Table 1. The reaction mix in a final volume of 50 µl

Reagenţi	**Volumul**
Buffer 10X	5 µl
SolQ	10 µl
dNTPs 10mM	4 µl
PPP1 Foward 20 pmoli/ml	1 µl
PPP4 Reverse 20 pmoli/ml	1 µl
TaqDNA Polymerase (5U/ml)	0,25 µl
H2O DNAsi şi RNAsi free	23,75 µl
ADN (extras din vaccin)	5 µl
Volum final	**50 µl**

The prepared mix was subject to a 5 min distorsion at 94ºC followed by 40 cycles of amplification, each cycle being followed in turn by a 30 seconds distorsion at 94ºC, a alignment phase at 58 ºC for 30 seconds and an elongation phase at 72 ºC for 45 seconds. At the end of the 40 cycles, the mix itself is exposed to a final extention for 7 minutes at 72 ºC.

2. Electrophoretic migration

At the end of the PCR process the obtained amplifier was subjected to electrophoretic migration using 2% agarose gel (100 mA, 400 V). Within the gel, 5µl of amplifier, together with 1µl of blue bromo phenol was added. The DNA amplifier was revealed by using ethidium bromide by viewing the gel on transilluminator (UV). Thus for each electrophoretic migration ti was used a quantitative marker of a known molecular weight (100bp DNA Ladder BioLabs, New England) as a negative control. The images were viewed after using Vision software Works®LS attached to GelDoc-It310 image analyzer (UVP, UK). The purification of the amplifier obtained through PCR, was performed using a NucleoSpin®Extract II kit (Macherey-Nagel, USA) which was intended to eliminate the amplified primers, nucleotides and other substances that were used to prepare the PCR mix. The end product obtained from the amplification reaction is brought to a volume of 100µl out of which: 5 µl of Buffer NE increased to every 45 ml of buffer and 50 µl of NT. The mix was placed in a tube equipped with a filter and centrifuged at 11,000 RPM for 1 minute. A 600 µl of Buffer NT3 is added further and centrifuged for 2 times,2 minutes for each cycle, thus allowing both washing and drying of the filter membrane. In order to prevent that the ethanol content within the washing substance will not interact during the rest of the processes, the tubes are incubated for 2-3 minutes at a 70ºC temperature and then centrifuged again at 11000 RPM's.

The quantification of the PCR products was performed using spectrophotometer (Abs 260/280), assessing an average of 5 readings made using BioPhotometer machine (Eppendorf, Germany) with a dilution of 2 µl sample and 58 µl water.

Results and Discussions

The amplification of the gene sequences that are conserved in B2L gene was done based on the extracted viral contained front the vaccine sample, the amplifier having a length of 594bp, this being visible within the gel after electrophoretic migration (Figure 1).

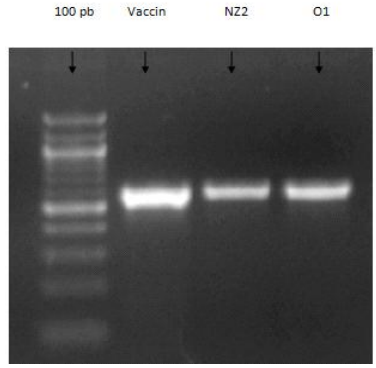

Figure. 1 Electrophoresis on 2% agarose gel, B2L gene (marker of 100 pb, Vaccin – positive sample, C.P (NZ2) – positive control, O1 – positive sample of Orf virus)

The B2L gene sequences that is encoding for the protein found in the PPV tire which is similar to the major envelope protein of vaccinia virus p37K, was used as a identifier for molecular diagnosis and identification of viral species.

Conclusions

1. In order to prevent the contagious ectima illness withing the youth animal population, Scabivax vaccine was used; the vaccine was used within the first days after birth for lambs and kids and on adults befor and after birth.
2. Through PRC method it was shown that the vaccine contains the NZ2 strain circulating in animals within eastern Romania and thus providing immunity for the Orf virus infection.
3. We recommend the use of this vaccine in the immunoprophylaxis of contagious ectima in sheeps and goats.

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RECOVERY OF NEUROLOGIC DEFICITS CAUSED BY POSTTRAUMATIC SPINAL HEMATOMA WITHOUT SURGERY DECOMPRESSION IN A DOG

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Abstract

A female golden retriever, 1.3 years old, weighting 28 kg, was presented at the Medical Clinic of the Faculty of Veterinary Medicine – Iasi with acute progressive tetra paresis and spastic urinary incontinence installed over 5 days. Neurological examination and imaging tests (X-rays, computer tomography and magnetic resonance imaging) established the diagnosis of thoraco-lumbar post-traumatic spinal hematoma. As the surgery for blood clot removal was impossible, the patient received symptomatic medication, and clinical signs evolved from tetra to spastic paraparesis. To restore motor functions lost due to traumatic impact, the dog underwent the physiotherapy program which had a major role in the patient's neurological recovery.

