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# SCIENTIFIC WORKS
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# SUMMARY

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## ANIMAL PRODUCTION, PUBLIC HEALTH AND FOOD QUALITY CONTROL



## EXPERIMENTAL MEDICINE

# FUNDAMENTAL SCIENCES

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# THE MORPHOLOGICAL CHARACTERIZATION AND THE ACTIVITY STATES OF THE EPITHELIAL CELLS PRESENT IN THE JENNET COLOSTRUM

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***Abstract***

*The colostrum secretion of different mammalian species contains heterogeneous cellular populations which come from lactiferous structures and from the general circulation, including various types of epithelial cells, respectively leukocytes. During the synthetis and milk-ejection process, in lactation are involved different types of epithelial cells, with a wide morphological and functional diversity. The aim of this research is represented by the cytomorphological investigation of the first jennet colostrum, which was drawn from healthy mammary gland. The colostrum samples were processed in stained slides, using Dia Quick Panoptic method and they were examined at the microscope and they were evaluated by using the milk cytogram technique (Ognean et al., 2011). The conducted studies have highlighted morphological elements and activity states necessary for the characterization of the cellular populations, regarding the morpho-functional description of the epithelial cells. The results revealed a significant percentage of the epithelial cells in the jennet colostrum. In the configuration of this cellular population, we have identified alveolar, squamous and columnar epithelial cells. The alveolar cells have prevailed among the epithelial cells and they have reflected the secretory activity of the alveolar epithelium. The squamous epithelial cells were distinguished due to their predominant polygonal cytoplasm and the punctiform nucleous, while the columnar epithelial cells were elongate, ovalar or polygonal, with a porous structure. A special category was represented by the atypical cellular structures, that were polymorphous and sometimes mixed with cytoplasmatic particles and cellular debris. The results were analysed in the context of encouraging the use of the mammary epithelial cells in different domains of the scientific research. In conclusion, the high content of the epithelial cells in the jennet mammary secretions represent a disposable resource for the molecular study of the mammary gene expression, lactogenesis, immunity or mammary cancer.*

***Key words****: jennet, colostrum, cytology, differentiation, epithelial cells.*

## INTRODUCTION

Despite the fact that, since the first description of the somatic cells of milk had passed almost 100 years (Donné et al., 1838; Jammes et al., 2002), nowadays we have not found any data available regarding the cytology of the jennet mammary secretions. In this context, we mention that this type of data abound in cows (Buehring et al., 1972), goats (Tateyama et al., 1988) or sheep (Lee et al., 1981; Ognean et al., 2016) and they occasionally appear in sows (Ognean et. al., 2011; Vlasiu et al., 2013), dogs (Meyer et al., 2010) or women (Gaffney et al., 1976).

The configuration and the distribution of the cellular populations in colostrum and milk are influenced by different physiological (species, physiological status, management policy) and pathological factors (mammary infections). Regarding the origin and the functions of the various types of milk cells, the most relevant studies have approached the morphological and the physiological criteria which has served as a foundation for their classification and description. The predominant cells in the colostrum and milk are represented by the components of the immune system (lymphocytes, neutrophiles and macrophages), and their involvement in the mammary gland defense totally justify the use of NCS (total number of somatic cells) as an indicator for the evaluation of the milk and mammary gland health in bovines and other species.

Together with the leukocytes, NCS include even more types of epithelial cells, which are present in milk during lactogenesis and lacto-ejection. Nowadays, a major scientific interest it is shown for the epithelial cells population. It is also noticeable the multiplication of the research which was based on the selection of

the milk epithelial cells and their characterization on the basis of the activity state and the viability level and differentiation (Meyer et al., 2010). The purpose of this study is to describe the epithelial cells population from the jennet colostrum, in order to acquire new applicable information for the evaluation of the experimental potential of the mammary epithelial cells.

## MATERIALS AND METHODS

The cyto-morphological studies were carried out on colostrum samples collected from 5 jennets, with healthy mammary glands.For the health evaluation of the mammary glands we have resorted to their clinical examination, followed up by a Contrast test (Rotaru and Ognean, 1998). The colostrum samples were harvested by manual milking, after a previous preparation of the mammary glands, based on the usual sanitation measures (washing the breast, removing the first milk jets and disinfecting the teats with sanitary alcohol). Therefore, in the first 3 days after parturition, we collected average samples , consiting of at least 3 fractions of the milking and representing 5 mL for each mammary gland . The colostrum samples were processed in stained slides by using the squash technique and Dia Quick Panoptic method and then, they were examined at the microscope and they were evaluated by using the milk cytogram technique (Rotaru and Ognean, 1998). The adjustment of this cytological method to the specific of the mammary secretion required some additional laboratory tests, besides the leucogram technique (from blood), such as: the preparation of the colostrum sediment, by centrifugating (10 minutes at 3000 g) 5 ml of colostrum sample, diluted in ratio of 1:4 with physiological serum; the removal of the grease and the preparation of the smears from the obtained sediment ; degreasing the smears, by dipping in xylene or methanol.

Some adjustments made to the specific of the milk secretion also required the procedure used for registering and analysing the data, consisting of the usage of the physiological criteria for the differentiation of the cellular subpopulations and the atypical nuclear cellular structures present in the mammary secretions.

In order to identify and classify the cells types we took into consideration the following morphological criteria: the aspect of the nucleus (shape, volume, segmmentation level); the distribution of the chromatic material; the presence of the nucleolus or nuclear corpuscle. Additional physiological assessments were made concerning the features of the cytoplasm and the evolution of the activity, based on the evaluation of the nucleus/cytoplasm ratio, respectively the volume and the cytoplasmatic tinctoriality. In the cellular configuration we have also observed the cellular conglomerates, that have

resulted from the intercellular agglutinations or grease, under the form of microconglomerates. The survey of the registered data has mainly pursued the morpho-functional description of the epithelial cells types and the documentary regarding the usage of these epithelial cells in various domains of scientific research.

## RESULTS AND DISCUSSIONS

Concerning the relevance of the squash technique and the Dia-Quik-Panoptic coloration, we consider that they have secured a good display and tinctoriality of the smears which were made from the colostrum; moreover,it has sustained their usage for the morphological exam of the colostrum physiological and pathological secretions, with the condition of degreasing them previously. The cellular content of the first colostrum has emphasized an extremely increased number and a high level of heterogeneous cellular populations. The overall configuration of the cellular population has revealed different types of epithelial cells, that come from lactiferous structures, respectively leukocytes (neutrophils, lymphocytes, macrophages), which come from general circulation. Other distinctive entities, with a high morphological and functional diversity have been discovered during the synthesis process and milk ejection, such as desquamated epithelial cells (Figure 1). In the following we have described and analysed the distinctive activity of the epithelial cells and the morphological criteria that has emphasized their features, respectively the differentiation of the cytoplasmic particles and the atypical cellular structures.

The obtained data underlined that the epithelial cells population, which has been identified in

the jennet colostrum, was represented by the alveolar, squamous and columnar epithelial cells.

The alveolar epithelial cells have morphologically one or two spherical nuclei, surrounded by a high volume of basophilic cytoplasm, with a circular or foamy aspect.

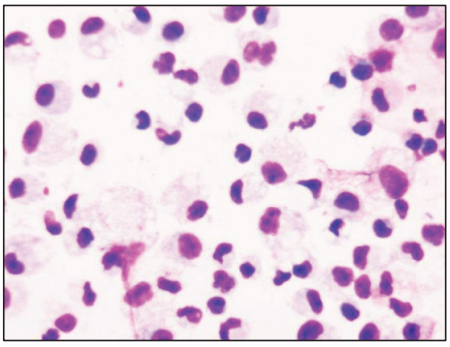

Figure 1. The overview configuration of the cellular population present in the jennet colostrum, including the alveolar cells which prevail among the other epithelial cells and different types of leukocytes (Dia Quick Panoptic Col.; x 100)

These alveolar cellular entities have different secretory activity states and they have prevailed in the epithelial cells population. The acinar cells were often difficult to be recognized because of their various morphological appearances and activity states (Figure 2).

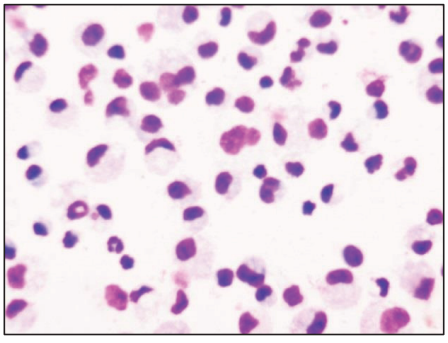

Figure 2. The predominance of the alveolar epithelial cells, in the jennet colostrum sediment, characterized by one or two spherical nuclei, surrounded by a poor basophilic cytoplasm, with a foamy aspect and grease vacuoles (Dia-Quik-Panoptic Col.; x 100)

The squamous epithelial cells have rarely been found, and their identification firstly required a differentiation from the atypical cellular structures. The main morphophisiolo-gical elements that are useful for the distinction of these cellular entities are the predominant polygonal cytoplasm and a small, punctiform and pyknotic nucleus (Figure 3).

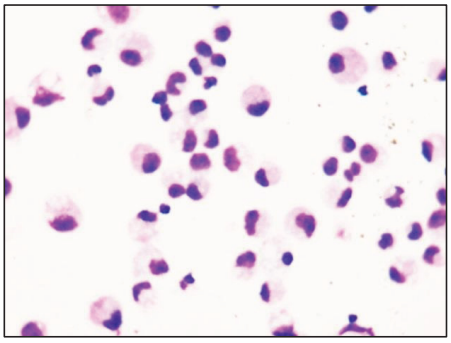

Figure 3. The presence of the squamous epithelial cells in the jennet colostrum, characterized by the polygonal cytoplasm and punctiform nucleus (Dia-Quik-Panoptic Col.; x 100)

The columnar epithelial cells were more frequent than the squamous ones. They are elongate, oval or polygonal, with a polygonal nucleus and a foamy structure (Figure 4).

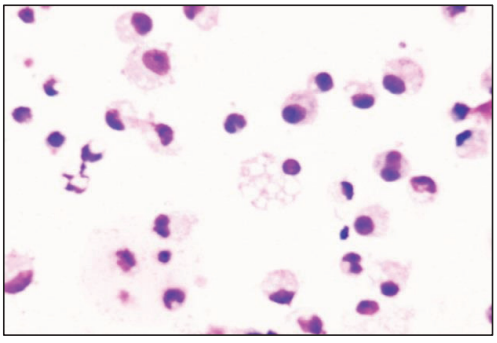

Figure 4. The presence of the columnar epithelial cells in the jennet colostrum, which are elongate,oval or polygonal, with an oval nucleus and a foamy structure (Dia-Quik-Panoptic Col.; x 100)

We consider that these epithelial cells often come from the epithelium of the mammary gland cistern and the galactophorous ducts.

The atypical cellular structures were emphasized such as polymorphic structures , which were frequently mixed with the cytoplasmic particles or anucleate cellular debris. They have a polymorphic and noticeable nucleus, surrounded by atypical morphological elements, more or less affected by the necrobiosis or apoptosis.

The mammary epithelial cells are present in

milk due to the desquamation of the lactiferous structures , that are often found in goats and other species with a predominant apocrine secretion (Taylor-Papadimitriou et al., 1981), than in cows and other species with a predominant merocrine secretion (Taylor-Papadimitriou et al., 1977). In goats and women, the increased frequency of the epithelial cells can be associated with the multiplication of the cytoplasmic particles and cellular debris (Schalm et al., 1971). In general, the cytoplasmic particles are anucleate and contain proteins (casein micelles) and lipids, without any pathological connotation. However, some of the particles may contain nucleate fragments. The origin and the function of the epithelial cells, which are strongly vacuolized and with a foamy aspect, were reported more than one hundred years ago in colostrum and during the dry period (Donné, 1838; Boutinaud and Jammes, 2002) and they were also the subject of several investigations. Therefore, some researchers regard them as desquamated epithelial cells (Gaffney et al., 1976), and others consider that they are similar to the macrophages from blood (Jensen et al., 1975; Lee et al., 1980). The epithelial cells from milk have diferent functions, including the transmission of the maternal immuno-globulins (IgA) by colostrum, a significant process of neonates immunity. The colostral immunity is a well-known mechanism in swines (Le Jan et al., 1996), leporids (Rosato et al., 1995) and ovine (Rincheval-Arnold et al., 2002).

The analysis of the cytological configuration of the colostrum reveals the augmentation of the phagocytic activity at the debut of lactation, which is very unstable and it requires the self defense mechanism of the mammary gland.

On the other hand, an increase of the PMN leukocytes at the end of colostrum period can be correlated with the lactiferous structures intensifying contact with various microbial agents, that colonize the mammary gland by ascendant way. The investigations conducted by Rotaru and Ognean (1998) had divided the cells which come from the ovine milk into leukocytes (65%) and cells with mammary origin (35%). Moreover, the increased frequency of the PMN leukocytes is associated with the evolution of different forms of mastitis.

It is widely recognised that the atypical cellular structures, the cellular debris and the acellular structures can be found in milk in cows, goats and sheep, with an increased frequency in goats (Jandal, 1996). This is a strong argument that reveals the limiting character of the indices of the mammary gland health based mainly on the somatic cells counting (NCS). The registered statistical analysis (Gonzalo et al.,1988) are relevant for the studies conducted on large sheep batches and they have established significant correlations between the increase of NCS and several factors, such as lactation period and the number of lactations.

The percentage of the epithelial cells varies during lactation and they are more common in milk than in colostrum, in women (Gaffney et al., 1976) and swine (Le Jan et al., 1996). For instance, in sows, epithelial cells represent 20-40% in colostrum and 60-90% in milk. A considerable decrease of the epithelial cells number takes place during the mammary involution (Le Jan et al., 1996). In general, the oscillations of the epithelial cells are not directly correlated with the NCS, which is more increased in the summer, mostly because of the microbial agents activity and not because of the high temperatures (Dohoo et al., 1984).

The flow cytometric analysis showed that 26% from the cells that come from the goat milk are epithelial cells, and they contributed to the removal of the dead cells. The trypan blue exclusion test of cell viability revealed that the human epithelial cells (90%) are more viabile than the epithelial cells which are present in goats milk (40%) (Boutinaud et al., 2002; Gaffney et al., 1976; Thompson et al., 1978). The apoptotic index (TUNEL test) emphasized that only 10% of the total amount of the milk cells could be apoptotic cells, because only 30% of the epithelial cells had an apoptotic DNA pattern. It was also suggested that the anucleate cytoplasmic particles could have remained unstained , and as a result, the value of the apoptotic index is lower than the cellular death rate established by the trypan blue exclusion test.

The epithelial cells represent the major component of the mammary secretion in women and swines (Buehring et al., 1972; Le Jan, 1996; Stoker et al., 1982). The majority of the species have a milk cellular population that consits mainly of leukocytes, including lym-

phocytes, neutrophiles and macrophages. They can be found in the mammary tissue and they are the primary cells of the host defense and immunity against any microbial agent (Le Jan, 1996). The macrophages prevail in bovines and ovine (35%-79%), and PMN leukocytes are predominant in goats (Rotaru and Ognean, 1998). The lymphocytes are present in the mammary secretions and they are responsible for the immunity self-defense of the neonates , especially human and swine newborns (Bertotto et al., 1990; Jain et al., 1989; Wirt et al., 1982). Furthermore, the research carried out by Rota et al., 1993 on 100 Verata breed goats underlined a more valuable increase of the lymphocytes in colostrum than in milk , in correlation with the role of the lymphocytes in the cellular immunity transmission in neonates. The macrophages release chemical mediators when they detect pathogens and as a result, they trigger the recruitment of the PMN leukocytes to the site of infection; their percentage increases from 5% to 25% in order to intensify the phagocytosis process. These arguments highlight that the percentage of the cellular structures varies depending on the species and it is different in colostrum from milk. Thus, it is estimated that the percentage of the macrophages in bovines is about 10% - 20% in colostrum and it is predominant in milk, where it represents 70% - 80% during the middle and late lactations stages. In contrast to that, the number of PMN leukocytes is higher in colostrum (50% - 80%) and lower in milk (1%) (Lee et al., 1980). Regarding the variations between different species, the higher number of PMN in the healthy goats milk can be associated with the low incidence of clinical mastitits in goats (1%). The conducted studies have showed that the increased number of the neutrophils in goats milk play an important part in protecting the animals from the mammary infections.

All data described above have revealed that the NCS oscillations can be correlated with the health condition of the mammary gland and respectively, with the quantity and the quality of the mammary secretion. Moreover, some factors such as stress, mammary involution or the immune response to an infection of the mammary gland lead to a massive requirement of the PMN leukocytes.

The quantitative and qualitative evaluation methods of the epithelial cells from milk provide useful information concerning the integrity of the mammary epithelium, the lactation stage or the effect of the milking method. Furthermore, the primary cultures of the epithelial cells from both colostrum and milk are relevant for the research of lactogenesis or galactopoiesis, mammary immunity mechanisms, cancer or mammary infections. In addition, the RNA structure that was drawn from the milk cells was eloquent for the mammary gene expression and it was also illustrative for the molecular study of the gene expression profile and its interraction with the environment.

## CONCLUSIONS

The cytogram of the colostrum sediment in jennet revealed the existence of a high percentage of the epithelial cells in the configuration of a diverse and well represented cellular population, which had also included the leukocytes (neutrophils, lymphocytes and macrophages), along with the cytoplasmic particles and the atypical cellular structures. The results of our research emphasized the presence of an increased amount of the epithelial cells in the jennet mammary secretions, that can easily represent an available resource of epithelial cells that are necessary for the mammary gland gene expression profile, lactogenesis, immunity or breast cancer investigations.

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# INFLAMMATORY LESIONS IN CASES OF BIRDS KEPT IN CAPTIVITY

**Iulia PARASCHIV[1], Andrei STOIAN[1], Bogdan TASBAC[1], Teodoru SOARE[1], Codrut VISOIU[2], Manuella MILITARU[1]**

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***Abstract***

*Exotic bird pathology comprises diseases, both infectious and non-infectious, incompletely studied so far as pathogenetic mechanisms, lesions identified and measures of management. The present study comprises a number of 33 cases of birds kept in captivity belonging to Corvidae, Fringillidae, Psittaculidae, Columbidae, Phasianidae and Apodidae families that were submitted to diagnosis after death of the birds. Out of these, 16 cases presented inflammatory lesions affecting different body organs and tissues. Results revealed frequent gross lesions of the lungs, liver and digestive tract. Histologic examination marked mainly lesions of fibrinous and necrotic pneumonia, necrotic hepatitis and catarrhal enteritis. Three cases were affected by chronic lesions of granulomatous inflammation located mainly in the coelomic cavity and digestive tract. Bacteriologic investigations revealed mostly Gram negative bacteria isolated from the lesions. Most frequent pathogens causing disease in the cases taken into study belonged to Salmonella, Escherichia, Pseudomonas, and Staphylococcus genera. In conclusion, results of inflammatory lesions revealed by gross and microscopic examination in correlation with microbiologic results represent a further step in evaluation of avian patients and risk of cross-contamination. Frequent affected regions in the body were the lower respiratory tract, liver and intestinal tract, suggestive for the type of contamination with the bacterial pathogens.*

***Key words****: exotic bird, bacteria, inflammation, pathology.*

## INTRODUCTION

Exotic avian pathology comprises a wide variety of diseases, which is due to behavioural, physiological, genetic and predisposing factors that contribute to different pathways of disease, both infectious and non-infectious. In addition, ornamental birds are at higher risk of developing long term diseases such as neoplasia or nutritional and metabolic disorders that can cause immunosuppression and bacterial pathogen invasion (Hoefer, 1997; Nemeth et al., 2016).

Reports on large number of cases examined revealed that post mortem diagnosis was frequently associated with infectious causes, due to husbandry conditions, overcrowded spaces, interference with wild birds and other causes (Lutful Kabir, 2010; Nemeth et al., 2016; Schmidt et al., 2003).

A variety of bacteria frequently cause enteritis and pneumonias in exotic bird species (Schmidt et al, 2003). Gram negative pathogens can be primary or secondary invaders (Lutful Kabir, 2010).

The present paper is aimed to focus on lesions and organs predisposed to react in case of a bacterial inflammatory process in cases of exotic and ornamental birds and bring benefits to both breeders and veterinarians in charge of these species and pathologists.

## MATERIALS AND METHODS

During 2014-2016 a total number of 33 cases of avian species was submitted to diagnosis at the Department of Pathological Anatomy.

The cases belonged to private owners and were classified in several groups, according family taxon, into *Corvidae, Fringillidae, Psittaculidae, Columbidae, Phasianidae and Apodidae.* All cases were submitted to full necropsy, histopathologic examination (H.&E., H.E.A., Ziehl-Neelsen and Gram stains), microbiologic examination (bacterial cultures) and for some cases by complementary examinations such as cytopathology on tissue imprints or pathologic liquids or radiography for coelomic cavity and joints.

## RESULTS AND DISCUSSIONS

Regarding epidemiologic data, all 33 avian corpses were submitted for complete necropsy evaluations and complementary examinations. Out of these, 16 cases were selected as being affected by inflammatory processes, the other 17 being affected by other lesions such as neoplasia, dystrophy or severe post mortem changes marked by autolysis.

The bird corpses examined in the present paper belonged to the following family taxon: *Corvidae* (one case), *Fringillidae* (one case) *Psittaculidae* (six cases), *Columbidae* (five cases), *Phasianidae* (one case) and *Apodidae* (two cases).

**Gross examination** of the bird corpses submitted to diagnosis in the present study revealed several lesions on different organs, affecting mostly either respiratory system or digestive tract.

On respiratory system there were lesions, both acute and chronic, mostly on lower respiratory tract on lungs and more rarely on air sacs. Lesions were identified as deposits of yellowish material on the pleural surface of the lungs in four cases of birds submitted to diagnosis. Other types of gross lesions were represented by whitish-grey lesions disseminated in the lung with various size, from 1 mm to 10 mm.

Regarding the digestive tract, lesions were identified mostly in liver and less often in the intestines.

The liver was affected in three cases of pigeons, four cases of parrots and the case of the pheasant *(Phasianus colchicus*) and the case of Eurasian siskin (*Carduelis spinus*). Lesions identified on gross examination were white foci of necrosis disseminated both on the surface and in the liver parenchyma. In five cases, gross lesions were represented by diffuse congestion or hepatomegaly.

Intestinal tract was affected in two cases of parrots by proventricular and ventricular dilation associated with whitish grey aspect of the mucosa in these regions. Five cases presented intestinal catarrhal secretion along with reddish aspect of the mucosal surface.

Other gross lesions were identified in one case of pigeon that presented increased size and whitish material in the humero-radio-ulnar joints. In this case, complementary examinations were done such as radiographic examination showing the presence of an inflammatory process inside the articular space. Afterwards, a fine needle aspiration was performed for cytopathologic examination and microbiologic culture of the liquid.

Overall gross lesions are presented in Table 1.

Table 1. Evaluation of gross examination of the bird cases affected by inflammatory processes

| Case data | Respiratory tract | Digestive tract | Other lesions |
|---|---|---|---|
| *Columba livia* | - | Hepatomegaly | Humero-radio-ulnar arthritis |
| *Columba livia* | Pulmonary congestion | Catarrhal enteritis, congestion | - |
| *Columba livia* | Fibrinonecrotic pneumonia | Hepatomegaly | - |
| *Columba livia* | Fibrinonecrotic pneumonia and thickening of air sacs | - | - |
| *Columba livia* | - | Liver necrosis | - |
| *Melopsittacus undulatus* | - | Catarrhal enteritis, congestion | - |
| *Melopsittacus undulatus* | - | Hepatomegaly | - |
| *Melopsittacus undulatus* | Pulmonary congestion | - | - |
| *Agapornis roseicollis* | Necrosis on right pulmonary lobe | Proventricular dilatation | - |
| *Psittacula krameri* | Necrosis on right pulmonary lobe | Proventricular dilatation | - |
| *Platycercus eximius* | - | Hepatomegaly | - |
| *Phasianus colchicus* | - | Hepatomegaly and discrete nodular lesions in omentum and adipose tissue | - |
| *Carduelis spinus* | Discrete granulomatous lesions and congestion | Diffuse liver and intestinal congestion, catarrhal enteritis | Hydropericardium, diffuse congestion in spleen and kidneys |
| *Corvus frugilegus* | - | Liver necrosis | - |
| *Apus apus* | Pulmonary congestion | Catarrhal enteritis, congestion | - |
| *Apus apus* | Pulmonary congestion | Catarrhal enteritis, congestion | - |

**Histopathologic examination** performed on tissues obtained from each case revealed inflammatory lesions of different degree for each of the 16 cases of birds.

Lungs were mostly affected by fibrinonecrotic pneumonia consistent of areas of detritus surrounded by inflammation with mixed heterophils and macrophages. Other identified

lesions were represented by congestion and oedema.
Bacterial presence was observed in the lung parenchyma both extracellular and intracellular in seven of the 16 cases affected by inflammatory lesions.
Liver lesions consisted of multifocal to coalescing necrosis in five cases, inflammatory reactions in the proximity of centrilobular areas in three cases and in one case in the margins of the organ. Other cellular changes were represented by binucleation, frequently located around the inflammatory processes and oxiphilia of the hepatocytes.
Three cases of birds submitted to diagnosis were affected by discrete mononuclear infiltrates in the areas of the centrilobular veins areas with nodular aspects of mononuclear aggregates. Other lesions identified in the liver were congestion and hemosiderosis.
Microscopic examination of proventriculus and ventriculus revealed significant changes in the cases of the two parrots affected by dilation. Histology of the stomach compartments in the two cases of birds with proventricular dilation revealed mucosal necrosis, frequent bacterial involvement due to overgrowth in the lumen.
Examination of intestinal samples revealed seven cases of birds affected by inflammatory processes, two more than the cases suspected at gross examination. The samples were collected from both small and large bowel and revealed three cases with catarrhal duodenitis, two cases of typhlitis and two cases with diffuse inflammation of both small and large bowel.

**Microbiologic examination**

Samples were obtained by fresh cut surface of different organs such as lung, air sac, small and large bowel, and by case proventriculus and articular fluid.
Historically, in exotic birds, any finding of a gram negative bacteria has been considered to indicate disease. However, organisms such as *E. coli* have been found in surveys of psittacine birds without clinical signs or lesions indicative of intestinal disease. Therefore, positive results in microbiologic examination need to be associated with gross and microscopic lesions to confirm the cause of the bird's death. Salmonellosis is another disease that can cause significant lesions and cause death of exotic species and is sometimes associated with wild bird feces or rodent contamination of food (Schmidt et al., 2003). In the case of the pigeon with articular inflammatory process, microbiologic culture of the liquid obtained by fine needle aspiration, isolated *Salmonella tiphimurium*, a frequent pathogen involved in pigeon pathology (Rosenthal et al., 2008).
Respiratory bacterial pneumonias occur either by inhalation of the pathogen or as part of a septicaemia process, sometimes secondary to malnutrition or viral infections (Schmidt et al., 2003).
Multiple scientific papers set the liver as a commonly targeted organ for systemic bacterial infections in birds. In this case, both gram positive and negative bacteria can cause hepatitis (Lumeij, 1996).
Results obtained in the present study are comprised in Table 2.

Table 2. Bacteriological test results of studied birds

| Bacterial isolates | Positive results |
|---|---|
| *Staphylococcus aureus* | 3 |
| *Streptococcus spp.* | 1 |
| *Pseudomonas aeruginosa* | 4 |
| *Escherichia coli* | 3 |
| *Salmonella typhimurium* | 4 |
| *Mycobacterium avium* | 1 |

Results of microbiologic examination revealed *Pseudomonas aeruginosa* and *Salmonella typhimurium* as most frequent bacteria affecting the cases taken into study, followed by *Staphylococcus aureus* and *E. coli*. Gram negative bacteria isolates, such as *E. coli*, *Klebsiella* sp., *Proteus* sp., *Salmonella* sp. and *Yersinia* sp. cause most systemic infections in exotic birds. In some cases, *Pseudomonas* sp. is a common isolate as a result of gut or respiratory infections or systemic invasion of the liver or other organs. Most frequent gram positive isolates are *Staphylococcus* and *Streptococcus* sp. and the mechanism of dissemination is through blood from chronic necrotizing skin lesions or by extension from adjacent air sac lesions (Randall and Reece, 1996; Schmidt et al., 2003).
The case (*Carduelis spinus*) that had a mycobacterial infection was tested both with special histopathology stains and microbiologic culture. Both evaluations showed disseminated infection on most internal organs, while at first, gross examination revealed mostly diffuse congestion in internal organs.

## CONCLUSIONS

Main inflammatory lesions identified were fibrinonecrotic pneumonia, necrotic hepatitis and catarrhal enteritis associated with frequent congestion of the organs.

Most frequent isolates in the present study by bacteriologic investigations were *Salmonella typhimurium, Escherichia coli* and *Pseudomonas aeruginosa.*

The affected regions of the body (lower respiratory tract, liver and intestinal tract) were suggestive for the type of contamination with the bacterial pathogens.

Inflammatory lesions revealed by gross and microscopic examination in correlation with etiological data obtained in bacteriological examination represent a further step in evaluation of wild and/or exotic birds.

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# THE INVESTIGATION OF THE VIABILITY OF THE Pt ELECTRODE IN SULPHITE CYCLIC VOLTAMMETRIC ASSAY

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***Abstract***

*This paper aims at the study of Pt electrode ability to act as working electrode in cyclic voltammetric sulphite assay. The analytical signal is represented by the intensity measured for the voltammetric peak, that corresponds to analyte anodic oxidation. The measured peak intensity depends linearly on sulphite concentration within the range 0.031 g/L – 4 g/L. The assay is characterized by a limit of detection of 12.4 mg /L, a relative standard deviation of 2.74%, and the process proved controlled by the analyte diffusion to the electrode.*

***Key words*** *cyclic voltammetry, platinum electrode, sulphite, anodic oxidation, linear potential sweep.*

## INTRODUCTION

Sulphite is a widely used preservative agent in foods and drinks, being incorporated in wines, beer, processed meat, fruit juices, jams and jellies (Pisoschi, 2014; Banu et al., 2000; Stan, 2007; Pisoschi, 2012).

It is recognized for its reductive and antibrowning activity. Its reductive properties constitute the basis of most assessment methods. Sulphite can be easily oxidized during its reaction with iodine, as follows:

$$SO_3^{2-} + I_2 + H_2O \longrightarrow SO_4^{2-} + 2\,HI$$

Excess iodine can be detemined by thiosulphate titration (Musagala et al., 2013):

$$I_2 + 2\,Na_2S_2O_3 \longrightarrow 2\,NaI + Na_2S_4O_6$$

Sulphite can be determined photocolorimetrically, with 5,5'-dithiobis 2-nitrobenzoic acid at 575 nm, allowing a linear range of analytical response between 0.10 şi 4.3 mg $L^{-1}$ (Li and Zhao, 2006). Fuchsin reagent can also be used in sulphite spectrophotometric assay (Leinweber and Monty, 1987).

Voltammetry and amperometry involve the current intensity measurement, at fixed or constantly varying potential. Amperometric assays employ sulphite oxidase-based biosensors, with enzyme immobilization on Prussian blue/polypyrrole nanoparticles composite, electrodeposited on an indium oxide working electrode (Rawal and Pundir, 2012).

Voltammetry at graphite (Lu et al., 1999), platinum (Skavas and Hemmingsen, 2007), modified glassy carbon electrodes (Wang et al., 2013). The linear analytical range for sulphite corresponded to 5 μM-0.41 mM, at 1 μM detection limit and a relative standard deviation value of 1.1%, at a glassy carbon electrode modified with graphene-chitosan and gold nanoparticles (Wang et al., 2013). Cyclic voltamograms at Ni–$Pt_{3layers}$ electrode proved the electrocatalytical potential of this material in sulphite oxidation (Enache et al. 2016).

The voltammetric sulphite electro-oxidation to sulphate was confirmed as an irreversible process (Lu et al., 1999).

## MATERIALS AND METHODS

A KSP potentiostat-galvanostat, laboratory made by Professor Slawomir Kalinowski, University Warmia and Mazury (Olsztyn), as well as the respective software Cyclic Voltammetry, were used for recording the cyclic voltammograms.

A Pt strip electrode Radelkis OP-0612P was used as working electrode. As reference, a saturated calomel electrode (SCE), Radelkis was used. The counter electrode was a Pt strip Radelkis OP-0612P electrode.

The stock solution of sodium sulphite (5 g/L) was prepared daily by dissolving $Na_2SO_3$ (Merck, ACS ISO,) in a 0.10 M KCl electrolyte solution (Chimopar, Bucharest, Romania).

Standard solutions of sulphite (as $Na_2SO_3$) subject to analysis, with concentrations comprised between 5 mg/L and 5 g/L were obtained by dilution of the stock solution with 0.10 M KCl solution. The volume of the analysed sample was 50 mL and all measurements were performed at $23^0$ C, using a 0.10 M KCl solution as supporting electrolyte.
Before each determination, the Pt working electrode was cleaned mechanically and electrochemically by applying a -1.5 V potential pulse for 3 seconds. For the cyclic voltammetric measurements, the potential was scanned within the range -100 to 1500 mV, with a 50 mV/s scan rate.
For the investigation of the influence of the scan rate, the potential sweep rate varied between 25 and 200 mV/s.

**RESULTS AND DISCUSSIONS**

Cyclic voltammograms were recorded, at sulphite increasing concentrations comprised between 5 mg/L and 5 g /L, using the Pt strip electrode. A representative cyclic voltammogram is presented in Figure 1.

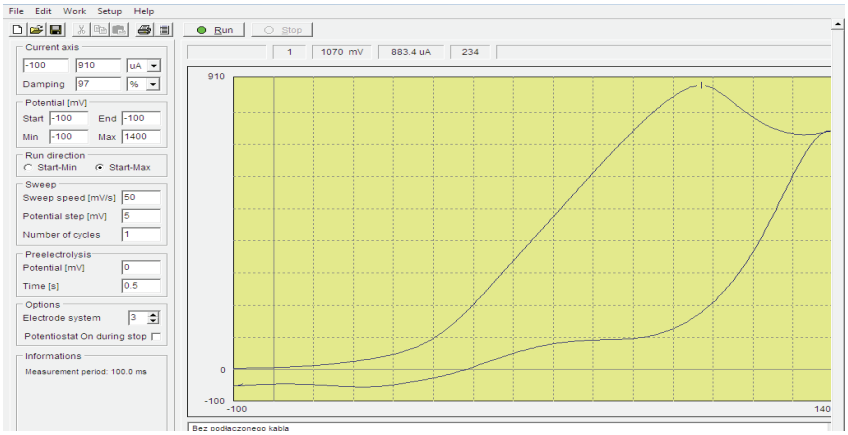


Figure 1: An illustrative cyclic voltammogram obtained for 1g/L sulphite solution, in the presence of KCl 0.1 M as electrolyte

The developed calibration curve (Figure 2) showed a linear range of analytical response corresponding to 0.031 g/L – 4 g/L.

The equation of the calibration graph corresponded to y = 423.57 x + 331.6, R2 = 0.9883.

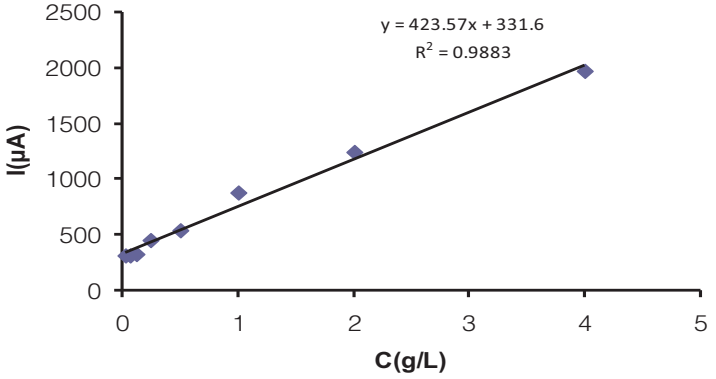


Figure 2: Calibration graph (obtained by plotting the peak current intensity in microamperes, versus concentration) obtained at sulphite voltammetric determination at a Pt strip electrode, in the presence of KCl 0.1 M as electrolyte .

The value of the relative standard deviation RSD was 2.74 %, calculated as 100 x standard deviation / the mean of determinations, c = 125 mg/L; n = 10).
The obtained detection limit was 12.4 mg/L, calculated as LOD = 3 s/m, where s represents the square mean error calculated for the KCl electrolyte solution as blank, and m represents the slope of the calibration graph.
The electroactive process proved diffusion-controlled observing Randles-Sevcik law, as shown by the linear dependence of the current intensity on the square-root of the potential-sweep rate (Figure 3).

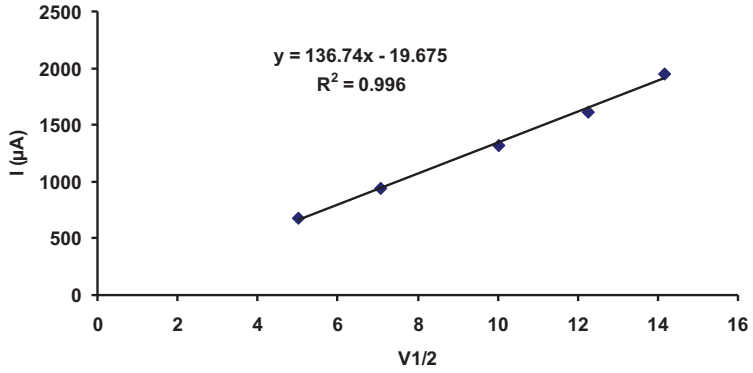


Figure 3: The dependence of the peak current intensity on the square root of the potential sweep rate, as obtained for 1 g/L sulphite, in the presence of KCl solution 0.1 M as supporting electrolyte

Hence, the magnitude of the analytical signal, considered as the measured current intensity at the voltammetric peak, is controlled by analyte diffusion from the solution to the electrode/solution interface.

## CONCLUSIONS

The anodic voltammetric peak corresponding to sulphite oxidation increases with analyte concentration. The linear range of analytical response corresponds to 0.031 g/L – 4 g/L sulphite concentration, with good correlation coefficient $R^2$= 0.9883.
This, along with a sensitivity value of 423.57µA/g/L (as given by the slope of the calibration graph), a detection limit as low as 12.4 mg /L and a relative standard deviation of 2.74%, proved the method's viability, and offers prospects for future studies regarding optimization and application.

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# THE EFFECTS OF FOODER SUPPLEMENTATION WITH ORGANIC SELENIUM ON HAEMATOLOGICAL AND BIOCHEMICAL MARKERS IN BROILER CHICKENS

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***Abstract***

*Selenium is an essential trace mineral that prevents the formation of pro oxidative free radicals. The role the selenium plays in the protection of hemoglobin against peroxidation is very well documented and researched. In order to perform this study, a number of 20 3-weeks-old broiler chickens of the Cobb breed were analyzed. The individuals were distributed into two even lots. The batching of the individuals was realized within the bio base of the Faculty of Veterinary Medicine in Bucharest. The duration of the experiment was of 4 weeks. Both the experimental batch and the control batch were fed a 21/1 ratio feed diet for broiler chicken, finishing period. The experimental batch's diet was additionally supplemented with 0.5 ppm of selenium, with 0.25 ALKOSEL R397 g/kg mixed fodder.*
*At the end of the experimental period, blood samples were taken by venipuncture of the cubital vein for hematological and biochemical laboratory analysis. The results were tabled and bio statistically interpreted. The experimental lot has shown significant increases in the values of the hematocrit (7.68%) and of the blood levels of white blood cells (14.01%), aspartate aminotransferase (8.19%), calcium (10.59%) and selenium (42.56%). They also showed a significant decrease in the values of blood protein levels (8.33%). The biochemical parameters influenced by the organic selenium supplemented feed lead to the prevention of the oxidative stress and a higher efficiency of fodder conversion rate*

***Key words:*** *biochemistry, haematology, broiler, selenium.*

## INTRODUCTION

Growth and immunity are negatively impacted by heat stress that induces physiological, hormonal and molecular alterations, as well as lipid peroxidation. (Donker et al., 1990). Baziz et al. (1996) observed that for each degree increase in temperature between 22°C and 32°C, broilers' feed intake and growth decrease with approximately 3.6 and 1.5%

Research has shown that supplementation of broilers' diets with antioxidants reduces the effects of heat stress on lipid peroxidation and metabolism (Cantor, 2003).

Selenium helps protect the hemoglobin against peroxidation by means of three enzymes: superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (Curcă, 2008; Răduță 2016).

Low levels of selenium can be responsible for the peroxidation of cellular membranes that lead to an increased production of prostaglandins.

Membranes peroxidation leads to many molecules, including the DNA molecule, being structurally damaged, favouring the development of neoplastic diseases (Ghergariu 1980; Curcă, 2005)

A deficiency in selenium is directly linked to disorders such as anaemia and/or haemolysis most frequently observed in rats, dogs, primates, but also chickens. (Curcă, 2005).

Supplementation of the feed with selenium can prevent the apparition of such conditions as myopathy effusion, bleeding diathesis etc.

Selenium in poultry diets protects them from pancreatic fibrosis and exudative diathesis (Combs, 1986).

Based on these reports, it is established that selenium is an essential nutrient for poultry. Due to regional variation in selenium content of natural feeds, broilers' diets are commonly supplemented with selenium.

Dietary and supplemental selenium is needed to form part of the amino acid selenocysteine, the only amino acid to have a selenium component. Selenocysteine is incorporated into at least 25 selenoproteins that have catalytic and antioxidant functions in the body. The chemical structure of selenocysteine, which is the 21st amino acid, is almost identical with the

structure of the amino acid cysteine (Okunlola et. al., 2015). The basic difference is that selenium has taken the place of sulfur in the molecule Allan et. al., 2000, Zelenka, 2005).
Selenium administered in its organic form (selenomethionine) improves its bioavailability, increasing the retention of selenium by the organism due to the fact that these amino acids are not excreted in urine (Surai, 2006, Okinlola 2015).

## MATERIALS AND METHODS

The experiment took place in the bio base of the Faculty of Veterinary Medicine of Bucharest and was conducted on two batches of broiler chickens of the Cobb 500 breed. Each lot contained 10 3-weeks-old individuals at the beginning of the experimental period (Figure 1).

Fig. 1. Broiler batch study group

Both the experimental group and the control group received the same feed regime: 21/1 ratio feed diet for broiler chicken, finisher stage. The fodder contained cereals, soybean meal, sunflower meal, corn gluten meal, calcium phosphate, calcium carbonate, salt, amino acids and vitamins and minerals premix.
The feed had the following nutritional values: crude protein – 17.20%, metabolizable energy – 3140 kcal/kg, methionine and cysteine – 0.65%, lysine 0 0.90%, calcium – 0.86%, phosphorus – 0.70%, choline – 0.004%, salt – 0.30%. It did not contain coccidiostats.
The experimental group had its feed supplemented with 0.25 g/kg M.F, 0.5 ppm organic selenium as ALKOSEL R397, obtained from Lallemand Animal Nutrition SA France. The control group received the normal feed.
After 30 days, at the end of the experimental period, blood was sampled by puncture of the cubital vein using EDTA anticoagulant 1-2 mg/ml of blood, for hematological analysis and for biochemical analysis were used clean dry tubes without anticoagulant (Figure 2).

Fig. 2. Blood sample collecting from the cubital vein for hematological and biochemical determinations

The RBC (red blood cells) count was performed using an automated Coulter Counter, CP-diff analyser ACT 5 Beckman. The WBC (white blood cells) count was performed within the laboratory of the department of Physiopathology of the Faculty of Veterinary Medicine of Bucharest, using the Burker hemocytometer and the Natt-Herrick dilution solution (Figure 3).

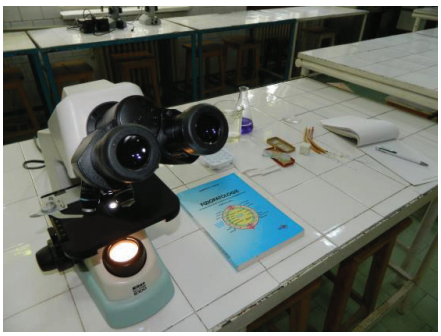

Fig. 3. The WBC count

The analysis of selenium, cholesterol, magnesium, calcium, ALT/GPT (alanine aminotransferase) and AST/GOT (aspartate aminotransferase) blood levels were performed by Bucharest's Institute of Animal Diagnostic and Health, using molecular absorption spectrometry. Glucose, proteins, ALKP/ALP (alkaline phosphatase), amylase and lipase blood levels were determined by the Laboratory of the Faculty of Veterinary Medicine of Bucharest, using specific kits for the aforementioned analysis. The kits were purchased

from S.C. Nova Group Investment S.R.L. The device used for these determinations was VeTTest, from Idexx Laboratory.
The results of these investigations were statistically calculated using ANOVA, a specialized statistics program. The data was processed by means of Microsoft Office 2010 software. The results were tabled, plotted and biostatistically interpreted (Table 1).

## RESULTS AND DISCUSSIONS

The individuals in the experimental lot, whose diet was supplemented with organic selenium, showed statistically significant increases of the haematocrit and of the blood levels of white blood cells (Figure 4).
Other statistically relevant increases were registered by biochemical markers such as AST, calcium and selenium.
The blood levels of proteins have decreased significantly. Other changes of some parameters of the individuals in the experimental group have been observed, but with no statistical significance (Figure 4 and Figure 5).
Lipase, ALT and magnesium blood levels tend to increase while the MCHC and the blood levels of ALKP, cholesterol and glucose tend to decrease, but none statistically significant.
Supplementing the feed with selenium favours erythropoiesis and therefore that will lead to a better oxygenation of the tissues. (Mertz, 1987; Curcă, 2005 and 2007; Răduță, 2011).
ALKOSEL R397 is one of the most efficiently utilized concentrated sources of additional dietary organic Se, favouring a greater activity of glutathione peroxidase, an antioxidant enzyme that reduces peroxides and other free radicals that could compromise cellular membranes (Edens and Gowdy, 2005). Apsite (1993, 1994, 2004) discovered that selenium metabolites in the organism stimulate the activity of glutamine peroxides, leading to the elimination of lipid hydroxyl peroxides present in cellular structures, decreasing the risk of oxidative stress. Such results were later obtained by other researchers.
Results showed that the haemoglobin blood levels only increased by 5.44%, as a result of a larger population of young erythrocytes, thereby influencing the increase of the MCHC (mean corpuscular haemoglobin concentration). This is by 1.26% higher than the results of the same analysis performed on the individuals in the control batch and it confirms a better load of the erythrocyte with haemoglobin (Smith and Picciano, 1987).
The high intensity of erythropoiesis leads to a larger number of young erythrocytes being developed by the bone marrow and, as a consequence, to a higher haematocrit, that suggests there is an increase in the cellular mass, detrimental to the plasmatic mass (Surai, 2002, Payne, 2005).
An increasing trend was observed in the MCV (mean corpuscular volume) values as well, due to the large number of young erythrocytes that have a lower volume than that of mature red blood cells (Tayeb, 2012).
In this experiment, the results obtained from the experimental group individuals were by 2.58% larger than those obtained from the control group individuals (Aristide Popescu L. and N. Aristide Popescu, 1990; Răduţă et. al., 2015).
The decrease of the MCHC of 1.29% can be explained by the larger quantity of circulating haemoglobin and by the MCV increase. The blood levels of white blood cells show significant decrease.
From a biochemical point of view, ALT blood levels tend to increase with 2.99%, compared to the control group.
Lipase blood levels in individuals whose diet has been supplemented with organic selenium tend to increase to 181.16 U/L. This is 32.62% larger than the results obtained by the control group individuals.
Other researchers showed that the blood levels of lipase also tend to increase, demonstrating that a selenium deficiency can lead to a poor absorption and to a low hydrolysis of lipids in the digestive tract, which result in a significant decrease of vitamin E absorption, whose low levels can lead to necrotising dystrophy of the pancreas (Poll, 1968; Apsite et. al., 1993; Mahan, 1995; Aye et. al., 1999; Agate et. al., 2000).
The association between an intense activity of lipase and AST and a decrease in the ALKP levels, leads to a higher permeability of the cellular membranes, especially that of the sarcolemma.
Enzymes are released from the cytosol into the blood circulation, which leads to the instatement of muscle degeneration, not perceptible

by the anatomopathological examination (Bansal, M.P. şi Kaur, T. 2002, Cornell University College of Veterinary Medicine, 2011, Hassan, S. 1990, Oster, O. şi Prellwitz, W. 1990, Pappas, A.C.et. all., 2004).