Key words: *dog, physiotherapy, spinal cord hematoma*

Introduction

The spinal injuries are the most frequent cause of the small animals' disability, and the most affected spinal segment is the thoracic-lumbar segment, particularly, for young dogs of medium breeds (Jeffery, 2010).

Patients with severe neurological impairment due to compression of spinal cord tissue need urgency surgery for decompression spinal nerve tissue and it must be enhanced functional recovery by recruiting nervous tissue (Jeffery, 2010).

In practice, there are situations when surgery may be detrimental for the patient' benefits and conservative therapy should be instituted immediately, although, only occasionally it leads to a complete neurological recovery (Kassem, 2010).

In case of spinal hematoma, similar to the human medicine, the decision to refuse the surgery of spinal cord decompression for removing the clot is based on the spontaneous recovery, but also non-surgical therapy has occasionally led to neurologic recovery. (Connolly et al., 1996; Dickman et al., 1988). More precisely, this decision is based on the neurological improvements that occur during the drug therapy of the early stages of haemorrhage, because the animals with vascular spinal pathology, commonly, show a fast and important improvement of the neurological deficits after the first week from the incident (Groen, 2004).

Thereby, the conventional therapy for patients with vascular myelopathies is based only on the physiotherapy and time, because the function recovery in the central nervous system does not occur by the neural tissue regeneration, but rather by the survival tissue that takes on the functions of those axons that have been damaged (Jeffery, 1999).

The recovery done by physical therapy techniques should not be made only to reduce the tissue damage extension in the spinal tissue or only to support the injured nerve cell function as a result of compression, but also to stimulate the capacity of the patient for regaining the locomotors function. To achieve these objectives, it is necessary that the

recovery program combines the physiotherapy techniques suitable for each patient in particular (Olby et al., 2005).

The prognosis of the animals with traumatic spinal cord is evaluated clinically. This is made particularly depending on the presence or the absence of the deep pain perception (Bagley et al., 1999; Olby et al., 2003) and the evaluation of the neurological progress of the patient during the physiotherapy program that is done using the recovery scores (Park et al., 2012).

The Olby score compared to other recovery scores (Griffin et al., 2009; Levine et al., 2009) includes a system that allows the assessment and the monitoring of the pelvic limb activity after thoracic-lumbar spinal injury. The system is represented by a numerical rating scale comprising 14 points and 5 stages (Olby et al., 2001).

Materials and methods

A female golden retriever, 1.3 years old, weighting 28 kg, was presented at the Medical Clinic of the Faculty of Veterinary Medicine – Iasi with acute progressive tetra paresis and spastic urinary incontinence appeared over 5 days, so it was diagnosed with post-traumatic thoracic-lumbar spinal hematoma.

After the 10 days of symptomatic treatment, the dog with spastic paraparesis was included in the program of physiotherapy for recovery of neurological deficits that occurred after the accident.

The recovery program was developed over a period of 12 weeks (W) and it included the methods of physiotherapy electrotherapy (low level laser therapy), the massage, the passive range of motion and the proprioception. (Table 1) (Figure 2,3,4,5,6,7,8).

Table 1. The recovery program

Physiotherapy techniques		Duration	Frequency	Benefits
Low level laser therapy		7 sessions	once a day	- the anti-inflammatory activity - the hematoma resorption - the local tissue bio stimulation
Massage		12 weeks	2 times per day	- the muscle relaxation - the microcirculation stimulation - the antalgic activity - the tissue heating before the exercise
Passive range of motion		12 weeks	2 times per day	- it supports the joint mobility - it heats the joints before the exercise
Proprioception	Balance board	6 weeks	once a day	- it stimulates the muscle proprioception and the static and dynamic stability
	Cavaletti rails			- it stimulates the ability and flexibility of the muscles, tendons and ligaments, it stretches the joints allowing the getting of a wide range of mobility so the patient be able to execute manoeuvres through tight spaces
	Pole weaving			- it encourage sides bending of the spinal column and it helps for the paraspinal muscle strengthening
Dancing		3 weeks	once a day	- it encourages the weight-bearing on the rear limbs
Sling		3 weeks	once a day	- it helps to provide support for maximal assisted standing of a patient