Experimental group individuals' amylase blood levels results were 179.83 U/L, lower by 0.73% than those obtained by the control group individuals.

Cholesterol blood levels reached 100.6 mg/dL of serum, 17.54% lower than those of the control group, whose feed was not selenium supplemented. This state can be explained by the improved lipid metabolism, without intermediary metabolites.

Blood glucose levels of the experimental batch registered a large decrease, reaching 203.8 mg/dL blood, which is 2,16% lower than the results obtained for the control batch.

Calcium and magnesium blood levels were larger for the experimental group, with 10.59% for the former, and 6.93% for the latter.

This indicates there is a link between selenium and the mineral metabolism (Upton, 2009).

One of the world's problems in raising broiler chickens is the development of the musculoskeletal disorders as a consequence of the high growth rate of the birds. Because of these disorders the chickens can no longer move and prefer abnormal decubitus positions, favouring the apparition of chemical burns on their limbs and pectoral muscles.

Pain and lack of movement lead to the stop of the growth process and therefore to a decrease of the body mass and ultimately, due to various complications caused by bacterial infections, to death (Avanzo J. 2001).

Table 1. Statistically modified parameters in the experimental group

| **Parameters** | Value average | Percentage average (%) | P value |
|---|---|---|---|
| Ht (%) | 35.72 | ↑ 7.68 | **.407** |
| WBC ( x $10^3$/µl) | 27.33 | ↑ 14.01 | **.0013** |
| GOT (U/L) | 117.5 | ↑ 8.19 | **.0236** |
| Calcium (µg /dl) | 10.176 | ↑ 10.59 | **.0044** |
| Selenium (µg /dl) | 31.62 | ↑ 42.36 | **<.0001** |
| Proteinemia (g/dl) | 3.3 | ↓ 8.33 | **.0213** |

P<0,05 – significant differences
P<0,01 – differences significantly distinct
P<0,001 –differences very distinct significant

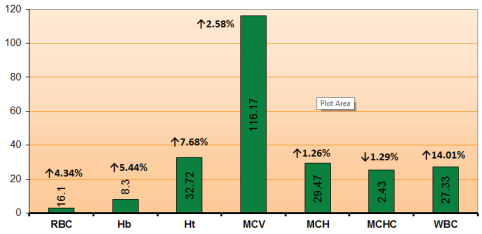


Fig. 4. Average values of haematological indices in the experimental group compared with the control group

**Legend:**
RBC – red blood cells (E x $10^6$ / µl)
Hb – Hemoglobin (g/dl)
Ht – hematocrit (%)
MCV – mean corpuscular volume ($\mu^3$)
MCH – mean corpuscular hemoglobin (pg Hb/Eritheremie)
MCHC – mean corpuscular hemoglobin concentration (g Hb/dl Erithremie)
WBC – white blood cells (x $10^3$/µl)

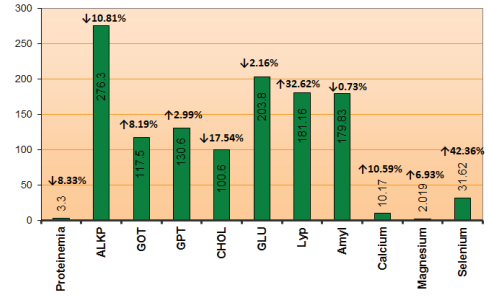


Fig. 5. Average values of biochemical indices in the experimental group compared with the control group

Legend:
Proteinemia – Proteinemia (g/dl)
ALKP – alkaline phosphatase (U/L)
GOT/AST – aspartate aminotransferase (U/L)
GPT/ALT – alanine aminotransferase (U/L)
Chol – cholesterol (mg/dl)
Glu – glucose (mg/dl)
Lyp – lipase (U/L)
Amyl – amylase (U/L)
Calcium – calcium (µg/dl)
Magnesium – magnesium (µg/dl)
Selenium – selenium (µg/dl)

## CONCLUSIONS

1. The conducted experiment demonstrates the beneficial effects of feed supplementation with selenium, by improving of some haematological and biochemical parameters.
2. Selenium has a biologically active role in the activity of the hematopoietic bone marrow and

therefore in the formation of new, young, red blood cells, in the experimental group the RBC average was with 4.34% higher then in the control group.

3. The increased blood levels of calcium, 10,17% and selenium, 42.36% lead to the improvement of the mineral metabolism and a better growth rate of the individuals in the experimental batch.

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# THE MACROSCOPIC MORPHOLOGY OF HEAD, NECK AND FORELEG LYMPH NODES AT COYPU (MYOCASTOR COYPUS)

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***Abstract***

*The coypu (Myocastor coypus) is a semi-aquatic mammal rodent, native from South America. Besides the fact that they are resistant to diseases and eat less, the coypu are useful for the precious fur and also for the tasty meat. After analyzing the bibliographic material it was noted that the data referring to the anatomy of this species are very sketchy, in particular those relating to the lymphatic system. For this reason I have chosen this topic, aiming to complement the existing data in the literature on the morphology of the muscular lymph nodes, at coypu. For the necessary investigations from this study, have been used corpses of coypu. After dissection it has been noted that existence of the mandibular, parotid, lateral retropharyngeal, rostral hyoidian, axillary and accessories lymph nodes. At the macroscopically level it was not possible to certainly determine the existence of superficial cervical lymph nodes, instead were described in detail the axillary lymph nodes, which is not found in this species bibliographic data. After dissection it was noted the presence of one lymph node, bilateral, at 40% of the dissected individuals (two specimens out of five). Their topography is the one characteristic to another domestic species, being situated caudal from the scapulo-humeral articulation, between the thoracic member and trunk, caudal to the emergence of the caudal cutaneous nerve of the forearm (the ulnar nerve).*

***Key words:*** *cervical region, coypu, dissection, lymph nodes.*

## INTRODUCTION

The advantage of growing coypu is that these rodent mammals are not picky eaters, the feeding being composed solely of vegetable origin (Barach and Hafner, 2002).
Besides the fact that they are resistant to diseases and eat less, the coypu are useful for the precious fur and also for the tasty meat. After analyzing the bibliographic material it has been noted that the data on the anatomy of this species are very lackluster, especially those related to the lymphatic system (Suntsova and Panfilov, 2009; Predoi,et.al., 2011; Predoi and Belu, 2001).
Besides a detailed study of the morphology of the linked mesenteric lymph nodes at this species, the other studies address to this subject in general, also the included photos are relatively simple schemes without any photographic images (Pérez et.al.,2008; Wood et.al., 1992; Hrițcu and Coțofan,2000).
For this reason I have chosen this topic, aiming to supplement the existing data in the specialty literature regarding the morphology of the muscular lymph nodes at coypu.

## MATERIALS AND METHODS

For the investigations of this present study, 5 corpses of adult coypu have been used . First of all, the animals were anesthetized and injected intradermally with colouring substance called the China dye. The China dye was filtered through a filter paper, attached to a funnel on a Berzelius glass for about 30 minutes.
The elective spots for the dye substance administration were at: the retroauricular region, the nose tip region, the commissure of the lips region, the dorsal cervical region, the ventral cervical region and the palmar pad (until forming a intradermal button).
For each injection used 0.3ml of substance have been used. After euthanasia, the investigations of the lymph nodes was performed through stratigraphical and regional dissection. The identification, the description and the approval of the structures has been made according to the NAV. -2005.

## RESULTS AND DISCUSSIONS

**The mandibular lymph center** is formed from two lymph nodes.
The proper mandibular lymph node is developed, rounded and slightly flattened latero-laterally with a diameter of about 10 mm (Figure 1-*1*).
It is located at the ventral edge of the masseter muscle, on the surface of the occipito-mandibular muscle.
The caudal edge of the lymph node is in contact with the cranial mandibular gland. The caudo-dorsal portion comes in contact with the much smaller **mandibular accessory lymph** node is much smaller (Figure 1-*1'*), which reaches near the linguo-facial vein confluence with the jugular vein.

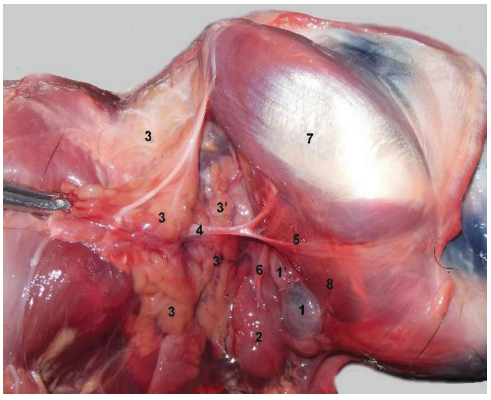


Figure 1. The morphology and topography of the mandibular lymph nodes at coypu
*1- mandibular ln.; 1'- mandibular accessory ln.; 2- mandibular gland; 3-the superficial portion of the parotid; 3'-the profound portion of the partoid gland; 4-jugular vein; 5-linguofacial vein; 6-mandibular gland duct; 7-masseter muscle; 8-occipitomandibular muscle. ln = lymph node*

**The parotid lymphcentre** is formed by one or two reduced lymph nodes that are very difficult to isolate from the parotid acini through macroscopical view (Figure 2-*1*). They are placed at the base of the ear: between the auricular cartilage and the dorsal edge of the masseter muscle, being differentiated only by the slightly darker colour of the gland. The dimension is of approximately of 1-3 mm in diameter.

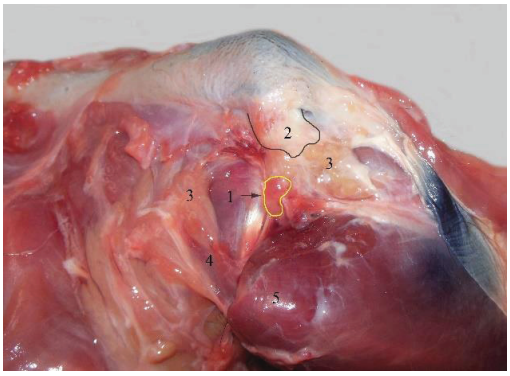


Figure 2. The Parotid lymph node morphology and topography at coypu: *1- parotid lymph node.; 2-the base of auricular cartilage; 3- portions remaining after removing the the parotid gland; 4- occipitomandibular muscle; 5- masseter muscle*

**The retropharyngeal lymph centre** is represented by the **lateral retropharyngeal lymph node**. It can be revealed after splitting the cranial insertion (on the occipital paracondilar process of the cleidooccipital muscle). It is extremely small, about 2 mm long and has relations with the vagosympathetic trunk, under the wing of the atlas (Figure 3-*3*). Much better represented is the **rostral hyoidian lymph node**, „hidden" in the medial occipito-mandibular muscle and lateral of the sterno-hyoidian muscle (Figure 3-*2*). The dimensions of this ovoid lymph node are approximately 10/5 mm. However the profound topography makes it difficult to approach. The literature does not reveal the presence of the **superficial cervical lymph nodes** at this species. However after the dissection through classic methods, some darker, smaller, monoliform formations were identified, interweaved in fat tissue in the prescapular region, at the medial trapezius muscle. To establish the existence of limfonodular structures harvesting and making histological preparations would be recommended,. Their analysis could clarify this issue. The lymphonodular groups belonging to the **profound cervical lymphocenter** couldn't be identified in any of the individuals.
**The axillary lymph centre** is represented by **the axillary accessory lymph nodes**. These are situated on the line connecting the shoulder with the olecranon thoracic angle, behind the tricipital line, on the surface of latisimus dorsi muscle. They are relatively easy to approach.(Figure 4-*1*).

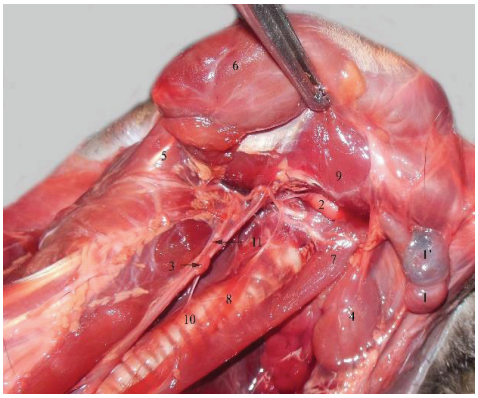


Figure 3. The morphology and the topography of lateral retropharyngeal and rostral hyoidian lymph nodes at coypu, after removing the parotid (original) *1- mandibular lymph node; 1'-accessory mandibular lymph node; 2-rostral hyoidian lymph nodes; 3-lateral retropharingeal lymph nodes; 4-mandibular gland; 5-atlas wing; 6-masseter muscle; 7-sternohyoidian muscle; 8-sternothyroid muscle; 9-occipitomandibular muscle; 10-tracheea; 11- vago-sympathetic cord in the vicinity of the common carotid artery.*

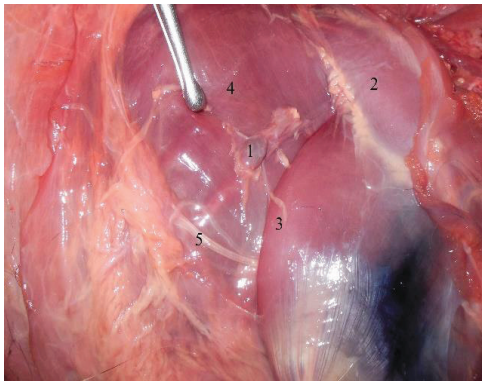


Figure 4. The morphology and the topography of the accessory axillary lymph nodes at coypu(original) *1- accessory axillary lymph nodes; 2-infraspinos muscle; 3-the caudal edge of the long portion of the triceps muscle, 4- latisimus dorsi muscle; 5- branches of lateral thoracic nerve.*

Contrary to some published literature that support the absence of **the proper axillary lymph nodes at this species**, after the dissection I have noted the presence of one lymph node, bilateral, at 40% of the dissected individuals (two specimens out of five).
Their topography is the one characteristic to domestic species, being situated caudal from the scapular-humeral articulation, between the thoracic member and torso, caudal to the emergence of the caudal cutaneous nerve of the forearm (the ulnar nerve) (Figure 5-*1*). The shape is globular and the diameter is of about 5 mm.

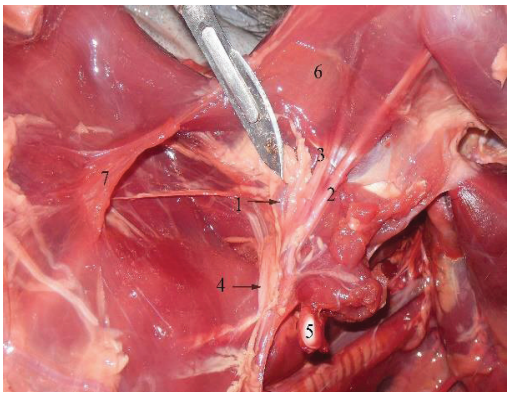


Figure 5. The morphology and topography of the proper axillary lymph nodes at coypu after opening the axillary region by cutting the clavicle and pectoral muscles (original): *1-proper axillary lymph node; 2-median nerve; 3-ulnar nerve, 4- radial nerve; 5-sectioned clavicle; 6 – long portion of the triceps; 7- latissimus dorsi muscle.*

## CONCLUSIONS

The mandibular lymph centre constantly appears formed from two lymph nodes easily to identify and isolate.
Parotid lymph node is very reduced, difficult to identify and isolate (the parotid gland acini).
Specific to the retropharyngeal lymph center is the rostral hyoidian lymph node, most easily identifiable in this group.
Constantly, at coypu, the profound cervical lymph node is not present.
Although in the scientific literature proper axillary lymph nodes are not described, I have found their existence bilaterally in 40% of the cases dissected.

Although the scientific literature does not reveal the presence of the superficial cervical lymph nodes at this species, at the medial face of the trapezius muscle, on the prescapular region, darker, smaller monoliforme formations, interwaved in fat tissue. To establish the existence of these lymphonodular structures, harvesting and making histological preparations is recommended.

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# DETAILED MORPHOLOGICAL DESCRIPTION OF THE LIVER AND HEPATIC LIGAMENTS IN THE GUINEA PIG (*CAVIA PORCELLUS*)

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***Abstract***

*The paper aimed to present the gross anatomy of liver and its ligaments in guinea pigs. The liver is located into intrathoracic part of abdominal cavity, having six separate lobes (right lateral, right medial, left lateral, left medial, caudate, and quadrate) but well connected one with each other. The falciform ligament which apparently divides the diaphragmatic surface of the liver in two territories –the right and left hepatic territories, was complete, being attached to the undersurface of the diaphragm and the dorsal surface of the abdominal wall at the level of the umbilicus. Its free edge contains the round ligament. The coronary ligament was well delineated being composed by an upper and a lower layer. Both the right and left triangular ligaments were present. The left triangular ligament was well developed connecting the left lateral lobe to the diaphragm. Cranial insertion of hepatorenal ligament was visualized on the ventral border of the caudate process, then run to the medial aspect of the right kidney, and to the descending loop of the duodenum. The liver is also attached to the stomach and to the duodenum by hepatogastric and hepatoduodenal ligament. A free edge of the hepatoduodenal ligament, whose cranial insertion was on the cystic duct, down along the common bile duct to be inserted on right lobe of pancreas, it was clearly visualized.*

***Key words****: liver, hepatic ligaments, anatomy, guinea pig.*

## INTRODUCTION

There is a general agreement about the presence of two main hepatic territories, right and left provided by portal vein bifurcation in mammalian liver (Rex 1888, McIndoe and Counseller 1927, Couinaud, 1954; Abdel-Misih et al., 20110; Bismuth, 2013; Fasel, and Schenk, 2013). The liver lobes, the number and their nomination, each with its own vascular and biliar system are still subject of debate, both in human and veterinary medicine. Also, the biliar system, especially the extrahepatic billiary tract shows anatomical differences within the same species. From rodents order, the most studied is rats liver due to their use as an experimental model in surgical hepatectomy (Higgins 1931; Madrahimov et al., 2006; Martins and Neuhaus, 2007; Martins et al., 2008). The rat liver is composed of four lobes and resembles the sector delimitation of the human liver (Kogure et al., 1999; Vdoviaková et al., 2016) and presents the same ligaments as in humans (Martins and Neuhaus, 2007). From caviomorphs, the description of chinchillas liver, point out the presence of four lobes with a little lobulation which is grossly visible at the surface (Lyon 2003, Spotorno et al., 2004). The gallbladder is located between the right and medial lobes, having 2-3 cystic ducts and a complex hepatic ducts system (Nowak et al., 2014). The guinea pigs liver was described having six lobes (Cooper and Schiller, 1975; Breazile and Brown 1976) and a well developed gallbladder. In rabbit, anatomical books described the presence of five liver lobes (Barone, 2009) while in scientific literature are reports who claim the presence of four liver lobes (Brewer, 2006) along the absence of the common hepatic duct. The hepatic ligaments in rabbits and nutria were detailed described by Perez (Pérez et al., 2005; Pérez and Lima 2007). The aim of this study is to describe the macroscopic anatomy of the liver and its ligaments in guinea pigs.

## MATERIALS AND METHODS

Ten adult guinea pigs, four male and six female (mean body weight 420±50g) were used. The Institutional Bioethics Committee of University

of Agricultural Science and Veterinary Medicine in accordance to Directive 2010/63 /EU of the European Parliament and of the Council on the protection of animals used for scientific purposes approved the study. Euthanasia was performing by administration of an overdose of isoflurane. The abdominal cavity was opened and the wall of it were carefully removed in order to visualize and to photograph the hepatic ligaments and lobulation.

## RESULTS AND DISCUSSIONS

### *Topograpy and surfaces*

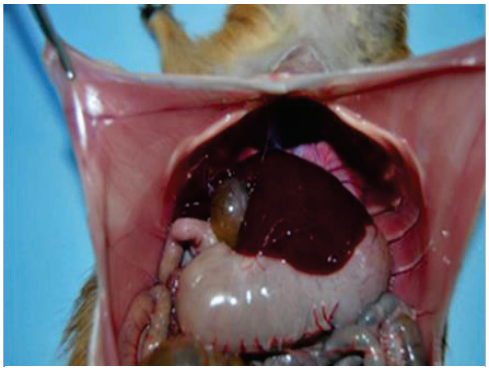

Figure 1. The liver topography. The liver occupied two thirds of the intrathoracic part of abdominal cavity

The guinea pig liver (*Hepar*) occupied two thirds of the intrathoracic part of abdominal cavity (Figure 1). It was multilobulated, having deep fissures, being composed of six lobes, light brown in colour and the average weight was 20.3 g. The liver mass represents 6% from the total body weight. The transverse diameter measured 9.8 cm ± 0.7 cm and the longitudinal diameter measured 6.2 ± 0.4 cm (Table 1).

Table 1. Liver morphometry

| | Weight | Transversal diameter | Longitudinal diameter | Depth |
|---|---|---|---|---|
| Liver | 20 g | 9.8±0.7 cm | 6.2±0.4 cm | 1.8±03 cm |

Concave diaphragmatic (*Facies diaphragmatica*) and convex visceral (*Facies visceralis*) surfaces were recognized. The visceral surface of the liver was in relation with the stomach, duodenum, pancreas, right colic flexure and right kidney (Figure 2). The liver lobes were clearly delimitated on the visceral surface and the porta hepatis (*Porta hepatis*) contained the principal's structures namely: hepatic artery, portal vein and hepatic ducts. On the diaphragmatic surface, the liver shows only four lobes (Figure 3).

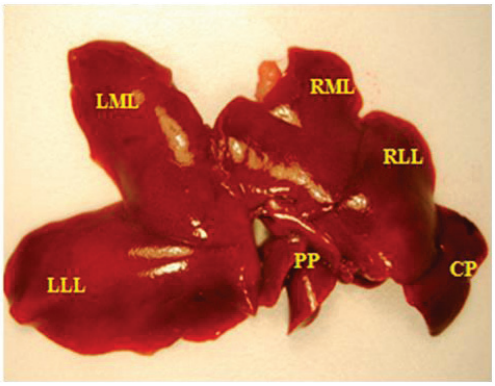


Figure 3. Diphragmatic surface of the liver. LLL-left lateral lobe; LML-left medial lobe; RML-right medial lobe; RLL-right medial lobe; CP-caudate process; PP-papillary process

The falciform ligament apparently divides the diaphragmatic surface in two territories –the right and left, each territory showing only two lobes on this surface. The dorsal margin (*Margo dorsalis*) was rounded and presents the imprint of inferior cava vein and esophagus. The lateral and ventral (*Margo ventralis*) margins were sharp, the lateral margins were interposed between the diaphragm and the hypocondrium and the ventral margin was in relation with the ventral surface of the stomach for the left lobes and with the duodenum and ascendant ansa of the proximal colon for the right lobes.

### *Liver lobes*

We considered that the portal vein and its branches, the billiary tract and the arterial supply of the liver divided the liver in right and left territories. The right lobe (*Lobus hepatis dexter*) was subdivided by a deep fissure into right medial lobe (*Lobus hepatis dexter medialis*), and right lateral lobe (*Lobus hepatis dexter lateralis*). On the diaphragmatic surface, from the medial border of the right medial lobe, the falciform ligament connects the liver with the diaphragm (Figure 4).

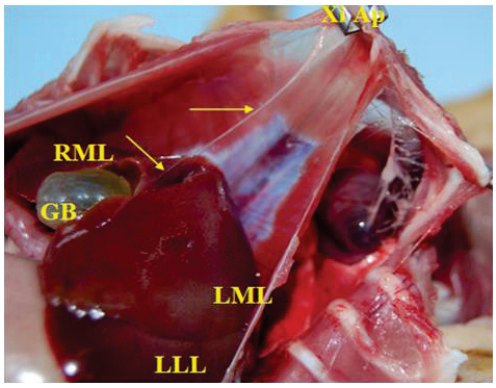


Figure 4. The falciform ligament connect the liver to the diaphragm. Posterior insertion was made on the aponevrotic region of diaphragm, being in contact with the upper layer of the coronary ligament

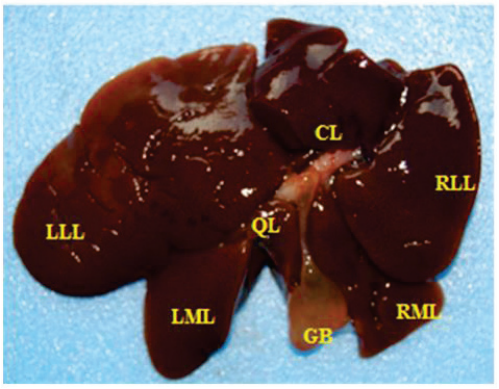


Figure 5. Delineation of liver lobes on the visceral surface. The most developed left lateral lobe - LLL; LML - left medial lobe; GB - gall bladder in gall bladder fosa between the quadrate lobe – QL and right medial lobe – RML; RLL – right lateral lobe; CL – caudate lobe.