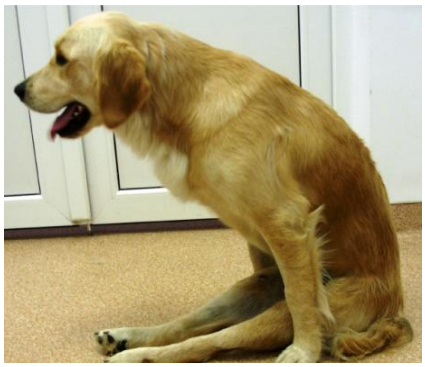

Figure 1. Golden retriever dog 1.3 years. Spastic paraparesis

Figure 3. The use of a sling to help provide support for standing

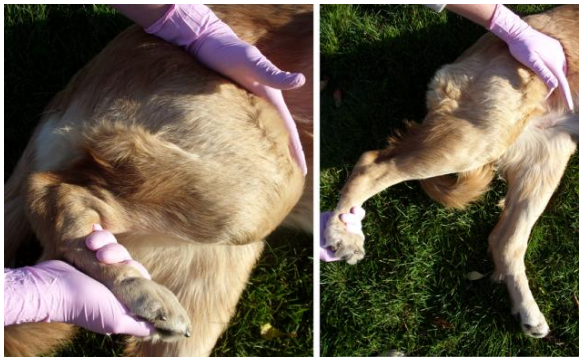

Figure 2. The passive range of motion

Figure 4. The spinal ataxia. The clinical aspect after 4 weeks

Figure 5. The use of a balance board under the patient's hind limbs it assists with the proprioceptive training

Figure 6. The dance encourages weight-bearing on the rear limbs

Figure 7. The pole weaving encourages side bending of the spinal column and it helps with the paraspinal muscle strengthening

Figure 8. The cavaletti rails help to increase the active flexion and extension of joints as the dog crosses the rails.

The laser treatment was applied using an IR 27 laser device Rolland series with the following features: soft-infrared laser emission, well equipped with a diode 27 W, wavelength 905 nm, frequency adjustable from 5 to 6500 Hz.

The laser therapy was applied and performed on the intervertebral spaces T9-T10, T10-T11, T11-T12, one side of the spine. Initially, the area was clipped and disinfected. After the contact points were made, the ultrasound gel was applied to facilitate the penetration of laser radiation with tissue. The parameters used were as it follows: the frequency - 900 Hz, the number of steps - 6 steps, the time step - 120 seconds, total time - 12 minutes. Therefore, it was applied an energy with the density of 64.8 J/cm ².

To assess the evolution of the neurological signs, the patient was examined before, during and after the physical therapy program and to highlight the stages of recovery of pelvic limb motility it was used the Olby score recovery.

Results and discussion

The medical history revealed an acute progressive evolution of clinical signs and after the symptomatic therapy; the neurological deficits were improved from the tetraparesis associated with urinary retention to the spastic paraparesis.

The objectives of the rehabilitation program included local tissue inflammatory process management and the support of damaged nervous tissue by using laser radiation and stimulation of ability to regain locomotor function (Olby et al., 2005).

The use of the laser therapy was based on multiple biological effects of laser radiation as changes in biomarkers of inflammation and the distribution of inflammatory cells, the reduction of the edema, haemorrhage and necrosis (Albertini et al., 2007; Correa, 2007; Honmura et al. 1992; Ionescu, 1999; Sattayut et al., 1999).

Some studies (Campana et al., 1999) have highlighted the similarity of the anti inflammatory effects of laser radiation resemblance to those of anti inflammatory substances as meloxicam. Moreover, the stimulating action of the laser radiation on the tissue and on the neovascular tissue with effect on the increasing of the local microcirculation it may lead to the local hematoma resorption within 2-4 days according to some studies (Ionescu, 1999).

The stimulation of the capacity of regaining motor function was based on therapeutic effects of manual physiotherapy methods (Tabel 1) and it was made by an appropriate

combination of these with exercises to stimulate the proprioception, the balance and the coordination (Olby et al., 2005).

Recording to the clinical course throughout the program of physiotherapy with recovery, the Olby score highlighted the fact that the patient has gone through all stages of recovery (I-V), accumulating a total of 10 points recovery (Figure 9).

The clinical course of the patient from the graphics (Figure 9) highlights the fact that the clinical major evolution of the patient was done in the first part of the physiotherapy program (W1-W5) followed by a stabilization period. The most points gained were recorded in W2-W4 with a total of 6 points recovery. Since W9 the clinical neurological evolution began to stabilize.