On the diaphragmatic surface of the right medial lobe a small groove corresponding to the inferior vena cava was noted. The ventral edge of the right medial lobe circumscribes the gallbladder fundus. In the left territory of the liver, (*Lobus hepatis sinister*) the left medial lobe (*Lobus hepatis sinister medialis*) and the left lateral lobe (*Lobus hepatis sinister lateralis*) were well delimited by a deep fissure. The left lateral lobe was the largest lobe and it was covered on the diaphragmatic surface by the left medial lobe. On the visceral surface, medial from the gallbladder, between the gallbladder fossa, porta hepatis and round ligament, the small quadrate lobe (*Lobus quadratus*) was visualised (Figure 5).

Dorsal from the porta hepatis, the connection pedicle of caudate lobe (*Lobus caudatus*) was seen, making the bond of this lobe with the right lateral lobe. The caudate lobe was composed of two parts: a well developed caudate process (*Processus caudatus*), behind to the right lateral lobe, having an obvious right kidney imprint and a papillary process (*Processus papillaris*) subdivided in two triangular segments, one of these segments reaching the small curvature of the stomach, on the right side of the esophagus. On the visceral surface, on the medial edges of the left lateral lobe and of the caudate process, small notches were visualized.

***Hepatic ligaments***

The falciform ligament (*Lig. falciformes hepatis*) was well developed under the diaphragm surface, appeared like a thin fold, had a slightly oblique position making the connection of the liver to the under surface of the diaphragm (Figure 6).

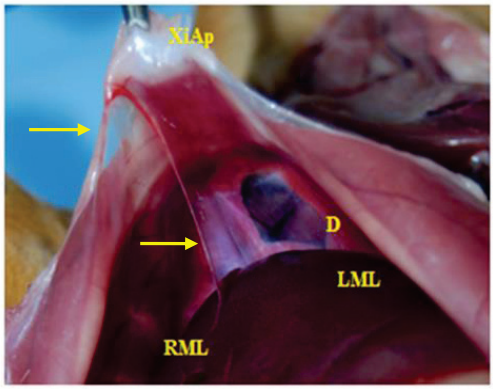


Figure 6 Complete falciform ligament in guinea pig. The falciform ligament extends from the xiphoid appendix, backward to the ventral abdominal wall -arrows

Its liver insertion was made by the union of the two folds from the diaphragmatic surface of the medial lobes, at the level of the main fissure. Posterior insertion was made on the aponevrotic portion of diaphragm and to the upper layer of the coronary ligament.

Anterior, the falciform ligament runs to the xiphoid appendix extending backward to the ventral abdominal wall. The falciform ligament was complete in all subjects. In the free margin of the falciform ligament, ascending from the umbilicus, the round ligament (*Lig. teres heaptis*) was visualised. On the visceral surfaces, the ligament provides a demarcation through its fissure (*Fissura lig. teres*) between the quadrate lobe and the left medial lobe of the liver (Figure 7).

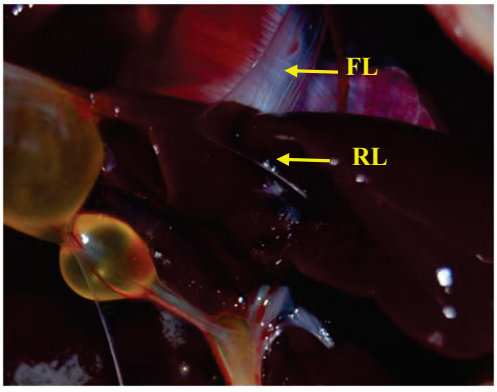


Figure 7. The round ligament – RL. On the visceral surface of the liver its fissure provides delineation between the quadrate lobe and the left medial lobe.

The coronary ligament (*Lig. coronarium hepatis*) bordered the inferior vena cava being the direct continuation of the falciform ligament on the dorsal margin of the liver (Figure 8).

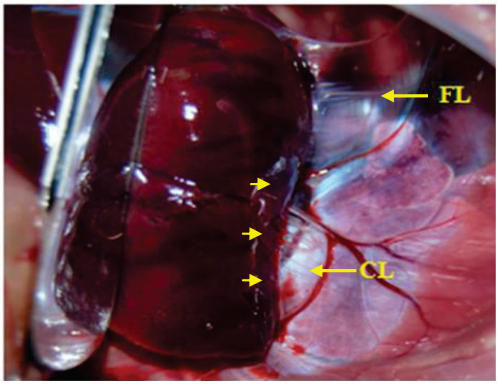


Figure 8. The coronary ligament – CL is a direct continuation of falciform ligament – FL, on the dorsal margin of the liver. The two layers of coronary ligament delineates a small bare area between them – small arrows.

It was composed of two layers, upper and lower layer which demarcates a small bare area between them, the upper layer being the direct continuation of the falciform ligament to the right. Dorsal and toward to the right, the two layers formed a short and tight right triangular ligament (*Lig. triangulare dextrum*). The left triangular ligament (*Lig. triangulare sinistrum*) was well developed in all subjects, having a conspicuous insertion on the lateral diaphragmmatic surface of the left lateral lobe including the dorsal edge of this lobe (Figure 9).

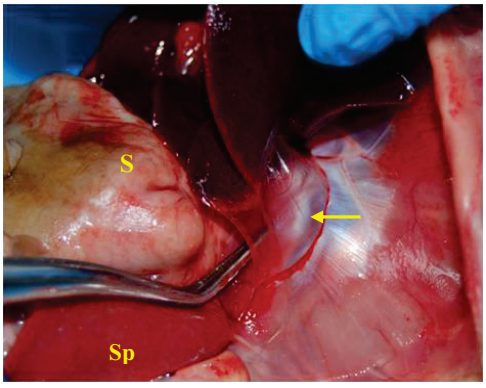


Figure 9 The well developed left triangular ligament connecting the lateral diaphragmatic surface and the dorsal edge of left lateral lobe to the diaphragm. S – stomach; Sp – spleen.

The hepatorenal ligament (*Lig. hepatorenale*) had a particular insertion. Cranial insertion of hepatorenal ligament was visualized on the ventral border of the caudate process, then run to the medial aspect of the right kidney, caudal insertion being on the terminal segment of ascending duodenum (Figure 10).

Hepatogastric ligament (*Lig. hepatogastricum*) was well individualized connecting the lesser curvature, near to the right side of the esophagus, with the papillary process of caudate lobe (Figure 11). The cranial part of the lesser omentum extend from the left side of the porta hepatis, near to the right side of the esophagus to the lesser curvature of the stomach, as the hepatogastric ligament. The caudal insertion of the hepatogastric ligament attaches the lesser curvature of the stomach to the papillary process of the liver, to the right of which the hepatogastric ligament continues as the hepatoduodenal ligament (*Lig. hepatoduodenale*) (Figure 11).

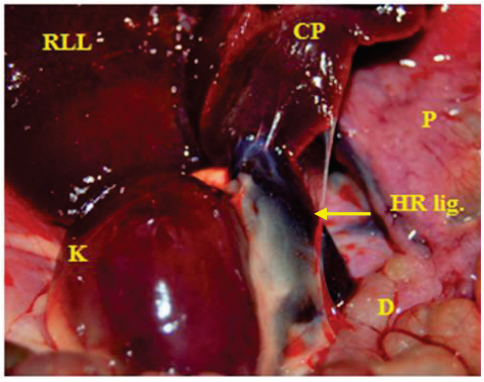


Figure 10 The hepatorenal ligament (arrow) connects the ventral border of caudate process- CP to the medial border of the right kidney – K; RLL – right lateral lobe; D – duodenum; P – pancreas.

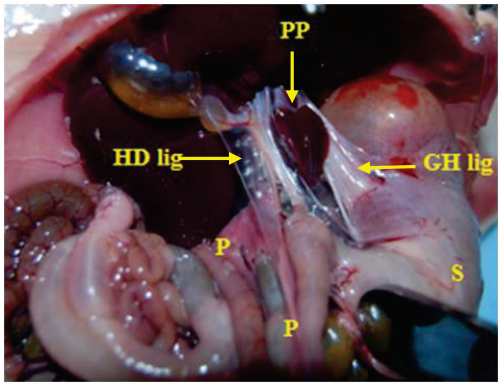


Figure 11 The hepatoduodenal ligament – HD lig. and the hepatogastric ligament – GH lig. P-pancreas; S – stomah; PP-papillary process.

In its thickened margin on the right side were identified three important structures: the common bile duct (*Ductus hepaticus communis*), hepatic artery (*A. hepatica*), and portal vein (*V. portae*).

These structures have the following relationship: the common bile duct lies ventrally and to the right, the hepatic artery lies ventrally and to the left, the portal vein lies dorsally to the above mentioned structures and the inferior vena cava lies more dorsally, behind the portal vein. Also, a free edge of this ligament whose cranial insertion was on the cystic duct, down along the common bile duct to be inserted on right lobe of pancreas, it was clearly visualized (Figure 12).

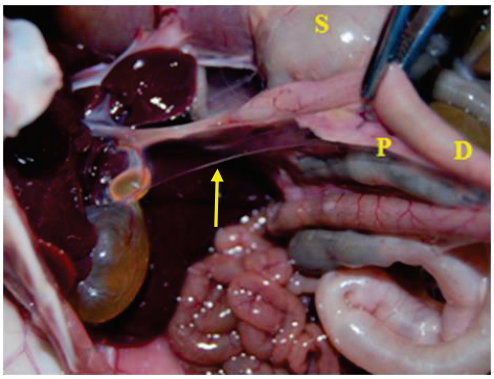


Figure 12 The hepatoduodenal ligament (arrow). Its caudal insertion was made on the right lobe of the pancreas – P. D – duodenum; S - stomach

The anatomies of liver and hepatic ligaments in domestic animals are well described in scientific literature (Barone 2009). Also, the liver, hepatic ligaments, biliary tract and gallbladder was intensively studied in humans both in terms of gross anatomy and vascularrisation (Aharinejad and Lametschwandtner, 1992; Lamah et al., 2001; Ellis, 2011; Mahadevan, 2014). Comparing to the humans liver, the most studied experimental model, from the rodent order is the rat liver – Wistar rat (Kogure et al., 1999). Studying the topographical anatomy, portal, arterial and biliary branching system, it was found that the rat liver is composed of four lobes: the left lobe, the middle lobe, the right lobe and the caudate lobe, the later three being subdivided (Martins and Neuhaus 2007; Martin et al., 2008). The same division was assessed in chinchillas (Nowak et al., 2014) and hamster (Nettlebad, 1954). The subdivision of the middle lobe was different between the mentioned species in the sense that the right medial lobe was the smallest in chinchillas, compare to the rat and hamster, in which the right medial lobe was the largest. Our results show that in guinea pigs there is no such a division of liver lobes, each of the six lobes being well individualized, the largest liver lobe being the left lateral lobe. This feature is in concordance with the description of nutria (*Myocastor coypus*) and rabbit liver (Perez and Lima, 2007; Stamatova et al., 2012). Concerning the liver anatomy in rabbit, our results are similar to Perrez et al 2007, who report the same division in two

territories- left and right- of the liver and presence of five lobes in rabbit. Regarding the differences of visualized lobes on the diaphragmatic and visceral surfaces, the literature is controversial, in terms of number of visualized lobes. According to Barone (2009) in rabbit, the diaphragmatic surface shows three lobes, the right undivided lobe covering the left medial lobe and the left lateral lobe. On the visceral surface the five lobes are described, the right, left medial, left lateral, quadrate and caudate lobes. The quadrate lobe was attached to the gallbladder fossa, without any further demarcation. The smallest lobe in rabbit was the quadrate lobe, this feature being recognized by the Stamatova in a study realized on twenty rabbits (Stamatova et al., 2012). Our results pointed out the location of the quadrate lobe in guinea pigs, in which the quadrate lobe was well visualized located in the left side of the gall bladder, between the gallbladder fossa, porta hepatis and round ligament. The caudate lobe in rabbits has a narrow attachement and because of this together with its projection, dorsally toward to the right kidney, in rabbits the torsion of this lobe has been reported (Taylor and Staff 2007; Wenger et al 2009; Stanke et al., 2011). Also, in rabbits the acute angle of the duodenum and liver compression due to hepatomegaly contribute to stomach distension in many cases, misinterpreted like gastric disorder instead of hepatic disease. Compare to nutria, in which the both kidney are in relation with the liver (Perez and Lima 2007) in guinea pigs only the right kidney make the renal imprint on the caudate process of the liver, similar with the most of rodents and reabbit.

Regarding the hepatic ligaments, there are some differences between the species belonging to the Rodent order and Lagomorphs. A whole large falciform ligament, extening to the ventral floor of the abdominal cavity from the abdominal surface of the diaphragm to the posterior surface of the right rectus abdominal muscle at the level of the umbilicus, it was found in rats (Martin and Neuhaus 2007), hamsters (Van Hoosier and McPherson 1987) and nutria (Perez and Lima 2007). The falciform ligament in guinea pigs was complete too. It made the connection between the medial lobes of the liver, diaphragm and xiphoid appendix extending on the abdominal wall up to the level of the umbilicus. Incomplete falciform ligament was reported in chinchillas (Nowak et. al 2014) and rabbits (Perez et. al 2005). On the visceral surface the fissure of the round ligament provide a demarcation for the quadrate lobe. The quadrate lobe was demarcated by the gallbladder fossa, porta hepatis and round ligament. This issue, in guinea pigs is the same with the reports regarding the quadrate lobe delineation in human's liver. A small coronary ligament in guinea pigs was well demarcated, being visible on the dorsal margin of the liver, making the connection of liver to the diaphragm, while in rabbits this ligament was almost indistinguishable (Barone 2009; Perez et al. 2005). The hepatorenal ligament in rabbit (Stan, 2014) and nutria had a long parietal insertion. In guinea pigs the hepatorenal ligament was inserted medial to the right kidney, toward to the right side of the mesoduodenum with caudal insertion on the decescending loop of duodenum, near to the ascending colon. Regarding the triangular ligaments, these ligaments vary both in presence and size. In guinea pig and rabbit the left triangular ligament is constantly present being well developed (Stan, 2014) and the right one is small and inconstant in some subjects, while in rats, hamsters and nutria, the triangular ligaments of liver are present on each side and with two layers for every ligament (Martin and Neuhaus 2007; Reznik et al 1979; Perez and Lima 2007). A particular feature of the hepatoduodenal ligament in guinea pig was its free edge with caudal insertion on the right lobe of the three lobes compound pancreas, issue that has been reported in other descriptions (Stan 2014).

## CONCLUSIONS

The guinea pig liver is divided by deep fissures in six lobes. The liver is connected to the under surface of the diaphragm and to the ventral abdominal wall by five ligaments: the falciform the coronary, and the two lateral peritoneal folds as right and left triangular ligaments and by the round ligament. Attachment of the liver to the stomach is made by the hepatogastric ligament and to the duodenum and pancreas by the hepatoduodenal ligament. In guinea pigs, the caudal insertion of hepatoduodenal ligament was

made on the right lobe of the pancreas. The hepatorenal ligament is well developed in guinea pigs, having a long insertion.

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# CLINICO-PATHOLOGICAL FINDINGS IN VECTOR-BORNE PATHOGEN CO-INFECTIONS IN DOGS, FROM BUCHAREST AREA

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***Abstract***

*Canine Vector Borne Diseases (CVBD) have a worldwide impact as some are of zoonotic concern and they lead to a variety of serious infections mostly classified by their vectors. The pathogens co-infecting the dogs are linked to their associated vector agents and with their natural habitat. Dogs with clinical signs compatible for VBDs should be tested for more than one pathogen as the signs may be often non- specific and they may vary from one individual to another. Co-infections may potentiate the disease pathogenesis, thereby changing clinical manifestations associated with singular infections. Seven cases were selected among dogs referred in the Veterinary Clinic, Faculty of Veterinary Medicine of Bucharest during of 2016, showing clinical signs compatible with VBD. They were serologically-positive for more than one pathogen. The seroreactivity revealed co-infections in dogs with four arthropod-borne pathogens: Dirofilaria immitis + Anaplasma spp. (3 dogs), D. immitis + Erlichia canis (2 dogs), E. canis + Borelia burgdorferi (1 dog ) and E. canis + Anaplasma spp. (1 dog). One dog, serological positive for D. immitis and A. phagocytophilum, was also positive for Babesia canis, detected in the blood smear. The present study emphasizes the chalenge of the diagnostic, therapeutics and management of co-infected dogs and illustrates the correlation between clinical aspects that the dogs are first presented with and the full panel of paraclinical investigations like imagistical (radiography, ultrasonography) and the blood analyses (haematology, biochemistry, citology and serology).*

***Key words:*** *Co-infection, canine vector borne diseases, dogs.*

## INTRODUCTION

According to WHO, there are more than 200 emergent and re-emergent zoonoses, of which almost 10 canine vector borne diseases (CVBDs), including Lyme disease, that appears to be the most common in Europe (WHO, 2014).

Climate change, together with increasing movement of dogs across Europe, have caused an increase in the geographical range of more vector borne diseases (Genchi, 2011b).

Among the vectors transmitting disease-causing pathogens, ticks play an important role as they can harbore multiple disease causing agents, sometimes completely different pathogens (Shaw et al., 2001).

The risk of exposure to ticks, mosquitoes and fleas is bigger for dogs. They can be infested with hundreds of ticks and sometimes with different tick species in the same time, therefore concurrent infections with multiple vector borne pathogens may occur (Otranto et al., 2009a).

Dogs are reservoir hosts for several arthropod-borne pathogens, some of which are of major zoonotic concern (Beugnet, 2009) and they can be infected with a large number of vector-borne pathogens such as *Hepatozoon canis, Ehrlichia canis, Anaplasma platys, A. phagocytophilum, Babesia canis, B. vogeli, Bartonella* spp, *Borrelia burgdorferi, Leishmania infantum, Dirofilaria repens* and *D. immitis (*de Caprariis et al., 2011*)*.

Some arthropods are competent vectors of more than one pathogen. Thus, dogs might be exposed to vectors infected with single pathogens at different points in time or to vectors concurrently infected with multiple pathogens, favoring the occurrence of co-infections (Otranto et al., 2009b).

Studies regarding seroprevalence, revealed that dogs from Romania are potentially at risk of major canine vector-borne diseases because of the relatively high prevalence rates of both mosquito and tick-borne pathogens in dogs (Ionita et al., 2012; Mircean et al., 2012).

The diverse tick fauna as well as the abundance of tick populations in Romania represent potential risks for both human and animal health (Ionita et al., 2016).

Anamnesis and search for specific clinical signs, along with laboratory results (biochemistry and hematology) are the key for aproaching an accurate diagnostic. These findings can be modified by the presence of a co-infection.
Therefore, in this study clinico-pathological findings from CVBDs co-infected dogs are described.

## MATERIALS AND METHODS

The study describes clinical and hematological findings in seven dogs that were presented in the Veterinary Clinic, Faculty of Veterinary Medicine of Bucharest during of 2016 and were positive for more than one vector borne pathogen.
Dogs showing clinical signs compatible for VBDs were subjected for routine clinical examination, followed by blood analysis (biochemical, hematological and serological investigations), radiography and ultrasonography.
Whole blood EDTA samples were collected and tested for some selected CVBDs using blood smears and serological tests (SNAP®4Dx® Plus from Idexx Laboratories). Li-heparine tubes were used for collecting blood and biochemistry analysis was performed from plasma.
The SNAP 4Dx Plus test is an in-clinic enzyme-linked immunoabsorbent assay (ELISA) commercial kit for the detection of *Dirofilaria immitis* antigen and antibodies for *Anaplasma phagocytophilum / Anaplasma platys, Ehrlichia canis, Ehrlichia ewingii*, and *Borrelia burgdorferi*.

## RESULTS

Serological evaluation revealed co-infections in seven dogs with four different arthropod-borne pathogens*: D. immitis* + *Anaplasma* spp. (3 dogs), *D. immitis* + *E. canis* (2 dogs), *E. canis* + *B. burgdorferi* (1 dog) and *E. canis* + *Anaplasma* spp. (1 dog). One dog, serological positive for *D. immitis* + *A. phagocytophilum*, *Babesia canis* was also positive, detected in the blood smear (Figure 1).
Dogs included in the study, displayed clinical signs compatible with VBDs, with the exception of one dog, presented for screening as a blood donor. The age ranged from 6 years to 14 years old. There were 5 mixed breed dogs, 1 Labrador Retriever and 1 Golden Retriever; among them, 4 were females and 3 were males.
Clinical signs in the dogs referred, included depression (2/7), fever (1/7), anorexia (2/7), weight loss (1/7), weakness (3/7), exercise intolerance (2/7), pale mucous membranes (1/7), lameness (1/7), coughing (3/7), respiratory difficulties (1/7), vomiting (2/7) and diarrhea (2/7).
The following laboratory abnormalities were registered: anemia, leucopenia, leucocytosis granulocytosis, trombocytopenia, eosinophilia, elevated liver enzymes, high blood urea nitrogen and high blood creatinine levels. (Table 1.)
Hematological parameters were determined, thus anemia and trombocytopenia were found in four dogs of seven, leucocitosis with neutrofilia in two dogs, eosinophilia in one dog and leucopenia in anoher two. Microfilaremia associated with *D.immitis* was present in three dogs of five serological positive, from the total seven.
In dogs with *Ehrlichia* spp. serological positive detected, quantitative and qualitative changes regarding leucocytes were observed as an inflammation response and antigenic stimulation (WBC >17 K/uL with Grans >13 K/uL).
Thrombocytopenia was present in four out of seven dogs, as a result of the development of anti-platelet antibodies in *E. canis* infections (three dogs) and in one dog co-infected with *Anaplasma* spp and *D. immitis* (PLT<175 K/uL).
The presence of triple infection, with *D. immitis, A. phagocytophilum, and B. canis* (fig. 1), was detected in one dog, mixed breed, male, 14 y.o with severe respiratory simptoms, anemia, leucopenia, and elevated liver enzymes.
One of the 7 dogs included in the study, was a 4 years old female, Labrador Retriever in a late stade of gestation, serological positive to *Ehrlichia* spp. and *Anaplasma* spp.
Dogs co-infected with *E. canis, B. burgdorferi and A. platys / A. phagocytophilum* were treated with doxycycline (10 mg/kg/day /PO) for more than 21 days.
In the case of the triple infection with *D. immitis, A. phagocytophilum, and B. canis,* the dog was treated with imidocarb dipropionate (4

mg/kg in a single dose) and supportive treatment was given to reduce the anemia. Doxycycline (10 mg/kg/day /PO) was also used. For co-infected dogs with *D. immitis*, a treatment with low dose ivermectine and microfilaricide combination was used.

## DISCUSSIONS

Canine tick-borne pathogens have been documented in several European countries and revealed that *A. phagocytophilum* and *Borrelia* spp share the same tick vector - *Ixodes ricinus* (Straubinger et al., 2008). Another example of a shared vector is *Rhipicephalus sanguineus*, transmitting *Babesia* spp., *Ehrlichia canis, A. platys* and *Rickettsia conorii*, leading to co-infections with vector-borne pathogens in dogs.

In Romania, in a serological survey two cases of co-infection with *A. phagocytophilum* and *E. canis* were reported (Mircean et al., 2012). In a similar study, three cases of co-exposure to *D. immitis* and *A. phagocytophilum* and one case co-exposed to *E. canis* and *A. phagocytophilum* were displayed (Ionita et al., 2012).

In Romania, tick fauna is very diverse, with up to 20 species of hard ticks identified, with the most abundant and frequent species reported *Ixodes ricinus, Dermacentor marginatus, Dermacentor reticulatus, Hyalomma marginatum, Rhipicephalus bursa* and *Rhiphicephalus sanguineus* (Mihalca et al.,2015). The tick species found more frequent parasitizing dogs in urban area of Buharest, were reported *R. sanguineus, D. reticulatus, and ocassionally I. ricinus* (Ionita et al., 2013).

Other vectors responsible for CVBDs are represented by mosquitoes (*Aedes* spp, *Anopheles* spp, and *Culex* spp), implicated in transmitting *D. immitis* and *D. repens*.