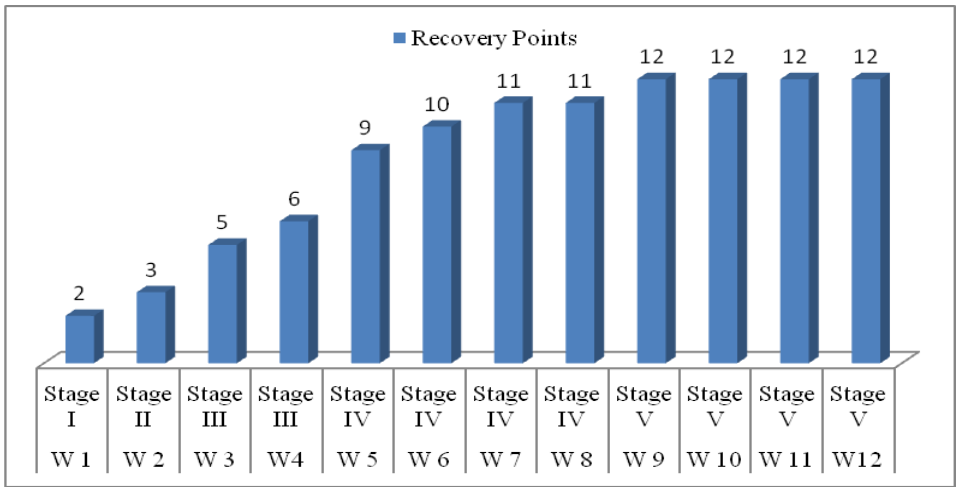

Figure 9. The graphic representation of the patient evolution

The evolution of the patient in the first 5 weeks can also be the answer of the young body of the patient, because the young nervous system has an extraordinary degree of plasticity expressed as spare capacity following the injury, this has already been demonstrated by some studies (Olby et al., 2003).

In another study on Olby regarding the time needed for recovery of the pelvic limb function of patients with severe spinal thoracic-lumbar, paraparesis and deep sensitivity it has been observed that it occurs in the first month after the injury was produced (Olby et al., 2003).

Generally, the thoracic-lumbar spinal injuries and especially the compressive injuries tend to affect more likely the white matter leading to myelin degradation, deformation of ion channels, obstruction of blood vessels and finally the destruction of the axon (Olby et al., 2005, Jeffery, 2010). Although in such cases, the surgical decompression can cause a dramatic reversal of the clinical signs (Olby et al., 2005), the possibility of recovering motor deficiencies from the first part of the recovery program reveals that the irritative thorn of spinal cord caused by the presence of hematoma have substantially reduced once with its resorption. Moreover, the support for the damaged nervous tissue survival as the main objective of the program of physiotherapy was well managed by the biological effects of the laser radiation.

The manual recovery methods that have completed the electrotherapy program played a key role in the regain the patient motor capacity. The massage and the passive range of motion performed daily contributed substantially to the reduction of muscle spasm, maintaining and fostering the mobility of joints and tendons limbs, but also it increased the range of motion even starting the 2nd week. The progressive exercise program always applied after muscle massage and the joint exercises heating made possible to assess the patient for stage III.

The use of a sling to support body had encouraged constantly the animal to maintain body weight, initially in resort only a few seconds. Encouraging the animal to support body weight, initially in resort and then in attempts to go it has become essential for the patient, the literature states that the weight must be supported initially between 75% and 100% then it must gradually decline such that it can provide enough support for the animal; support necessary for realization of the exercises and the activities which it cannot perform before (Millis, 2004).

The exercises to stimulate the proprioception, the balance and the motor coordination were applied gradually since the second half of the recovery program and they were continued daily until the end of the program according to the literature (Millis, 2004) that recommends their performance until the animal has a normal or near normal gait. During these exercises, the patient recovery gained 2 points, but made possible its transition from stage IV to stage V. Nevertheless, the therapeutic exercises proved to be the most important aspects of the physical recovery because they have contributed to the clinical evolution of the patient.

The choosing of the recovery score is a decision of the veterinarian because it is variable depending on each patient. For example, though the Olby score with 14 motor points is qualified as system that was developed the possibilities of scoring and monitoring of pelvic limb function following a thoracic-lumbar disorders, it could not assess the postural reactions, the limb asymmetry or the evaluation of the thoracic limbs as well as other recovery scores therefore in this study could not follow closely these issues.

Conclusions

The first five weeks of the program of physiotherapy are the most important for recovery of lost neurological deficits due to trauma.

The electrotherapy techniques must be applied in the early days of physiotherapy according to therapeutic targets and then it must be combined with increasing sequential manual techniques.

The adequate heating of tissues by massage and passive range of motion provides patient the relaxation and effective fitness for a program of exercises well supported.

The key for the success of the recovery program is represented by the correct application of the techniques and exercises to maintain a consistent level of daily activity, because only several short sessions per day are more effective than one long session in the first part of the recovery.

The choosing recovery score is a decision of the veterinarian depending on the patient's neurological condition.

The registering of the clinical course of neurological patient before, during and after recovery program is very important both for the doctor and for the owner, because the doctor is the only person who can decide the fate of the animal.

The collaboration between the veterinarian and the owner of the animal is the key to success of physiotherapy because recovery requires time, patience and dedication to the paralyzed patient.

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