Therefore, different combinations of vector-borne pathogens and their effect on the host, should be further investigated, as the possibilty of multiple vectorial capacity can occur. Co-infections might put the clinician in difficulty, as their expression vary from sick dogs to clinical healthy ones.

Serological assays do not differentiate between current and previous infections, when it comes for the detection for antibodies; Therefore, other confirmatory test are needed (e.g. PCR). Future studies should add new insights regarding molecular charecterization of vector-borne pathogens occuring in Romania.

The results in the present study supports the geographical expansion of canine vector borne diseases in Romania and that there is a challenge for the practioners when it comes for co-infections with CVB pathogens.

In this study, the hematologic results in infections with *Anaplasma* spp and *E. canis* were similar to those in other studies, as Mylonakis et al. reported (2004). Simultaneous infection with *E. canis* and *Anaplasma* spp in dogs resulted in a more pronounced anemia (HCT 23 % with HGB 6 g/dL) and thrombocytopenia compared to the single infection with either pathogen.

Also, co-infection with *E. canis* and *Anaplasma* spp appeared to result in a more persistent infection (Mylonakis et al., 2004).

A study conducted by Latrofa et al.(2016), sustained vertical transmission of *A. platys* during the early stages of gestation, and throughout its entire course, thus increasing the importance of screening for CVBDs in dogs.

Previous studies have shown that naturally infected clinically ill dogs, suspected of having either Lyme disease, granulocytic anaplasmosis, or both diseases, were nearly twice as likely to have antibodies to both *Borrelia burgdorferi* and *A. phagocytophilum* as compared to healthy dogs from the same region, suggesting that exposure to more than one pathogen may increase the possibility of disease expression (Beall et al., 2008).

Epidemiological studies performed in Europe, evaluated the seroprevalence of *A. phagocytophilum* in dogs between 3 to 57%, but serological cross-reactivity with other *Anaplasma* spp. *(A. platys)* can potentially cause an overestimation of the true seroprevalence (Sainz et al, 2015). In Romania, values regarding seroprevalence for *Anaplasma* spp varied from 5,5% to 16% (Mircean et al, 2012; Ionita et al., 2012).

More specific diagnostic methods, such as polymerase chain reaction (PCR) are necessary, due to cross-reactivty, particulary among members of the same genus ( Pantchev et al., 2010).

**Table 1. Clinical signs, laboratory abnormalities and diagnostic test results in seven dogs co-infected with canine vector borne diseases causing pathogens**

| Nr. crt. | Dog's data | | | Clinical signs | Serology (Snap 4Dx Plus) | Biochemistry | Haematology and blood citology | Ultrasounds | Radiology |
|---|---|---|---|---|---|---|---|---|---|
| | Breed | Age (year) | Gender | | | | | | |
| 1 | Golden retriever | 4 | F | weakness, vomiting, modified mamary glands discharge | *Ehrlichia* spp. + *Anaplasma* spp. + | no abnormalities | Mild anemia<br>PLT ↓<br><br>Microfilaremia | Gestation | Arthtritic degenerescence of the hip joint |
| 2 | Mongrel | 12 | M | Coughing, vomiting | *Ehrlichia* spp. + *Borrelia burgdorferi* + | ALKP ↑ (477 U/L)<br>GGT ↑ (25 U/L) | ESR ↑ (11,3) | Hepatomegaly<br>Splenomegaly | Congestive thorax |
| 3 | Mixed breed | 10. | F | Depression, Diarehea, anorexia, respiratory difficulties | *Ehrlichia* spp. + *Dirofilaria immitis* + | ALT ↑ (156 U/L) | Anemia<br>Leucocitosis with neutrofilia<br>Trombocitopenya<br>Microfilarilaremia | Enlarged aorta | Cardiac hypertrophy<br>Congestive pneumonia |
| 4 | Mixed breed | 13 | F | Coughing, depression | *Ehrlichia* spp. + *Dirofilaria immitis* + | No abnormalities | Leucopenia<br>PLT ↓ | Ventricular hypokinesia<br>Cardiac arrhythmia | Pulmonary reactivity |
| 5 | Mixed breed | 14 | M | Coughing, weakness, pale mucous membranes exercise intolerance and lameness | *Dirofilaria immitis* + *Anaplasma* spp. + | ALT ↑ (163 U/L)<br>AST ↑ (130 U/L)<br>ALKP ↑ (680 U/L)<br>Glu ↓ (65 mg/dL)<br>BUN ↑ (32 mg/dL)<br>CREA ↑(2.7 mg/dL) | MCHC↓ (28.1g/dL)<br>Hct ↓ (32,7%)<br>Hgb↓ (11.1 g/dL)<br>Wbc ↓ (5.70 K/uL)<br>PLT ↓<br>*Babesia* spp. +<br>Anemia | Splenomegaly<br>Hepatomegaly<br>Gastritis<br>Bile sludge | Cardiac hypertrophy<br>Interstitial lung pattern |
| 5 | Mixed breed | 6 | M | Weight loss, Anorexia, Fever, Diarrhea, Exercise intolerance | *Dirofilaria immitis* + *Anaplasma* spp. + | TP↓ (5.5 g/dL)<br>GPT↑ (173 U/L)<br>ALKP ↑ (212 U/L) | Mild anemia<br>Microfilaremia | Mild cardiac dilatation of the right ventricle and right atrium | Pulmonary congestion |
| 7 | Labrador | y.o. | M | Screening for blood donor Asymptomatic | *Dirofilaria immitis* + *Anaplasma* spp. + | No abnormalities | Grans↑(12.70k/uL)<br>Neu ↑(11.14 K/uL)<br>Eos ↑ (1.71 k/uL)<br>Microfilaremia | No abnormalities | No abnormalities |

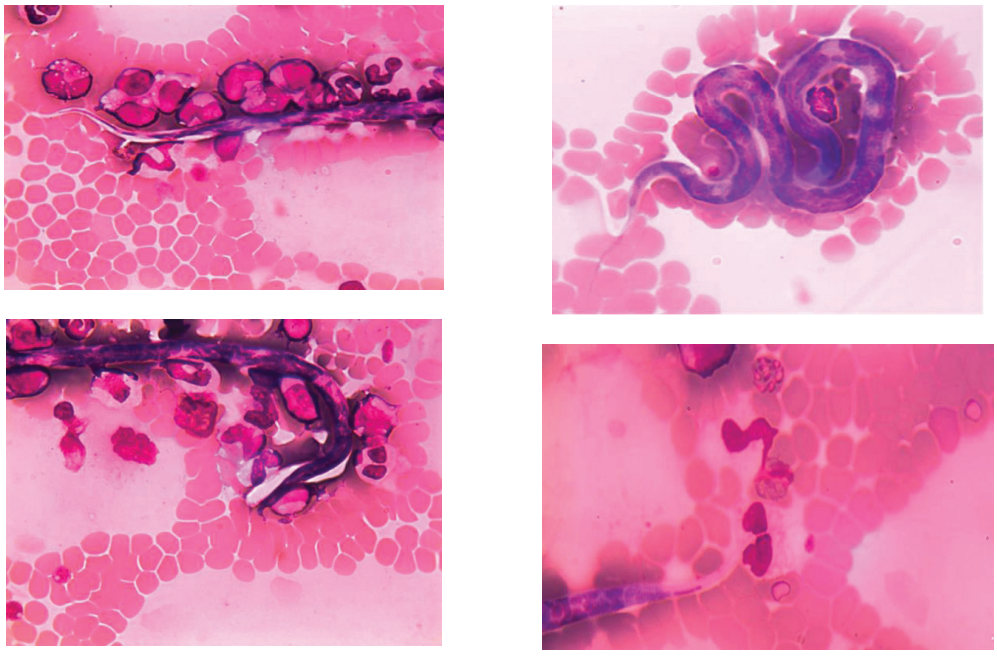

Fig. 1. Blood smears of dog showing microfilaria and merozoites of large *Babesia* spp - a case with triple infection (*B. canis, D. immitis* and *Anaplasma* spp)

## CONCLUSIONS

This study emphasizes the clinical difficulties associated with assigning a specific clinical sign or haematological abnormality to a particular canine vector-borne disease.
Monitoring the response for treatment is very important in dogs with severe hematological abnormalities and multiple infections, to improve the animal clinical status before treating for a specific vector-borne pathogen.
Assigning a specific treatment, needs a complete diagnostic aproach, remainig challenging to distinguish, disease from previous exposure to one or more vector-borne pathogens.

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# ELECTRORETINOGRAPHY: SELECTION OF PATIENTS AND PERFORMING THE TECHNIQUE

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***Abstract***

*Electroretinography (ERG) is an objective test that evaluates the retinal function. This paper wants to present to the veterinary Romanian scientific community the importance of using this diagnostic tool for clinicians dealing with blind patients. Candidates for ERG were selected from ophthalmological patients presented with blindness because it was difficult to establish a definitive diagnosis using only ophthalmological examination. All patients underwent general anesthesia. ERG was performed using the HMsERG unit, with preprogrammed included protocols. The protocols used in this study were Short protocol for the cataract surgery candidates and ISCEV protocol for the other patients. ERG confirmed the results obtained on chromatic pupillary light reflex evaluation. ERG proved useful in the following cases: diagnosing retinal inherited disorders, differentiating between retinal and postretinal causes of blindness, evaluation of retinal function in cataract surgery candidates. ERG objectively assesses the retinal function and should be used on a larger scale by practitioners dealing with blind patients.*

***Key words****: electroretinography, retina, blindness, cataract.*

## INTRODUCTION

The flash electroretinogram (ERG) is an electrodiagnostic test that assesses the function of one part of the central nervous system, the retina (Gelatt et. al, 2013) and it is described as the electrical response recorded when the retina is stimulated by flashes of light (Maggs et al., 2008).

This technique is useful for the early diagnosis and prognosis of inherited progressive retinal atrophy (PRA) or characterizing retinopathy due to other causes.

ERG is also useful when it is performed before cataract surgery as well as in diagnosing specific blinding disorders in dogs, such as sudden acquired retinal degeneration, optic neuritis or cortical blindness (Narfstrom et al., 2002).

Although ERG is a widely used technique in veterinary ophthalmology, this paper wants to present to the veterinary Romanian scientific community the importance of using this diagnostic tool for clinicians dealing with blind patients.

## MATERIALS AND METHODS

Candidates for ERG were selected from the patients presented with blindness in the Ophthalmology Department of the Faculty of Veterinary Medicine of Bucharest, during December 2016 – February 2017. They were divided into 3 groups, based on clinical findings: partially blind patients, completely blind patients and cataract surgery candidates. After clinical assessment, serological and hematological examinations were performed.

Ophthalmological examination included Schirmer tear test, fluorescein test, visual testing (menace response, cotton ball test, obstacles test), tonometry, pupillary light reflex, chromatic pupillary light reflex, ophthalmoscopy. In some cases complementary diagnostic tests have been used, such as ultrasound and MRI.

The chromatic pupillary light reflex (chromatic PLR) is a useful method for detecting PLR abnormality in sudden acquired retinal degeneration (SARDS), progressive retinal atrophy (PRA), or optic pathway disease, thus distinguishing between these 3 diseases that all present with blindness (Terakado et al, 2013; Yeh et al., 2017).

Therefore, evaluation of the chromatic PLR in blind patients can suggest a possible diagnosis prior to ERG or MRI examination.

Prior to anesthesia, both pupils were dilated by applying one drop of 1% tropicamide (Tropicamida 1%; Rompharm, Romania) and phenylephrine 10% (Fenefrin 10%; UnimedPharma, Slovakia) three times, with a 10 minutes interval between instillation of the drops.

The patients were fully anesthetized in order to prevent artifacts due to involuntary muscles movement (Ekesten et al., 2013).

Dogs were premedicated with dexmedetomidine (2-10 mcg/kg) (Dexdomitor 0,5 mg/ml; Orion Pharma, Finland) and butorphanol (0.2-0.4 mg/kg) (Butomidor; Richterpharma, Austria), delivered intramuscularly. Induction and maintenance were achieved using propofol initial bolus (1 mg/kg) (Norofol 1%; Maravet, Romania) and a constant rate infusion of 0.1-0.5 mg/kg/min intravenously.

Cats were premedicated using dexmedetomidine (0.01 mg/kg), ketamine (2 mg/kg) (Anestamine 100 mg/ml; LeVet Pharma, Holland) and butorphanol (0.2 mg/kg) administered intramuscularly. Induction and maintenance were achieved using propofol initial bolus (4 mg/kg) and a constant rate infusion of 0.6 mg/kg/min.

Local nerve blocks of the auriculopalpebral and zygomatic nerve were performed on all patients, using lidocaine (1-2 mg/kg) (Xilina 10mg/ml; Zentiva, Romania).

Topical anesthesia was induced using 0.4% oxybuprocaine hydrochloride (Benoxi 0.4%; UnimedPharma, Slovakia).

Each patient was placed in sternal recumbency. Eyelids were kept open during the examination with a lid speculum (Barraquer eye speculum; Acrivet, Germany) and globes were centered with 1-2 conjunctival stay sutures.

ERGs were recorded using the Handheld Multispecies ERG system (HMsERG, RetVetCorp, USA) consisting of the unit body, a mini Ganzfeld dome and three electrodes (active, reference and ground electrodes).

Retinal signals were recorded using a contact lens active electrode (ERG-jet; Fabrinal, Switzerland) that was placed on the cornea after applying artificial tear gel (Hypromeloza P 0.5%; UnimedPharma, Slovakia), in order to improve conduction. The reference and the ground electrodes were skin needles electrodes (Stainless steel subdermal electrode; OcuScience, USA) that were placed approximately 2 cm caudal to the lateral canthus and in the midline on the top of the skull, respectively. Electrodes were kept in a stable position using a piece of tape. All 3 electrodes were connected to a preamplifier and the signals were amplified with a bandpass of 0.3 to 300 Hz, provided by the HMsERGunit.

Impedance and baseline tests were performed before ERG recordings. The electrode impedance was maintained under 5 kΩ and the baseline test under 25 microvolts.

A handheld mini Ganzfeld dome, positioned approximately 1 cm to the eye, without touching the animal, provided light stimuli and background adaptation.

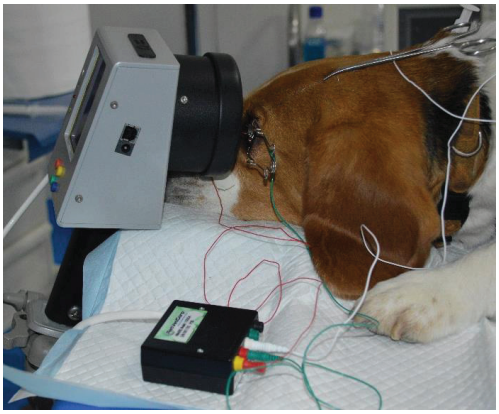

Figure 1. ERG setup using the HMsERG device.

The HMsERG system software package has several preprogrammed protocols for evaluating retinal function. We used the ISCEV Protocol for patients with partial or complete blindness and the Short Protocol for cataract surgery candidates.

Before performing the ISCEV Protocol, the patients were dark adapted for 20 minute. For the Short Protocol, the patients were light adapted. All lights in the examining room were turned off at the ERG test initiation.

According to the HMsERG user's manual, for rods' function evaluation, responses for scotopic ERG procedures were recorded using low intensity stimuli (0.01 cd.s/$m^2$ of light stimuli), scotopic standard intensity responses

(Std R&C) using 3 cd.s/m$^2$ of light intensity for stimulus, scotopic higher intensity responses (Hi-Int R&C) using 10 cd.s/m$^2$ of light intensity to stimulate both rods and cones. For cones' function evaluation, responses for photopic ERG procedures were recorded using light stimuli of 3 cd.s/m$^2$, scotopic higher intensity responses (HiCones) using 10 cd.s/m$^2$ of light intensity, flicker responses using 3 cd.s/m$^2$ of light intensity for standard flicker (Std. Flicker) and 10 cd.s/m$^2$ of light intensity for higher intensity flicker (Hi-Int. Flicker).

ERG data for each patient were recorded bilaterally. ERG recordings were analyzed by measuring the amplitude and implicit time for each a- and b-wave ERG component, as well as the b/a ratio.

The a-wave amplitude was measured from the baseline to the a-wave trough, the b-wave amplitude was measured from the a-wave trough to the b-wave peak. A- and b-wave implicit times are calculated from the light stimulus onset to the a-wave trough and b-wave peak, respectively. Amplitude of the cone flicker response represents the average of the amplitudes from the trough to the positive peak in at least 3 responses in the train. Implicit time of the cone flicker response is calculated as the average of the times from the light onset to the positive peak in at least 3 responses in the train. (Ekesten et al, 2012). For the scotopic low intensity responses and photopic 30 Hz flicker responses, only the b-wave amplitude and implicit time were measured.

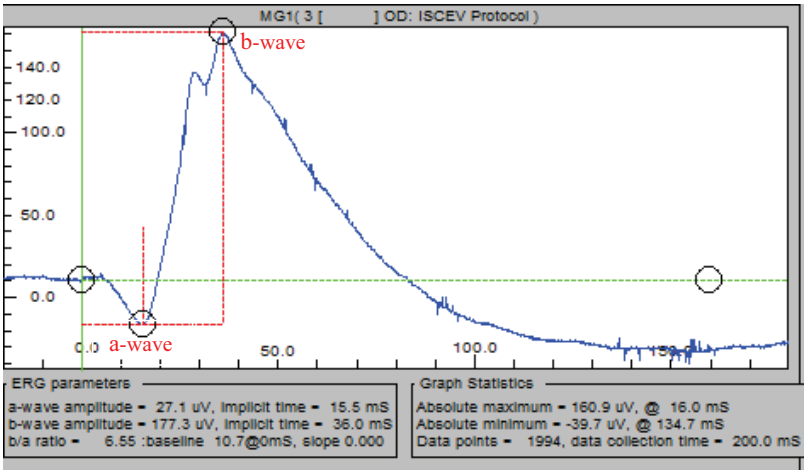


Figure 2. A- and b-wave parameters in a normal ERG, DSH, 4 years old.
The vertical green line represents the stimulus onset

## RESULTS AND DISCUSSIONS

Although different from other anesthetic protocols usually used for ERG recordings, the protocol that we used assured appropriate anesthesia for ERG examination. None of the patients were intubated during ERG evaluation. Because atipamezole can be used to counter the effect of dexmedetomidine if needed and because propofol is a short action drug, we believe that this protocol is safe. Furthermore, if after ERG recordings, further cataract surgery or MRI examination is needed, the anesthesia can be maintained by intubating the patients.

Local anesthetic blocks provided good anesthesia, no blinking was recorded, thus reducing the artifacts to minimum.

The HMsERG device has built in protocols based on the International Society for Clinical Electrophysiology of Vision (ISCEV) guidelines. We chose the Short Protocol for the cataract patients, due to the fact that we only needed confirmation whether the retina is functional or not and because of the shorter time necessary to perform it. The ISCEV protocol was used in the cases where we needed a more complex investigation of the retina, separating the rods' responses from the cones' responses.

All results were compared with the normal ERG recorded in our clinic for the same species and age group and also compared to specialty literature data (Figure 2).

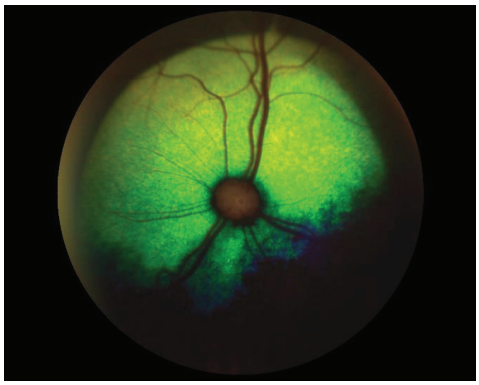

Figure 3. The normal fundus of a cat (DSH, 4 years old)

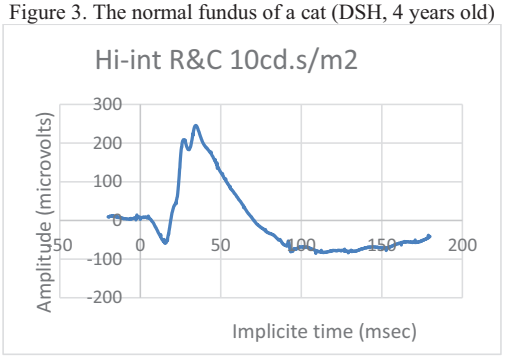


Figure 4. Normal ERG of the same cat

For the selected ophthalmological patients included in this paper, except for the cataract surgery candidates, it was difficult to establish a definitive diagnosis using only ophthalmological examination.

In patients which were presented with partial or complete blindness and with ophthalmoscopic modifications, ERG was used to confirm the absence of retinal function, establishing the diagnosis of early or late onset retinal degeneration (Figure 5 and Figure 6).

Two of the patients presented with acute blindness, complete mydriasis, unremarkable fundus examination and unresponsive pupils to the chromatic PLR suggesting optic neuritis. For these patients, ERG recordings proved to be an invaluable tool, diagnosing decreased retinal function, suggesting combined autoimmune retinal and optic nerve disease (Figure 7 and Figure 8).

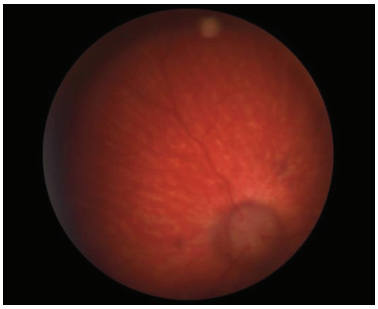

Figure 5. Fundus examination in a blind 6 months old Husky revealed vasculature attenuation.

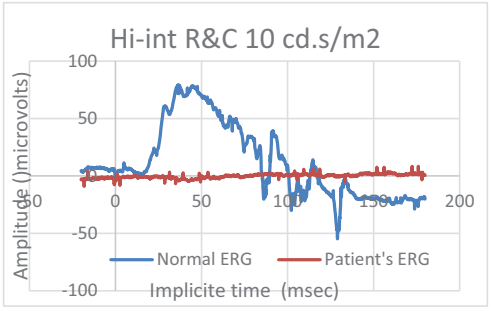


Figure 6. The electroretinogram of the Husky confirmed absence of retinal function, consistent with early onset retinal degeneration (PRA)

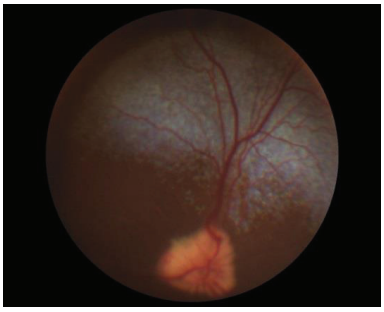

Figure 7. Normal fundus appearance of a blind 7 years old French Bulldog

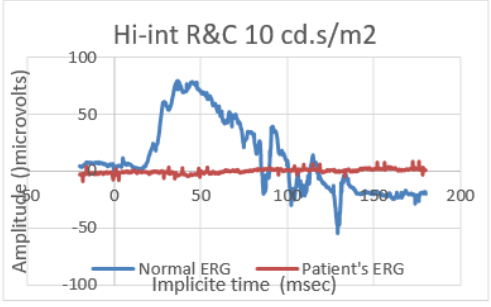


Figure 8. The electroretinogram of the French Bulldog showed decreased retinal function, consistent with an autoimmune mediated retinitis

One patient presented with partial blindness. Visual tests were negative in the affected eye, pupils were mydriatic in ambiental light and the chromatic PLR was normal in both eyes. An ERG was performed and it showed normal retinal function. The MRI examination confirmed a postretinal cause of blindness, tumor of the coroidal plexus and ventricular hydrocephalus.

Five patients that were candidates for cataract surgery, were first examined by ERG. A good candidate for surgery has normal retinal function (established by chromatic PLR and ERG) and the lens in a normal, anatomical position. The retinal function responses that were recorded helped us in selecting the better candidates for surgery, as the ones with low a- and b-wave amplitudes and implicit time were excluded. One of these patients had normal, promt and complete chromatic PLR, but due to the low retinal function recorded by the ERG, it was excluded from cataract surgery (Figure 9).

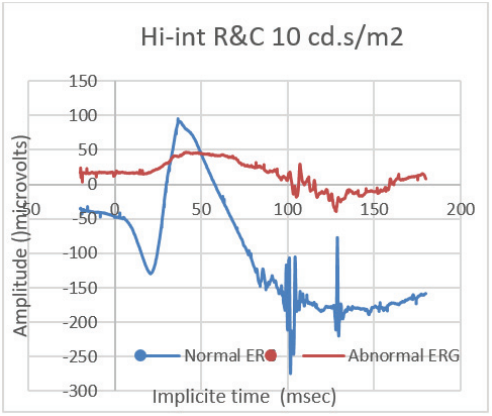


Figure 9. Differences between ERGs of two cataract surgery candidates. If the electric response of the retina is good, the patient is then operated (blue line). If the electric response of the retina is low, the surgery is not recommended (red line)

## CONCLUSIONS

Electroretinography objectively evaluates the retinal function.

Candidates presented with blindness were diagnosed with retinal inherited disorders, differentiating between retinal and postretinal causes of blindness.

Evaluation of retinal function of cataract surgery candidates proved very useful in deciding whether the surgery is recommended. ERG should be used on a larger scale by practitioners dealing with blind patients.

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# BACTERIAL BIOFILMS AS WOUND HEALING DRESSING – A REVIEW

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***Abstract***

*Tissue engineering and regenerative medicine promote skin regeneration through biomaterials that are easy to provide. Lately, many studies showed that bacterial biofilms can ensure the necessary conditions for proper healing. Several bacteria (Acetobacter spp., Lactobacillus spp., Azotobacter spp.) produce extracellular polysaccharides (cellulose, kefiran, alginate) organized in biofilms with different chemical structures. All have properties that grant medical application: cartilage and bone repair, nerve surgery and arterial stent coating. Bacterial cellulose, alginate and kefiran biofilms seem to have the qualities needed as wound healing dressings, but their characteristics and availability vary widely. The aim of this study was to summarize the current state of art on bacterial biofilms to discriminate among their specific properties and application in wound healing management. The comparison was focused on obtaining techniques, physiochemical characteristics, advantages and disadvantages of use. Cellulose, alginate and kefiran showed good results in wound healing processes, but it seems that cellulose and kefiran are the most used. Biocellulose can be obtained in multiple ways (such as stationary or agitated culture) thus the protocol varies depending on available laboratory equipment. Both cellulose and kefiran have high biocompatibility, kefiran presents antimicrobial activity as well, while cellulose can incorporate drugs. Alginate has all the properties of a wound dressing material, but it is difficult to obtain. In conclusion, bacterial cellulose seems to be the most suitable for local covering of wounds. It is studied extensively on laboratory animals and it is currently used in human medicine. However, there seems to be a lack of case studies on wound management of small animals, mainly cats and dogs.*

***Key words****: alginate, bacterial biofilms, cellulose, kefiran, wound healing, cellulose.*

## INTRODUCTION

The main function of the skin is to protect the body against the environment and major disorders (chronic infection or necrosis).Wound healing is linked to growth and regeneration. Tissue engineering and regenerative medicine employ materials that support and accelerate healing (Nasrabadi and Ebrahimi, 2011). Thus, wound therapy remains a clinical problem and a proper, efficient management is required. The proper treatment needs to promote rapid healing and generate functional tissues (Sulaeva et al., 2015). New approaches are being developed for acute and chronic wound that avoid complications. Wound dressings and medication form an important segment of the global pharmaceutical market (Patel et al., 2012).The global market attempts to offer a variety of wound dressings for proper wound management based on different types of materials – natural or synthetic. Applicable in different forms – films, hydrocolloids and gels, they can contain drugs and bioactive substances that can accelerate wound healing process (Sulaeva et al., 2015).

Thus, the dressing of choice must ensure the necessary conditions: a moist and clean environment, blood and excess exudates absorption, infection prevention, optimal temperature, non-adhesive and rare changes (Boateng et al., 2008). Materials must be safe, biocompatible, biodegradable and non-toxic. A variety of materials meet these qualities, such as chitosan, collagen, gellan gum and bacterial biofilms (Mokhtarzadeh et al., 2016).

Biofilms are bacterial-synthesised exopolysaccharide organised into long polysaccharidic chains of sugars (glucose or galactose) or sugar derivates arranged in branches (Chawla, 2009). Their formation is an essential stage in the survival of bacteria (Sabra et al., 2001). Biocellulose is a non-toxic, hypoallergenic, non-biodegradable material, with a unique nanofiber and porous structure. These properties make it a perfect wound dressing

(Rajwade et al., 2015), being one of the best scaffold for repairing and remodelling large areas of injured skin (Mogosanu and Grumezescu, 2014).

Alginate is used for a variety of application including pharmaceutical, and biotechnology industries (Schmid et al., 2015). Usually dressing removal produces pain and destroys regenerative tissue, but alginate gels have an advantage over other scaffolds, like cotton or viscose gauze: they adsorb exudates, which prevent the fibres from sticking to the wound. Alginate gels also provide moisture and prevent drying, which benefits wound healing. They also have haemostatic properties and good permeability for oxygen that supports rapid healing (Hoefer et al., 2015).

Kefiran is an exopolysaccharide extracted from kefir grains and has superior dressing qualities. It has antibacterial, antitumoral and antifungal properties (John and Deeseenthum, 2015) and together with satisfactory mechanical resistance and good appearance makes it suitable as wound scaffold (Zolfi et al., 2014). Its applications are, however, limited by high water permeability which can be improved by incorporating hydrophobic compounds (Ghasemlou et al., 2011b).

There are many studies that describe biofilms and their main characteristics (Mogosanu and Grumezescu, 2014), but there is a lack of pertinent comparison among them. They are usually used in human medicine as wound healing materials (Mogosanu and Grumezescu, 2014) and they were largely tested on laboratory animals (Lee and Mooney, 2012; Hoefer et al., 2015; Kwak et al., 2015; Majid et al., 2016).

Studies on bacterial biofilms properties and biocompatibility reveal that they could be successfully used in veterinary medicine. However, they are not yet introduced in current veterinary practice. The three biofilms were studied extensively by many authors in respect to their mechanical properties, healing and wound dressing properties but they overlooked comparing them in regard to their use on clinical cases in wounds management of small animals. Thus, the aim of this review is to critically analyse the current knowledge on biofilms as wound dressings for veterinary use. The main bacteria species involved in biofilm production, their growth conditions and discrimination among the properties of bacterial biocellulose, alginate and kefiran are presented.

## BIOFILM-PRODUCING BACTERIA AND OBTAINING TECHNIQUES

**Biocellulose** (BC) is an exopolysaccharide synthesised by a variety of bacteria: Gram-negatives such as *Rhizobium*, *Aerobacter*, *Agrobacterium*, *Salmonella*, *Escherichia*, *Rhodobacter*, *Acetobacter, Pseudomonas*, *Gluconacetobacter, Alcaligenes*, *Azobacter*and Gram-pozitive *Sarcina ventriculi* (Huang et al., 2013; Sulaeva et al., 2015). Compared to plant cellulose, it has superior mechanical properties and a unique structure that makes it suitable for wound dressing (Rajwade et al., 2015). *Acetobacterxylinum*, *A.hansenii* and *A. Pasteurianus* produce high yields of BC (Chawla, 2009), but only species of *Gluconacetobacter* are economically efficient (Ul-Islam et al., 2015). Relatively high levels of exopolysaccharides are produced from various sources of carbon and nitrogen (Chawla, 2009). The main stains producing BC and their cultivation conditions are systematically presented in Table 1.

The morphology is conditioned by the activity and fermentation ability of bacteria (Huang et al., 2013). Static culture was initially used, but the thickness varied a lot (Ul-Islam et al., 2015). Agitation techniques were designed to increase the yield and quality of biocellulose to commercial requirements (Czaja, 2004).

Agitated cultures of BC forma thick layer of small irregular or spherical pellets (Ul-Islam et al., 2015). The nanofibers get attached as they are synthesized through the medium, forming a deformed mass of cellulose (Huang et al., 2013). The use of high-speed agitators is a third technique used to increase the yield of BC. Static and agitated cultures cannot ensure the optimal oxygen distribution and mixture of the media. High-speed agitated cultures are produced in reactors, where oxygen is at ideal values and nutrients can be added at any time. The rotation speeds prevent the formation of BC conglomerates (Ul-Islam et al., 2015). Different strains of *Acetobacter xylinum* are commonly used to produce a reasonable amount of biocellulose from a variety of carbon sources.

Table 1. Main bacterial stains producing bacterial cellulose

| Bacteria species | Medium | Carbon source | Supplement | Type of culture | Temper-ature | Culture time | pH | Reference |
|---|---|---|---|---|---|---|---|---|
| *Gluconacetobacter swingsii* spp. | Hestrin and Schramm | glucose | - | static | | | | |
| | sugar cane juice | glucose, fructose, sucrose | - | static | 28°C | 13 days | 3.5 | Castro et al. (2011) |
| | pineapple peel juice | glucose, fructose, sucrose | - | static | | | | |
| *Gluconacetobacter xylinum* BRC-5 | Hestrin and Schramm | glucose | - | static | 30°C | 14 days | | Cai and Kim (2009); Kim et al. (2010) |
| *Acetobacter xylinum* FF-88 | coconut milk | sucrose | - | static | - | 10 days | 3 | Nakagaito et al. (2005) |
| *Acetobacter xylinum* TISTR 975 | yeast extract powder | glucose | - | agitated | 30°C | 24 h | 6 | Maneerung et al. (2008) |
| *Acetobacter* spp. A10 | Hestrin and Schramm | glucose | - | static | 32°C | 9 days | 6.7 | Kwak et al. (2015) |
| *Acetobacter xylinum* NQ5 | Hestrin and Schramm | glucose | 0.1% cellulose enzyme (*Trichoderma resei*) | static | 28°C | 3 days | - | Czaja (2004) |
| | | | | agitated | 28°C | 7 days | - | |
| *Acetobacter xylinum* X2 | green tea powder | sucrose | - | static | - | 7 days | 4.5 | Wan et al. (2007) |
| *Acetobacter xilinum* NBRC 13693 | Hestrin and Schramm | fruit juice | disodium hydrogen phosphate | - | 30°C | 14 days | 6 | Kurosumi et al. (2009) |
| | Hestrin and Schramm | sugar reagent (glucose, fructose, sucrose) | nitrogen | | | | - | |
| | - | fruit juice | - | | | | 6 | |
| *Acetobacter xylinum* E25 | Hestrin and Schramm | glucose | - | static and horizontal fermenters | 30°C | 48-64 h | 3.22 | Krystynowicz et al. (2002) |
| | Hestrin and Schramm | glucose | ethanol 1% | | 30°C | 48-64 h | 3.12 | |
| | Yamanaka | sucrose | - | | 30°C | 48-64 h | 3.61 | |
| | Yamanaka | sucrose | ethanol 1% | | 30°C | 48-64 h | 3.28 | |
| | Yamanaka (optimized) | sucrose, fructose | fructose, yeast extract, ammonium sulfate | | 30°C | 48-64 h | 4.98 | |
| | Yamanaka (optimized) | sucrose, fructose | ethanol 1%, fructose, yeast extract, ammonium | | 30°C | 48-64 h | 4.78 | |

It can be produced by various culture methods to produce reasonable economic quantities and to achieve desirable mechanical properties.

**Alginate.** Bacterial alginate was discovered by Linker and Jones back in 1964, by extracting exopolysaccharides from a *Pseudomonas aeruginosa* mucoid stain (Hoefer et al., 2015). Alginate is an anionic linear polymer formed by β-1,4-linked mannuronic acids and α-L-guluronic acid. The molecular mechanisms involved in biosynthesis is extensively studied (Hay et al., 2014). Microbial alginate is restricted to the *Pseudomonas* and *Azotobacter* species. More efficient large scale production is specific to algae. However, bacterial alginate has constant composition and yield, thus optimised larger scale production would make it a more desirable product (Sabra et al., 2001; Schmid et al., 2015).

*Pseudomonas* and *Azotobacter* have virtually identical genes involved in alginate biosynthesis, but the process differs. Alginate production is influenced by 12 genes (algD-algA) under strict control of alginate promoter (algD) which encode enzymes involved in precursor synthesis and encoding proteins that modify the alginate structure as travelling the periplasm (algI, algJ, algF, algL, algV and algG) (Remminghorst and Rehm, 2006). The production of bacterial alginate could be expanded by expressing biosynthesis genes and inactivate negative regulators (Schmid et al., 2015). Genetic engineering of *A.vinelandii* can control the molecular weight, degree of acetylation, monomer composition and sequence structure of alginate (Remminghorst and Rehm, 2006). Thus, new techniques must be developed to obtain alginate with optimal properties and yields.

*Azotobacter vinelandii* is cultivated on Burk`s medium (Hoefer et al., 2015). The pH is adjusted to 7 ± 2 with $NaOH_2$ (Gómez-Pazarín et al., 2016) or HCl and autoclaved for 15 min at 121°C (Hoefer et al., 2015). Cultures are grown at 29°C for 72h (Gómez-Pazarín et al., 2016) in an orbital incubator with a 25mm shaking diameter. Carbon sources (sucrose and glycerol) are then added. The cultures are grown at 30°C for 48h. Favourable development conditions are supplemented by growing under strict oxygen control (Hoefer et al., 2015; Gómez-Pazarín et al., 2016). After 48h the bacteria is incubated in the shaker at 30°C to dissolve the cell-associated alginate and then the suspension is diluted with NaCl. The bacteria are separated by centrifugation at 4°C for 40 min. Then, by adding ice-cold ethanol, the alginate in the supernatant is precipitated and collected by repeated centrifugation. Alginate is washed 2 times with ethanol before drying overnight (Hoefer et al., 2015).

Future biotechnological research should aim at improving bacterial production stains by genetically engineering to obtain alginate suitable for high value wound dressings (Schmid et al., 2015; Mokhtarzadeh et al., 2016).

**Kefiran** is a heteropolysaccharide soluble in water, isolated from kefir grains and produced by several *Lactobacillus* species: *L. kefiranofaciens*, *L. parakefir*, *L. kefirgranum*, *L. parakefir*, *L. kefir* and *L. delbrueckii* subsp. *bulgaricus* (Vinderola et al., 2006; Patel et al., 2012). It contains glucose and galactose in approximately equal amounts and it encapsulates acetic acid bacteria and yeasts, involved in the fermentation process. Visco-elastic properties of acid milk films are improved by glycerol (Patel et al., 2012).

First the kefir grains - the starter cultures - are kept until they are cultured, in skimmed milk, at room temperature (Ghasemlou et al., 2011a). Kefir grains are obtained by growing the *Lactobacillus* spp. in lactic acid whey broth (LAW). The pH is adjusted to 5.5 with liquid DL-lactic acid syrup. Distilled water is added and the solution is boiled for 30 min. The precipitate is centrifuged for 25 min at 4°C. Fermentation occurred at 25°C, under anaerobic conditions and the pH is adjusted daily at 5.5 with KOH (Vinderola et al., 2006). The kefir grains are usually collected when they reach a 2 cm diameter (Shahabi-Ghahfarrokhi et al., 2015).

The polysaccharides are extracted by dissolving kefir grains in boiling water 1:100 for 1h (Ghasemlou et al., 2011a; Zolfi et al., 2014) or 1:10 for 30 min (Shahabi-Ghahfarrokhi et al., 2015; Blandon et al., 2016) and agitated. Then the mixture is centrifuged for 15 min at 20°C (Ghasemlou et al., 2011a; Zolfi et al., 2014). The polysaccharides are precipitated by adding equal volume of 96% cold ethanol and kept

overnight at -20°C (Zolfi et al., 2014). Then the mixture is centrifuged again for 20 min at 4°C to separate the precipitated carbohydrate. The precipitates are washed with water for removing impurities. The process is repeated three or four times. The resulting solution is concentrated precipitated polysaccharides and is hereafter called kefiran (Vinderola et al., 2006; Ghasemlou et al., 2011a; Zolfi et al., 2014; Shahabi-Ghahfarrokhi et al., 2015; Blandon et al., 2016).

Film preparation begins with weighing the amount of film-forming kefiran aqueous solution, with different concentrations. Then glycerol is added to the mixture as a plasticizer at various levels (15-35%) (Ghasemlou et al., 2011b) or equal amount of glycerol to that of kefiran (Zolfi et al., 2014). The mixture is then agitated using a magnetic stirrer for 15 minutes. The filmogenic solutions obtained are cast in Petri dishes and dried at 30°C for 30 min (Zolfi et al., 2014) or at 40°C for 6h (Piermaria et al., 2011) in a ventilated oven to remove the air bubbles (Zolfi et al., 2014; Sulaeva et al., 2015). The resulted films are removed from the plates and stored at 20°C and humidity at 75% (Piermaria et al., 2011).

Plasticizer must be added into the film to achieve flexibility otherwise they are fragile and cracked during drying (Ghasemlou et al., 2011b; Zolfi et al., 2014). Ghasemlou et al. (2011) used different concentrations of Kefiran (1%, 2%, 3%) and showed that biofilms containing 2% were taken easily from plates, but, on the contrary, films with 1% were thick and difficult to handle (Ghasemlou et al., 2011b).

## BACTERIAL BIOFILMS PROPERTIES

**Biocellulose** is produced as a gel at the interface of air-liquid of the proper medium. Culture time and carbon source in the medium influence the thickness of BC (Ul-Islam et al., 2015). Scanning electronic microscope (SEM) studies reveal a 3D network structure with 30 to 100 nm fibre diameter (Yang et al., 2012) and 120-160 nm pore size (Shahmohammadi Jebel and Almasi, 2016). Bacterial cellulose has a porous structure, which gives it water absorption properties (Ul-Islam et al., 2015) up to 350% its own weight in 24h with a water vapour transmission rate of 112.14 g x $m^2$/h (Kwak et al., 2015). The tensile straight ranges widely between 12.13 MPa (Kim et al., 2010) and 450 MPa, the strain reaches up to 12.53% and the crystallinity about 17.63% (Kwak et al., 2015).

Drying method influences the structure of biofilm: an uniform pores distribution and greater number of pores are present when freeze-drying biocellulose compared to air-drying (Rajwade et al., 2015).

The biodegradability of biocellulose was studied *in vitro* by immersing the membrane for 8-12 weeks in phosphate buffered saline solution at 37°C temperature and 7.25 pH. The studies showed a modest fragmentation of the film and formation of woolly aggregates (Rajwade et al., 2015).

Biocompatibility of cellulose was studied as a substitute for dura mater membrane in dogs (Rajwade et al., 2015). Research showed no pathological inflammation when implanting cellulose in the nasal dorsum of rabbits, and the results showed little fragmentation of the biofilm after 6 months (Rajwade et al., 2015). Other studies were made on rats, by implanting subcutaneous BC (Kalia et al., 2011). There were no signs of biodegradability after 12 weeks of implantation (Rajwade et al., 2015).

Biocellulose has unique mechanical properties such as ultrafine 3D network structure, with various pore geometry, is highly purified, has high water absorption ability (over 100 times its own weight) and high crystallinity.

**Alginate** has a smooth and uniform surface, with an ordered fibre structure, resulting in transparent biofilms that can easily be removed from plates (Zhang et al., 2015). SEM studies showed a porous microfiber structure (Mogosanu and Grumezescu, 2014). The tensile straight of bacterial alginate is 6.51 MPa (Zhang et al., 2015) in contrast to 2.6 MPa of algae alginate (Hoefer et al., 2015). The ability to absorb exudates is an important feature of alginate. A comparison between bacterial and algae alginate was studied by immersing both biofilm types in a 0.9% saline solution, containing calcium. After 30 min, both biofilms turned into hydrogels, but bacterial alginate absorbed a larger amount of solution. This changed their microscopic structure, the fibres almost disappeared. Marine alginate kept its

fibre structure and absorbed less saline solution (Hoefer et al., 2015). Alginate is highly soluble in water (~99.5%), but the solubility can be reduced by adding lipids (Zhang et al., 2015). In contrast, Mogosanu and Grumezescu (2014) observed a porous structure, no adhesive properties and water absorption up to 20 times its weight.

The pH can influence viscosity: it increases with the decrease of pH, reaching a peak at pH 3-3.5 (Hay et al., 2013). Alginate did not show any bacteria-inhibition properties (Zhang et al., 2015), but it can retain and inactivate bacteria inside its structural matrix (Spasojevic et al., 2016). By adding antibacterial agents this disadvantage can be removed (Zhang et al., 2015). The antimicrobial activity of alginate-lignin compound was tested on bacteria active in chronic wounds: *Enterobacter cloacae*, *Escherichia coli*, *Salmonella enterica*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Micrococcus flavus*, *Listeria monocitogenes* and *Staphylococcus aureus.* It was concluded that lignin has little antimicrobial activity, but in association with alginate, the effect is synergetic (Spasojevic et al., 2016).

Alginate forms strong thermostable gels by interacting with various cations, especially $Ca^{2+}$. This aspect grants encapsulation properties. It is suitable for medical delivery systems because it is permeable to liquids and small molecules (i.e. drugs) (Mokhtarzadeh et al., 2016).

The biocompatibility of alginate was largely investigated *in vivo* and *in vitro* studies (Lee and Mooney, 2012; Spasojevic et al., 2016), but there are disagreements about the effect of its composition on tissue response. Some studies show that alginate can be immunogenic and can induce cytokine production (Lee and Mooney, 2012), in contrast, others observed no such effect (Spasojevic et al., 2016). The immunogenic response could be assigned to remaining impurities because highly purified alginate induced no body reaction in animal tissues (Lee and Mooney, 2012). Alginate-lignin compound revealed no cytotoxic effect when tested on cervix carcinoma and human conjunctival epithelial cells. Furthermore, no damage on wounds or nearby skin was observed when tested *in vivo* on sterile wounds induced by incision on rat skin (Spasojevic et al., 2016). Similarly, no important inflammatory reaction was noticed when alginate gel was subcutaneously injected to mice (Lee and Mooney, 2012).

Alginate yield can be increased by genetic modification of bacteria strains but even so it cannot reach a reasonable economic scale. Alginate forms transparent gel like films with a fibre porous structure, good mechanical properties and the ability to absorb exudates. It has a good biocompatibility and although it has no antimicrobial activity it has the ability to incorporate drugs, fact that substitutes this lack.

**Kefiran.** Studies reveal the use of polysaccharides to prepare films with different properties increase significantly. Kefiran finds increasing use because of its texture and promising mechanical properties. Biofilms have good appearance although are highly permeable to water vapour and the control of moisture in wound healing is a desirable propriety (Ghasemlou et al., 2011a).

SEM reveals that kefiran biofilms have smooth uniform surface, with compact structure, after being plasticized with glycerol (Ghasemlou et al., 2011b). The structure of kefiran can be changed by varying the concentration of glycerol (Piermaria et al., 2011) which makes the biofilm more compact (Piermaria et al., 2009). An increased amount of plasticizer increases the moisture content from 17.95% to 37.04%. The plasticizer acts as a water scavenging agent: the plasticity increases with the increase of water content (Ghasemlou et al., 2011b).

An increasing polysaccharide concentration increases the film thickness from 1.9±1.2 μm to 2.1±1.3 μm (Piermaria et al., 2009). Sugar and polyols, used as plasticizer, lead to thicknesses varying from 22 to25μm, while sucrose generated a 31μm film (Piermaria et al., 2011).

Transparency is an important propriety, pure kefiran biofilms transparency varies between 2.714±00.15 A600/mm (Piermaria et al., 2009) but also depends on the plasticizer used, ranging between 1.88 A600/mm to3.30 A600/mm (Piermaria et al., 2011).

Glycerol influenced the mechanical properties of kefiran film as well. A considerable tensile straight was shown in films with no glycerol and lower elongation at break (Piermaria et al., 2009). Thus, plasticizers affect the tensile

straight and elongation at break: tensile straight decreases with an increase of glycerol (Ghasemlou et al., 2011b). The tensile straight of pure kefiran ranged from11.18±2.2 MPa (Ghasemlou et al., 2011b) to 40.92±7.83 MPa (Piermaria et al., 2009). The plasticized biofilm had a variable tensile straight depending on glycerol concentration 8.85±1.64 at 15% and 5.04±2.1 at 35% (Ghasemlou et al., 2011b).

Elongation at break was 116.69±14.48 % in glycerol enriched kefiran compared to 2.70±0.47% in pure film (Piermaria et al., 2009). Another study observed 39.56± 11.13% in pure kefiran biofilms and as high as 162.45 ± 6.09% in films containing 35% glycerol (Ghasemlou et al., 2011b). Thus, plasticized biofilms have elongation values higher than cellophane (20%) or polystyrene (1%), but much lower than low-density polyethylene (500%) (Ghasemlou et al., 2011b).

The water solubility of kefiran depends on temperature. It is relatively soluble at 25 to 37°C and totally dissolved at 100°C (Ghasemlou et al., 2011b). Adding glycerol increased solubility (Piermaria et al., 2009; Ghasemlou et al., 2011b).

X-ray diffraction patterns revealed that the degree of crystallinity was less than 3.1% and no significant differences were observed among biofilms with different plasticizers (Piermaria et al., 2011).

Kefiran biofilms are extremely permeable to water vapour, which limits its applications (Ghasemlou et al., 2011b). To remedy this disadvantage hydrophobic compounds are often incorporated in biofilms to enhance water barrier properties.

Lactic and acetic acids in kefiran could induce antibacterial and wound healing activity (John and Deeseenthum, 2015). Natural antibiotics and inhibitory substances (lactic acid, acetic acid, bacteriocins, reuterin, hydrogen peroxide) from kefiran have good action over pathogens (Rahimzadeh et al., 2015).

Kefiran biocompatibility was tested in several studies (Huseini et al., 2012; Majid et al., 2016) it decreased blood pressure and cholesterol, also slowed tumour growth. It was used as an oral antigen and conferred systemic immunity by releasing cytokines into the blood (Patel et al., 2012).

Kefiran films find increasing use in wound healing management with satisfactory mechanical properties and good appearance. It is permeable to water vapour fact that limits its application since the control of moisture is a desirable propriety. Hydrophobic compounds are added to remedy this lack. Kefiran has antimicrobial activity because of lactic and acetic acids in its composition.

## BIOFILMS AS WOUND DRESSING MATERIALS

**Bacterial cellulose** was first described as a wound dressing material back in the early 1980s (Sulaeva et al., 2015). The perfect wound dressing material has a unique 3D nanofiber network, with a porous structure and different pore size. The structure can be modified by varying the carbon source, pH, temperature, culture time or production method. The best choice seems to be wound scaffold (Rajwade et al., 2015) because it is a never-dried membrane, with exceptional mechanical strength and physiochemical properties (Mogosanu and Grumezescu, 2014).

Biocellulose is a suitable scaffold material for chronic wounds, being a non-degrading material. It deteriorates very slowly in the body because of its crystallinity and lack of enzymes able to digest the glycosidic bonds (Rajwade et al., 2015).

Bacterial cellulose is usually used as healing dressing for chronic wounds because it reduces pain and accelerates healing. It stimulates granulation and epithelisation processes (Mogosanu and Grumezescu, 2014).

Sprague Dawley (SD) rats with inflicted burn skin injuries were treated for 15 days with biocellulose films and gauze dressing (Kwak et al., 2015). The severity score of skin injury was lower in the BC group throughout the study, the thickness of dermis and epidermis was significantly higher, as well, angiogenesis was pronounced, many new blood vessels were observed and a remarkable level of collagen was expressed in the group treated with BC (Kwak et al., 2015).

Biocellulose can incorporate different active molecules like vitamins, enzymes, antioxidants, drugs, fact that expand its qualities (Mogosanu and Grumezescu, 2014).

**Alginate** is used as wound dressing because of its haemostatic properties in bleeding and burn wounds, being a very absorbent natural fibre (Mogosanu and Grumezescu, 2014). Alginate can absorb body fluids or water up to 20 times its own weight. Hydrophilic alginate biofilms area moist environment, which is perfect for proper wound healing. Films have a porous structure and no adhesive properties, so a second dressing is needed to secure and protect the biofilm (Mogosanu and Grumezescu, 2014). *In vivo* and *in vitro* studies showed that calcium mediates wound healing, by supporting the fibroblast production, and alginate dressings contains calcium ions. Further *in vitro* studies (Lee and Mooney, 2012) revealed that the mobility of fibroblast did not increase. This suggested that calcium ions released from alginate dressings can increase only some cells involved in the process of wound healing (Lee and Mooney, 2012). Other studies concluded that alginate activates human macrophages to generate tumour necrosis factor (TNFa), this induced inflammatory responses - an important step in injury healing (Lee and Mooney, 2012; Mogosanu and Grumezescu, 2014).

Different composite alginate materials were obtained by adding compounds that increased the antimicrobial properties and wound healing properties: zinc, silver, chitosan (Mogosanu and Grumezescu, 2014).The alginate biofilms became firmer by adding guluronic acid and alginate-mannuronate gel become softer and more flexible as they absorbed wound exudates (Boateng et al., 2008).

Alginate can be used as a proper wound dressing because it forms gels and it is highly absorbent in contact with wound exudations. Alginate gel is very hydrophilic (Mogosanu and Grumezescu, 2014), this restrains wound secretions, but also protects the tissue from microbial contamination. Alginate forms a protective gel-like biofilm in contact with the exudates and blood in wounds, it also maintains optimum healing temperature and a favourable moisture, ensuring proper healing (Boateng et al., 2008; Lee and Mooney, 2012). Alginate has gelling properties because of the calcium ions in its composition. Calcium also forms crosslinks with alginic acid polymers that lead to a slow degradation of the biofilm. These properties make alginate an ideal scaffold in wound healing management (Boateng et al., 2008; Hoefer et al., 2015).

**Kefiran** can produce films that have satisfactory mechanical characteristics, but are very permeable to water vapour, fact that limits its application (Ghasemlou et al., 2011a).

Kefiran is reported to have wound healing, antimicrobial, antifungal and antitumoral properties (Ghasemlou et al., 2011a).

Kefir films have great potential in wound healing management increasing epithelisation, scar formation and decreasing inflammation (Nasrabadi and Ebrahimi, 2011). Kefir extracts also hastens wound healing by stimulating the immune system against pathogens (Rahimzadeh et al., 2014).

A remarkable shorter healing time and decreased wound size was noticed in biofilm containing kefir extracts fermented for 96 h, compared to lower fermentation time (Huseini et al., 2012; Rahimzadeh et al., 2015).

Wound healing experiments were made on Wistar rats with induced diabetic cutaneous injuries. The results showed that the group treated with kefiran presented an increased inflammation and an improved accelerated healing process, compared to control groups (Majid et al., 2016). Similar studies were made, on induced thermal wounds. The results showed that the inflammation decreased, scar formation and epithelisation increased significantly (Nasrabadi and Ebrahimi, 2011; Huseini et al., 2012; Rahimzadeh et al., 2014). Other studies on rats with burn injuries revealed that kefiran had better wound healing properties than sulfadiazine treatment or clostebol-neomycin emulsion (John and Deeseenthum, 2015).

Kefiran could be the best choice as a wound dressing material due to its antibacterial properties, ability to accelerate wound healing and reduce inflammation.

## CONCLUSIONS

Biocellulose is synthesized by a variety of bacteria species, in contrast to alginate or kefiran that are produced by *Pseudomonas* or *Azotobacter* and *Lactobacillus* species. BC can be produced at economic scale, depending on the culture method. Alginate yield can be increased by genetic modifications of bacteria

strains, but even so, it does not reach commercial level. Kefiran biofilms are easy to obtain, from common bacteria species, but glycerol must be added to obtain desired properties.

Cellulose biofilm has a 3D nanostructure with porous structure, good tension straight and low elongation at break. Also, it can absorb exudates, it is a never-drying material and it is not biodegradable. Neither cellulose, nor alginate have antibacterial properties, but active agents can be encapsulated and delivered. Alginate absorbs water, it has encapsulation properties, but it can generate immunogenic responses, if it is not highly purified. Kefiran films can be manipulated only if plasticizer is added; this also gives good tensile straight and low elongation at break. Kefiran has good antibacterial properties due to lactic and acetic acids. Both alginate and kefiran are soluble in water and lipids should be added in their structure.

Based on the ease of obtaining, the main properties, biocompatibility and its unique structure, biocellulose should be the best choice as a wound dressing material.

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# CRYSTALLOIDS/COLLOIDS RATIO FOR FLUID RESUSCITATION DURING ANESTHESIA

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***Abstract***

*Crystalloids and colloids are first options for fluid resuscitation. Crystalloids expand extracellular volume, while colloids (synthetic and natural) exert a high oncotic pressure and expand volume by oncotic pressure. Many clinical studies advocate the use of crystalloids versus colloids. Greater fluid volumes are required to meet the same targets with crystalloids than with colloids, but there is a heterogenity among studies. Crystalloids' effect may lead to extracellular fluid accumulation, increased gastrointestinal wall edema, pulmonary edema, especially in patients with cardiac or renal dysfunctions. While low dose colloids preserve hematocrit and coagulation, there is a risk of abnormal hemostasis if high doses of colloids are administered. This study presents researches results regarding crystalloids/colloids ratio for fluid resuscitation during anesthesia.*

***Key words:*** *Crystalloids, colloids, anesthesia.*

## INTRODUCTION

Crystalloids are solutions of electrolytes that can pass freely outside of the vascular space while colloidal macromolecular solutions are kept inside the vascular space for a longer period of time. Fluids are used during anesthesia for maintaining the homeostasis, loss covering or fluid resuscitation. There is no evidence from randomized controlled trials that resuscitation with colloids reduces the risk of death, compared to resuscitation with crystalloids, since the use of hydroxyethyl starch might increase mortality (Perel P. et al. 2013). Since colloids are not associated with an improvement in survival rate (Annane D. et al. 2013), this study aims to present the results after the use of colloids and crystalloids for a group of anesthetized patients.

## MATERIALS AND METHODS

This study compares the results of fluid administration (crystalloids and colloids) during anesthesia for 123 cases (dogs), from the small animal clinic of the Faculty of Veterinary Medicine Bucharest (June 2015-December 2016). Patients with sepsis, renal dysfunction, severe liver disease or coagulopathy were not included in this study. The average rate for fluid therapy during anesthesia for all cases, without any loss of fluids was 3-5 ml/kg/hour of normal saline solution (0.90% NaCl, 308 mOsm/L) (Costea R, 2015). Fluid resuscitation protocol was necessary in 9 anesthesia cases, complicated with hypovolemic shock. During hypovolemic shock a fluid therapy protocol was administered, consisting of a bolus of isotonic crystalloid (20-30 ml/kg, given in 20 minutes) followed by a bolus of colloids (hydroxyethyl starch- Voluven, 6% HES 130 / 0.4.- 5 ml /kg, in 5-10 minutes).
The algorithm was repeated at 10-20 minutes, until the patient was stabilized, within the limits of maximum doses (maximum 80 ml/kg for NaCl 0.9% and 10-20 ml/kg for HES).

## RESULTS AND DISCUSSIONS

Fluid therapy was given continuously during anesthesia, at 3-5 ml/kg/hour (NaCl 0.9%) for all cases. Fluid therapy protocol for hypovolemic shock consisted in a bolus of crystalloids followed by a colloid bolus, respecting doses and dosing interval until the patient is stable (Bansch P, 2015). A bolus of NaCl 0.9%, 20-30 ml/kg was followed by a bolus of 5 ml/kg HES. This protocol was necessary for a number of 19 cases during

anesthesia (15.4% of total cases). In 13/19 cases the protocol was continued with a second administration of crystalloid/ colloid bolus. Five of this cases needed a total of 3 administrations (Table 1). For each case a crystalloid/colloid ratio was calculated and then a group ratio was obtained (Table 2). The protocol was repeated for each case until clinical status was stabilized, taking into consideration the following:

- normal heart rate (60-100 bpm)
- normal perfusion (CRT = 1,5 seconds)
- powerful and constant pulse
- mean arterial blood pressure (>60 mm Hg)
- urinary output (1-2 ml/kg/hour)
- HTC > 25%

Table 1-clinical data

| No. of cases | NaCl 0.9% ml/kg/h mean values | HES 5 ml/kg | Mean time until clinical stabilization |
|---|---|---|---|
| 6 | 18.9 ml | 5 ml | 18.9 minutes |
| 8 | 37.2 ml | 7.8 ml | 48.8 minutes |
| 5 | 57.1 ml | 19.2 ml | 87 minutes |
| 104 | 4.7 ml | - | - |

Table 2-clinical data

| No. of protocols administrated | | Total ml | | NaCl 0.9%/ HES |
|---|---|---|---|---|
| | | NaCl 0.9% | HES | |
| 6 cases | 1 | 1268 | 450 | 2.81 |
| 8 cases | 2 | 3776 | 1397 | 2.7 |
| 5 cases | 3 | 4032.5 | 1420 | 2.83 |
| 19 | | 9076.5 | 3267 | **2.77** |

Fluid volumes required to meet the same clinical targets for resuscitation with crystalloids (37.73 ml/kg mean total amount for 19 cases) exceeded colloids (10.66 ml/kg mean total amount for 19 cases), with an estimated ratio of 2.77 (2.73–2.83).

## CONCLUSIONS

Greater fluid volumes are required to meet the same targets with crystalloids than with colloids with an estimated ratio of 2.77 (2.73–2.83). Colloids were not used frequently compared to crystalloids. Only for 19 cases (15.4% OF total cases) a colloid/ crystalloid protocol was necessary for fluid resuscitation. When a colloid/ crystalloid protocol was established, clinical status was assessed as normal after a mean time of 49.41 minutes. Further studies are needed to establish a correlation between the crystalloids/colloids ratio and the mean time of patient clinical stabilization.

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# CLINICAL AND THERAPEUTIC ASPECTS IN SOME SKIN DISEASES IN DOGS AND CATS

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***Abstract***

*In veterinary medicine, skin diseases, regardless of their etiology, record an increased frequency, mostly because humans are sharing the same lifestyle with their quadrupeds. The purpose of this paper was to present the evolution of skin pathology in dogs and cats, in conjunction with the reactivity of the body towards the pathogenic germs also towards the conditioning or opportunistic pathogens. For the identification and diagnosing of dermatitis, in a private veterinary clinic there were examined 18 animals (12 dogs and 6 cats), which showed skin lesions and intense itching. There were diagnosed 5 cases of dermatitis with parasitic etiology, 7 cases of allergic dermatitis (of which 2 cases showed secondary bacterial otitis), 1 case of autoimmune etiology and 5 cases of infectious etiology. In the case of bacterial dermatitis, good results were obtained with treatments based on antibacterials selected by antibiogram, with antipruritic substances, and topical therapeutic baths. Bacteriological examination on special culture media revealed the presence of mainly Staphylococcus species in the pathological material collected from the skin lesions. In the case of parasitic dermatitis, favourable results were obtained by applying spot-on pipettes containing Moxidectin and by administering oral or otic Ivermectin. In allergies, the treatment was effective after allergen elimination, administration of antipruritic substances, followed by therapeutic baths to ensure decontamination of body surface area.*

***Key words****: dermatitis, food allergy, antibiotics, Staphylococcus, demodicosis.*

## INTRODUCTION

Since ancient times, the pharmacy and therapeutics played a very important role in the evolution of human civilization, meaning about seven thousand years of searching, research and enhancement of products of vegetal, animal and chemical origin, all in the service of life itself. Mankind has always tried to find remedies and with the aid of pharmacology it managed to fight these diseases, thereby restoring the health of animals.

Nowadays, the pharmacy is leading a struggle to adapt to the current pathology and is designed to be up to date with it by finding and establishing specific remedies.

In veterinary medicine, skin diseases, regardless of their etiology, record an increased frequency, mostly because humans are sharing the same lifestyle with their quadrupeds. Thus, a particularly complex issue is the diagnosis and indication of a corresponding pharmacological therapy for these kinds of disorders (Guaguere et al., 2008; Koch et al., 2012).

The purpose of this paper was to present the evolution of skin pathology in dogs and cats, in conjunction with the reactivity of the body towards the pathogenic germs also towards the conditioning or opportunistic pathogens. The diagnostic methods are very important, without them couldn't be possible to develop a topical or systemic treatment (Miller et al., 2013).

## MATERIALS AND METHODS

For the identification and diagnosing of dermatitis, in a private veterinary clinic there were examined 18 animals (12 dogs and 6 cats), which showed skin lesions and intense itching.

During the dermatological examination, the sequence of the work methods included the following:

a) recording the history data related to the disease, treatments previously performed

and their results, along with information about the animal's lifestyle;
b) the dermatological examination that allows the localization and sets the injuries' appearance;
c) examination using Wood lamp;
d) sampling for direct microscopic examination;
e) sampling for inoculation on appropriate medium cultures;
f) presumptive clinical diagnosis (Rhodes and Werner, 2011).

Depending on the case, in order to make a definite diagnosis, the following additional tests were performed:

- lesions' amplification with a magnifier;
- video-otoscopic examination;
- interpreting the trichogram;
- superficial-scrape examination;
- deep-scrape examination;
- otic smears examination, direct smears and smears obtained after the aspiration with a fine needle;
- scotch test.

In order to identify the bacteria and fungi grafted on the skin, they were inoculated on specific culture mediums. Afterwards, using these cultures, the sensitivity of isolated bacterial strains to various antibiotics products was tested.

In case of food allergy, the trial consisted of two steps: using a hypoallergenic diet and afterwards a food rechallenge (Cosgrove et al., 2015). The exclusion diet involved feeding the animal a new food that has not been used before, in order to identify the food components that caused an allergic response (Guaguere et al., 2008).

Depending on the situation, intradermal testing, serological testing, patch testing, and skin biopsy were performed.

## RESULTS AND DISCUSSIONS

The analysis of the results presented in Table 1 shows that in the 18 cases, analysed for finding the etiologic diagnosis, there were diagnosed 5 cases of dermatitis with parasitic etiology, 7 cases of allergic dermatitis (of which 2 cases showed secondary bacterial otitis), 1 case of autoimmune etiology and 5 cases of infectious etiology (Figure 1).

In addition to primary typical infections, in 12 cases the bacterial infections were secondary.

Table 1. Patients included in study: species, breed, age, sex, diagnosis, healing time

| Case no. | Species Breed | Age Sex | Diagnosis | | Healing time | |
|---|---|---|---|---|---|---|
| | | | Primary | Secondary | Primary | Secondary |
| 1 | Feline/European | 11 years, (F) | Food allergy | Superficial pyoderma | 4 months | 2 months |
| 2 | Feline/European | 4 years, (F) | Food allergy | Superficial pyoderma | 4 months | 2 months |
| 3 | Canine/Half-breed | 4.5 years, (M) | Discoid lupus erythematosus | Superficial pyoderma | - | 2 weeks |
| 4 | Canine/Labrador | 10 months, (F) | Generalized demodicosis | Deep pyoderma | 4 months | 1.5 months |
| 5 | Canine/Westie | 2 years, (M) | Atopic dermatitis | Superficial pyoderma Otitis | - | 1 month |
| 6 | Canine/Amstaff | 10 months, (M) | Atopic dermatitis | Superficial pyoderma | - | 3 weeks |
| 7 | Canine/Bichon | 10 months, (F) | Generalized demodicosis | Superficial pyoderma | 10 months | 3 months |
| 8 | Canine/Chow -Chow | 4 years, (M) | Sarcoptic mange | Superficial pyoderma | 7 months | 2 months |
| 9 | Canine/Akita Inu | 4 years, (M) | Superficial pyoderma | - | 2 weeks | - |
| 10 | Canine/Westie | 5 years, (M) | Generalized demodicosis | Deep pyoderma | 6 months | 2 months |
| 11 | Feline/European | 7 years, (F) | Deep pyoderma | - | 1 month | - |
| 12 | Canine/Bucovina Shepherd | 5 years, (F) | Deep pyoderma | Lick granuloma | 2 months | 1.5 months |
| 13 | Canine/Half-breed | 7 years, (F) | Deep pyoderma | Lick granuloma | 1 month | 1.5 months |
| 14 | Feline/European | 10 months, (F) | Ear mites | - | 2 weeks | - |
| 15 | Feline/European | 6 years, (F) | Flea saliva allergy | Superficial pyoderma | 1 month | 2 weeks |
| 16 | Feline/Persian | 11 years, (M) | Food allergy | Superficial pyoderma | 4 months | 2 months |
| 17 | Canine/German Shepherd | 3 years, (M) | Deep pyoderma | - | 1 month | - |
| 18 | Canine/Mops | 4.5 years, (M) | Food allergy | Deep pyoderma Otitis | 4 months | 2 months 1 month |

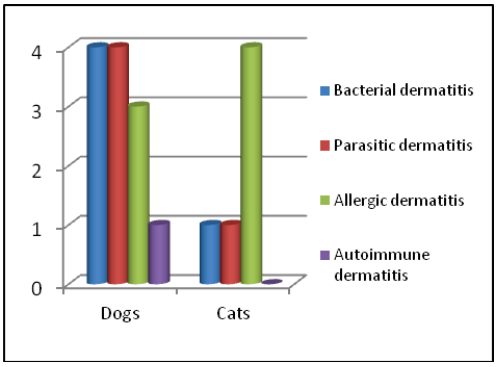


Figure 1. Types of dermatitis in dogs and cats included in the study

Antipruritic therapy was administered in two ways: for a few days to improve the quality of the patients' life or during the whole period of hypoallergenic diet (in case of patients with food allergies), excepting the last 14 days, to see if the remission of itching was present (Table 2). In the cases with atopic dermatitis, the antipruritic therapy can be applied lifelong, at a minimum dose.

The antibiotic therapy in dermatology is administered for at least 14 days in an infected surface, sometimes reaching 2-3 months in a deep infection. It is necessary to continue the antibiotic treatment for another week after healing of the lesions.

In the case of food allergies, good results were observed after food diets with cooked food which has not been used before, this type of diet being more appropriate than the commercial food because it has no additives and the risk of contamination is low.

Atopic dermatitis has been studied in 2 dogs with different breeds and ages. In order to establish the diagnosis, the animals were subjected to intradermal testing after eliminating the suspicion of food allergy.

Table 2. Treatments administered to patients involved in the study

| Case no. | Antibiotherapy | Antipruritic | Antiparasitic | Topical | Diet | Others |
|---|---|---|---|---|---|---|
| 1 | Cefovecin | Prednisone | Moxidectin | Chlorhexidine | Cooked food | - |
| 2 | - | Prednisone | Moxidectin | Chlorhexidine | Cooked food | Hydrocortisone aceponate |
| 3 | Marbofloxacin | Methylprednisolone | - | Sunscreen cream | - | Azathioprine (Imuran) |
| 4 | Cefalexine | - | Moxidectin Ivermectin | Benzoyl peroxide | - | Allerderm |
| 5 | Pradofloxacin | Oclacitinib (Apoquel) | Spinosad (Comfortis) | Chlorhexidine | Cooked food | Itraconazole Vetoskin Allerderm Immunotherapy |
| 6 | Cefalexine | Oclacitinib | Flumethrin/ Imidacloprid | Chlorhexidine | RC Anallergenic | Immunotherapy |
| 7 | Amoxicillin and clavulanic acid | - | Moxidectin Ivermectin | Benzoyl peroxide | - | Vetoskin |
| 8 | Cefalexine | - | Moxidectin | Chlorhexidine | - | Vetoskin |
| 9 | Cefalexine | Methylprednisolone | Fipronil | Chlorhexidine | Purina HA | - |
| 10 | Ciprofloxacin | - | Moxidectin Ivermectin | Benzoyl peroxide Betadine Mibazon | - | Vetoskin |
| 11 | Amoxicillin and clavulanic acid | - | Moxidectin | Chlorhexidine | Purina HA | - |
| 12 | Ciprofloxacin | - | - | Chlorhexidine Betadine Mibazon | - | - |
| 13 | Cefalexine | - | - | Chlorhexidine Betadine Mibazon | - | - |
| 14 | - | - | Ivermectin | EpiOtic | - | - |
| 15 | - | Prednisone | Moxidectin | Chlorhexidine | Cooked food | |
| 16 | Pradofloxacin | Prednisone | Moxidectin | Chlorhexidine | Cooked food | Allerderm |
| 17 | Cefalexine | Methylprednisolone | - | Chlorhexidine | - | - |
| 18 | Marbofloxacin | Oclacitinib | Spinosad | Chlorhexidine EpiOtic EasOtic | Cooked food | Allerderm |

Allergy to flea saliva is easy to treat, external monthly disinfestation with antiparasitic substances being enough, this way the suspicion of a new reinfestation can be eliminated (Bowman, 2014). Pruritus was partially controlled by the use of antipruritic substances, especially at the beginning of the disease.

Of the 5 cases of parasitic etiology, three dogs showed generalized demodecosis with intercurrent pyoderma. Demodecosis particularly affects dogs between of 3 and 8 month sof age, but it was also found in a dog of 5 years age (Mueller et al., 2012). The treatment of generalized canine demodicosis lasted between 4 and 10 months. Good results were obtained using moxidectin spot-on pipettes that had acted as induction followed by administration of ivermectin till the second negative control. The treatment was completed with therapeutic baths based on benzoyl peroxide which had the role of follicular cleaning and bacterial decontamination.

When treating sarcoptic mange, good results were obtained by applying spot-on pipettes based on moxidectin and the bacterial skin infection was treated with systemic antibiotic therapy, topical antibiotic therapy and therapeutic baths with chlorhexidine.

The cat that had ear mites was treated with otic ivermectin. Good results were obtained after two weeks of treatment, and to prevent bacterial or fungal infections, antiseptic solutions were applied in the ears.

In the case of autoimmune dermatitis, lesions were strictly located on the face and have worsened during the summer due to UV radiation (Day, 2012).

From the skin lesions, *Staphylococcus* species were mainly isolated, but also *Escherichia coli* and *Streptococcus* (Table 3).

Table 3. The results of bacteriological/fungal exams and the sensitivity to antibacterials

| Case no. | Bacterial culture | Antibiogram (sensitivity) | Fungal culture |
|---|---|---|---|
| 4 | *Staphylococcus spp* | Cefalexine | - |
| 5 | *Staphylococcus spp* | Pradofloxacin | |
| 8 | *Staphylococcus spp* | Cefalexine | - |
| 9 | *Staphylococcus spp* | Cefalexine | - |
| 10 | *Staphylococcus spp; E. coli* | Ciprofloxacin | Negative |
| 11 | - | - | Negative |
| 12 | γ and β-hemolytic *Streptococcus* | Ciprofloxacin | - |
| 13 | *Staphylococcus spp* | Cefalexine | - |
| 16 | *Staphylococcus spp* | Pradofloxacin | - |
| 18 | *Staphylococcus spp* | Marbofloxacin | - |

## CONCLUSIONS

Of the 18 cases of dogs and cats diagnosed with dermatitis, 38.9% had allergic etiology, 27.8% had bacterial etiology, 27.8% had parasitic etiology and 5.5% had autoimmune etiology.

In the case of bacterial dermatitis, good results were obtained with treatments based on antibacterials selected by antibiogram, with antipruritic substances, and topical therapeutic baths. Bacteriological examination on special culture media revealed the presence of mainly *Staphylococcus* species in the pathological material collected from the skin lesions.

Parasitological examination revealed the presence of Demodex, Sarcopes, and Otodectes parasites. In the case of parasitic dermatitis, favourable results were obtained by applying spot-on pipettes containing Moxidectin and by administering oral or otic Ivermectin.

In allergies, the treatment was effective after allergen elimination, administration of antipruritic substances, followed by therapeutic baths to ensure decontamination of body surface area.

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# HYDROCEPHALUS IN FEMALE FRENCH BULLDOG CASE PRESENTATION

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***Abstract***

*The patient named Kim, a female French Bulldog was presented to the doctor since the age of two months. She attended a deworming and vaccination complete scheme. Over the time, she went through an episode of paraparesis which led to the diagnosis of lesions in the T3-L3 column section (resulting from RX). After a few months, neurological signs have emerged from the forebrain. After performing the clinical and neurological examination, an investigation that would exclude some conditions (differential diagnosis based on the acronym "VITAMIND"), a brain MRI was performed. The diagnosis was hydrocephalus. As a result, this case brings together two anomalies: one at the brain level and the other from the spine. Each new sign the owner described, was completed every time with clinical and neurological examination witch led to a correct neurological diagnosis. The treatment was initiated immediately and was adjusted according to patient response to one of the prescription medications (acetazolamide).*

***Key words****: neurologic, hydrocephalus, abnormality, spine, acetazolamide.*

## INTRODUCTION

Hydrocephalus is an active distension of the ventricular system of the brain resulting from an inadequate passage of CSF from its point of production within the ventricles to its point of absorption in the systemic circulation.

Loss of brain parenchyma may result in a secondary increase in ventricle size, which has been termed as compensatory hydrocephalus or hydrocephalus ex vacuo.

A congenital predisposition exists in many miniature breed dogs, Bulldogs and Boston Terriers.

The condition may be congenital due to obstruction of ventricular drainage (often at the level of the mesencephalic aqueduct) or decreased absorption of CSF due to dysfunction of the arachnoid villi, or it may be the result of secondary obstruction due to acquired disease (e.g. neoplasia, infection or inflammation).

Hydrocephalus may be secondary to CSF overproduction (e.g. choroid plexus tumor [rarely]) or increased viscosity of CSF due to elevated CSF protein content seen with some tumors and the 'dry-form' of FIP in cats (Lahunta and Glass, 2009).

Hydrocephalus is described here because it involves the accumulation of excessive amounts of CSF in the brain or cranial cavity. In fact, the correct definition of hydrocephalus is any incase in the volume of CSF, which means that it is not always related to the cause of any neurologic signs.

A number of terms have been used over the years in reference to hydrocephalus, with varying usefulness (Platt and Garosi, 2012):

- internal hydrocephalus is a ventricular distention with CSF accumulation.
- external hydrocephalus is a subarachnoid space distension with CSF accumulation. This is also referred to as hydrocephalus ex vacuo.
- non-communicating hydrocephalus is a ventricular dilation due to an intraventricular obstruction of CSF flow preventing the communication between the ventricular system and the subarachnoid space.
- communicating hydrocephalus is a ventricular dilation secondary to an extraventricular obstruction of CSF flow.

- normotensive hydrocephalus is associated with an increase in CSF pressure.
- hypertensive hydrocephalus is associated with an increase in CSF pressure.
- the two major categories of hydrocephalus are compensatory and obstructive.

Hydrocephalus results in diffuse cerebral and/or brainstem signs due to cortical compression and elevated ICP. Most commonly, animals have altered mentation, inappropriate behavior, cortical blindness and seizures.

A ventrolateral strabismus is common. Hydrocephalus may be asymptomatic in milder cases. Congenitally affected animals often have a skull deformity (dome-shaped) and persistent fontanelles (Platt and Garosi, 2012).

Although developmental obstructive hydrocephalus occurs sporadically in all breeds of dogs, there is a much higher incidence in the toy and brachycephalic breeds, especially in the Chihuahua, Pekingese, Pug, Boston terrier, Yorkshire terrier, Pomeranian and English or French Bulldog.

This disorder is uncommon in cats. Despite the presumed fetal genesis of the obstruction, clinical signs may not be evident at birth.

Most will be observed by 3 months of age, some between 3 and 12 months, and rarely beyond 12 months. Some dogs exhibit no clinical signs despite markedly enlarged lateral ventricles with significant cerebral atrophy.

This suggests that the clinical signs may be related to the level of CSF pressure, which can be quite variable in these dogs (Lahunta and Glass, 2009).

The most common clinical signs observed are prosencephalic in origin because of the severe expansion of the lateral ventricles, with compromise of the cerebral tissue and compression of the diencephalon (Lahunta and Glass, 2009).

## MATERIALS AND METHODS

The patient was presented to the doctor at the Veterinary Faculty on April 5th 2015. The following investigations were performed:

- anamnesis (history);
- clinical and neurological examination;
- cardiologic exam;
- ophthalmological examination;
- biochemical examination;
- NH3 and bile acids;
- hematological examination;
- abdominal ultrasound;
- 4DX Test;
- performed an X-ray on the spinal column T3-L3 (Figure 1)
- toxoplasmosis test – IgG and IgM (Figure 2).
- cerebral MRI.

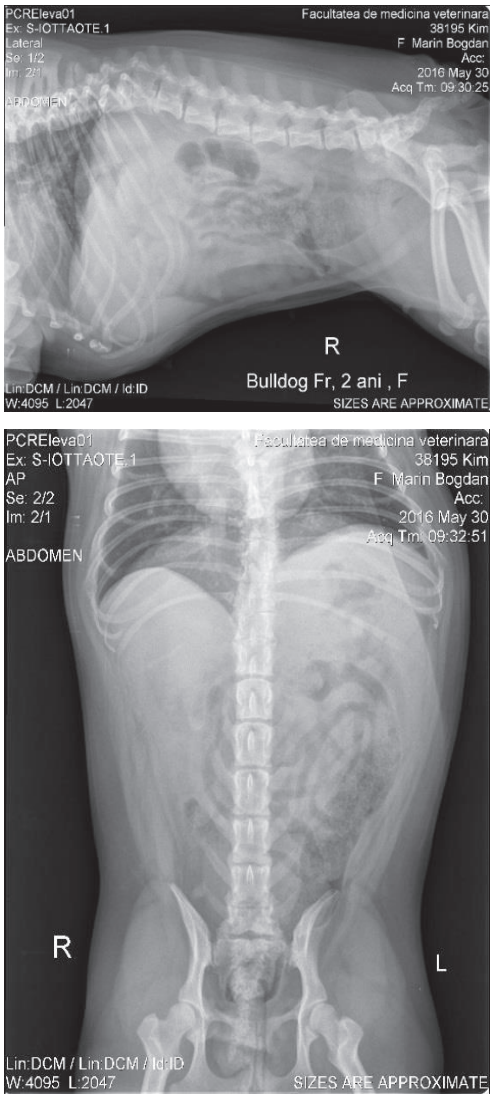
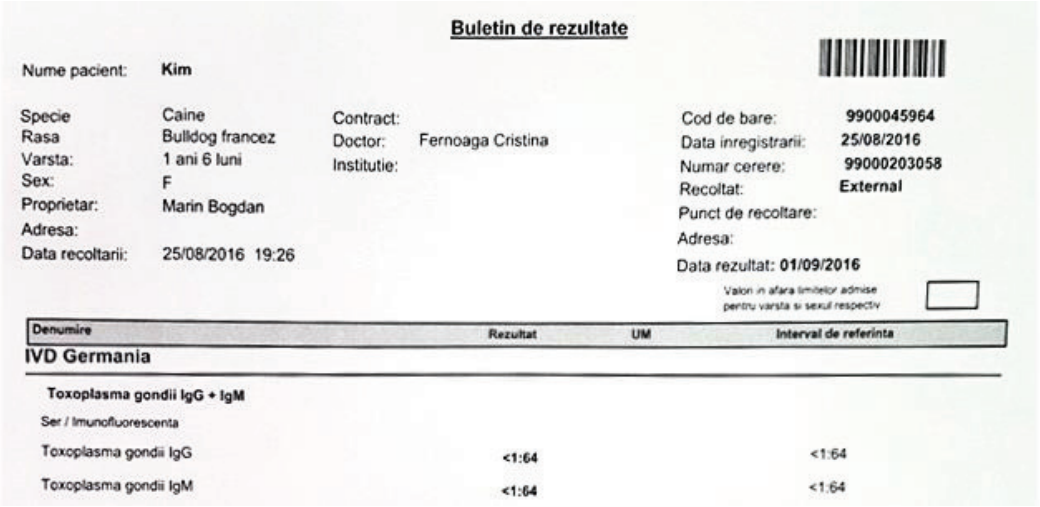



**Figure 1**. T8-T10-T13 Hemivertebras. T7-T9 united spinous processes. Spinous processes sclerosis at T11-T10. Deposition of new bone on the surface of the ventral vertebral bodies at T12-L2 level. Transitional vertebra - S1 (S1 vertebra lombarization), (dr. Nicolae Tudor permission)



**Buletin de rezultate**

Nume pacient: **Kim**

| | | | | | |
|---|---|---|---|---|---|
| Specie | Caine | Contract: | | Cod de bare: | 9900045964 |
| Rasa | Bulldog francez | Doctor: | Fernoaga Cristina | Data inregistrarii: | 25/08/2016 |
| Varsta: | 1 ani 6 luni | Institutie: | | Numar cerere: | 99000203058 |
| Sex: | F | | | Recoltat: | External |
| Proprietar: | Marin Bogdan | | | Punct de recoltare: | |
| Adresa: | | | | Adresa: | |
| Data recoltarii: | 25/08/2016 19:26 | | | Data rezultat: | 01/09/2016 |

Valori in afara limitelor admise pentru varsta si sexul respectiv

| Denumire | Rezultat | UM | Interval de referinta |
|---|---|---|---|
| **IVD Germania** | | | |
| **Toxoplasma gondii IgG + IgM** | | | |
| Ser / Imunofluorescenta | | | |
| Toxoplasma gondii IgG | <1:64 | | <1:64 |
| Toxoplasma gondii IgM | <1:64 | | <1:64 |

**Figure 2**. Toxoplasmosis test- negative IgG and negative Igm

## RESULTS AND DISCUSSIONS

In the Medical Clinic there has been presented a French Bulldog female of two and a half years old, named Kim. She was adopted by her owners when she was 2 months old and she followed the vaccination program according to age, being dewormed internally at 4 months and externally every month. She grew and developed normally.

She received good quality food and her playing partner was a cat. At 6 months she had a 2 week period when she experienced a secretion in the right ear (unilateral otitis), but it was treated (with an ear drops solution containing an anti-inflammatory and an antibiotic) and got cured.

The first signs of abnormal manifestations appeared at the age of 8 months, as follows:

- heavy walking on back legs (paraparesis);
- modified proprioception on both hind limbs: delay on flexion-extension test;
- normal spinal reflexes;
- hind limbs show contracture and spinal ataxia;
- light kyphosis;
- doesn't climb stairs up or down.

Neurological diagnosis: affection on the spinal cord T3-L3.

The administered treatment was Prednisolon 0,8 ml; then 10 days she underwent treatment with Aflutop 1 ml/day, IM.

After 10 days she showed a complete recovery. Maintenance treatment has been done with Arthrovet HA 1cps / day, MSM / 12 h , K9 Complete Motion 1 / day and Ganoderma for 3 months.

A month after begining the treatment, she showed heavy walking, no pain when touching the spine and no proprioception changes. The treatment continued for another 3 months.

She had not shown neurological manifestations for 4 months. One month after stopping the previous treatment, in March 2016, the owner noticed that she had begun insistently licking on both forelimbs. She did not show any lesions or alopecia.

She resumed treatment with Arthrovet HA and K9 and added Pentoxiphiline at 50 mg / day to improve peripheral circulation.

Also in March 2016, she was sterilized with inhalatory anesthesia. She did not show any problems during surgery and the recovery was fast.

Although she followed a treatment with Atopivet 1 cps / day for 30 days, she followed a diet with z/d Hill's and had general baths with chlorhexidine 2 times / week for 4 weeks. Until July 2016 the licking persisted.

Since July 2016 changes in behavior have appeared. The owner described the changes as: the " crisis" began in June, at first very violent but with short duration. The crisis was represented by a very loud growl, barks and

heavy breathing. The crises were started throughout the day but at night intensified. The crisis worsened and in early September they were continuously. If at first a crisis was between 5 and 15 minutes, at the beginning of September they sometimes lasted all night. The only days in witch the Bulldog did not have any crisis were those in witch she had been subjected to anesthesia for MRI and after she began a treatment with prednisone and furosemide after seizures came back again with intensity increasingly higher. and it noted that there is almost the same hour when crises arise are 19-20 pm, 2-3 am, 6-7 am. If she is kept locked in a room one day she has no seizure and that if we found in our presence the ”crisis” is stronger.

As a way of reaction, they are combined: the shaken powerful head, lets her head down on the floor, stretches her neck up and aside, has uncontrolled movements as if she catches a fly, and especially very violent ”crisis”.

Now it seems she has no reaction to treatment as the crises are continuing in the same characteristics.”

When this pacient was examined, the result of the neurological exam was:

- moments of nervousness;
- looking in an exact spot (fixed locations);
- catching “flies”;
- sensation of “pinching” and jumping from a spot even when calm.

Focal epileptiform crises were suspected.

These had taken place only inside the house, outside having normal behavior. It was established a treatment with Gabapentin 10 mg/kg 2 times/day.

During the check-up from August 2016, the owner informed us that for the previous signes, she was supporting herself with her muzzle on the ground.

She continued the treatment with Gabapentin and performed a cerebral MRI in September 2016.

The results were:

- bilateral ventriculomegaly.
- suspection of a slight enlargement of the mesencephalic aqueduct. No loading with contrast.

Conclusions: hydrocephalus, most probably congenital (Figure 3).

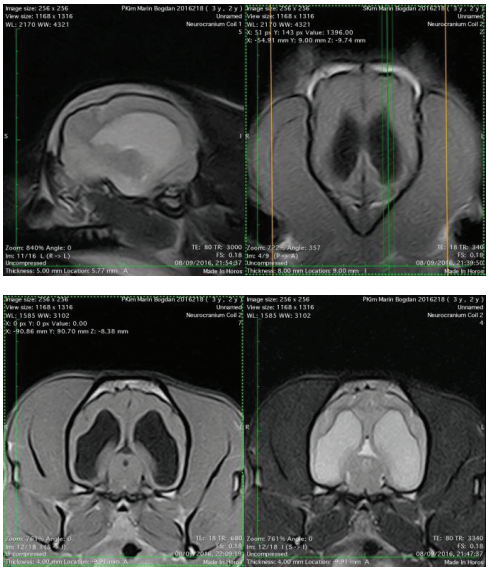


**Figure 3**. Bilateral ventriculomegaly hydrocephalus (dr. Florin Grosu permission)

The established treatment:

- Furosemid 40 mg 1 mg/kg /day;
- Prednisone 1 mg/kg/day;
- Omeprazole 10 mg/day (1mg/kg/day);
- Aspacardin ¼ cpr/day;
- Diazepam rectally when needed;
- Acetazolamide 10 mg/kg x 2 times/day.

After two weeks she came back for a check-up. Administering acetazolamide was stopped because the owner told that Kim was becoming very violent (according to the prospectus) after this medicine. Because the ”crises” had become very frequent and lengthy (approximately 30 minutes) Levetiracetam at 10 mg / kg / 12 h , orally, was added to the treatment.

There have been added to the treatment:

- Phospholipids and ornytine;
- Omega-3 500 mg / day.

The ”crises” have lost in intensity over time, but in December she was having ”crises” between 6 and 9 pm, especially before having administered Diazepam rectally.

If Diazepam was not administered rectally, the crises would become more frequent and strong, therefore Diazepam was administered rectally 1-2 mg / day in the afternoon.

Fenobarbital at 4 mg / kg / day was added in order to remove Diazepam from the treatment.

Ever since the outside temperature was low, Kim has felled much better. She still had crises

but were light and weak in intensity. She sometimes had up to 4 crises per day but was joyful, present and not aggressive.
For the neurologic examination from January 2017, changes were only noticed on cranial level: delayed reaction “of attention” (menace) at both eyes, eyelid reflex incomplete in both eyes.

## CONCLUSIONS

The medical history is very important and the data taken from the owner helped to establish a correct diagnosis.
The neurological examination was performed for each condition separately and was resumed on every recontrol. In the neurological observation sheet were noted the results of every done examination.
In this case, Kim had two different neurological diseases: one located in the brain (cerebral hemispheres) and another in the spine.
For the differential diagnosis the acronym "VITAMIND" was used and thus the anomaly as the cause of the neurological signs in this case was chosen.
To obtain a correct diagnosis, a MRI was performed, which confirmed the presence of hydrocephalus.
The treatment for hydrocephalus was established according to the literature, but due to a reaction of the particular patient when taking acetazolamide, the treatment was adapted.

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# ANESTHESIA DURING GESTATION AND ITS EFFECTS ON NEWBORN VIABILITY

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***Abstract***:

*During the gestation period, canine females go through physiological changes which can influence the way the anesthetics are absorbed, metabolized and excreted. Furthermore, the pharmacokinetics of the drugs vary from mother to newborn. The drugs administered will pass through the placental barrier and carry across from dam to fetuses. For this reason, choosing the right anesthetic protocol for cesarean section can represent a challenge for a doctor, who has to keep in mind the well-being of both the dam and the fetuses. While choosing the anesthetic protocol to be used, the anesthetist will have to consider all of the above criteria, seeking to minimize the cardiological, vascular and neurological depression of the fetuses. The purpose of this paper is to discuss the different anesthetic protocols which can be used, and to assess the benefits, as well of the disadvantages that each of the available medications and methods can present. The options of anesthetic procedures being considered during the caesarean section in canines are represented by general anesthesia or local anesthesia combined with general anesthesia. Of equal importance are the preoperative assessment and the potential recovery time of the mother, which can influence the immediate maternal care given to the newborns.*

***Key words***: *anesthesia, dog, effects, gestation, viability.*

## INTRODUCTION

During pregnancy, all the surgical non-emergency interventions that need an anesthesia should be postponed. Both elective and emergency cesarean sections are commonly used in preventing or treating dystocia (Ryan, Wagner, 2006). The major goal of anesthesia during cesarean section (CS) is to minimize the effects of anesthetic drugs in order to reduce fetal respiratory, cardiovascular and neurological depression as well as assuring the delivery of live, vigorous puppies. It is equally important to provide adequate analgesia to the dam and prevent anesthesia-related complications such as hypotension, hypoventilation, hypoxemia, hemorrhage and hypothermia, which will increase morbidity and mortality in both mother and puppies (Paddleford, 1992; Kraus, 2016). The risk of complications in pregnant females is also increased by the physiological changes the mother goes through. (Traas, 2008; Vullo et al., 2014). Some of the changes that occur during pregnancy, in cardiovascular function, respiration and in other systems may affect anesthesia. Understanding them may aid in planning a safe anesthetic protocol for pregnant patients (Kushnir, Epstein, 2012). Another criterion is the differing pharmacokinetics and pharmacodynamics between the fetus and dam. (Ryan, Wagner, 2006). The selection of an anesthetic protocol should be optimized for both dam and fetus so that there is minimal neurologic and cardiorespiratory depression (Luna SP, 2004; Wiebe, Howard, 2009). The risk of anesthesia is represented by the fact that the drugs used cross the placenta and the blood-brain barrier, leading to a variable extent of newborn depression (Pascoe, 2001; Raffe, Carpenter, 2007; Clarke et al. 2014; Vullo et al., 2014). Therefore, several studies have been performed to determine the optimal anesthetic protocol during caesarean sections.

## MATERNAL PHYSIOLOGIC CHANGES

In mothers, the changes during pregnancy are hormonal, electrolyte and electrocardiographic, which are physiological and can be related to eutocia or can predispose to dystocia (Simões et al., 2016). The nature of cardiovascular changes, include increased blood volume, relative anemia, increased cardiac output,

increased cardiac work, and decreased peripheral vascular resistance. Other changes are respiratory, represented by decreased FRC, decreased total lung volume, increased minute ventilation and oxygen consumption, and decreased PaCO2 (Ryan, Wagner, 2006; Lemke, 2007; Branson, 2007). Dams and fetuses have an increased metabolic demand, leading to a maternal blood volume increases of approximately 40%. Plasma volume increase is proportionally greater than the increase in erythrocytes, leading to hemodilution and relative anemia (Seymour, 1999; Pascoe, Moon, 2001). A greater number of fetuses will translate into increased anemia (Kaneko et al., 1993). Pregnant females have elevated gastric acidity. Also, the gravid uterus causes increased intra-abdominal pressure, leading to reduced gastric and lower esophageal sphincter tone. This, in turn, will make regurgitation during anesthesia and possible aspiration or esophagitis more likely (Pascoe, Moon, 2001). Pregnancy induces minor alterations in hepatic function. Plasma protein concentration decreases slightly, but total plasma protein is increased because of the increase in blood volume (Tranquilli et al., 2013). Bilirubin concentration remains the same, while serum enzyme concentrations and sulfobromophthalein sodium retention are increased. Plasma cholinesterase concentration decreases. Despite these alterations, overall liver function is generally well maintained (Ralston, Shnider, 1978). Renal plasma flow and glomerular filtration rate progressively increase, paralleling the changes in blood volume and cardiac output. Due to increases in renal clearances, blood, urea and creatinine levels are all lower than in non-pregnant animals (Tranquilli et al., 2013). The physiological changes that occur during pregnancy significantly alter the pharmacology of most drugs, such as uptake, distribution and disposition of anesthetic agents and must be considered carefully when selecting anesthetics (Raffe, Carpenter, 2007; Wiebe, Howard, 2009).

## PLACENTAL TRANSFER OF DRUG

The primary role of the placenta is to act as an interface between dam and fetus. Canines have an endotheliochorial and zonary type of placenta (Miglino et al., 2006; Furukawa et al., 2014). Most anesthetics cross the placenta and the blood-brain barrier of the fetus. The permeability of the placenta differs depending on type of placenta and the physicochemical properties of the drugs. The endotheliochorial placenta present in canines allows close maternal-fetal contact which facilitates the passive passage of drugs. Placental transfer of drugs can occur by several mechanisms; by far, the most important is simple diffusion. Diffusion across the placenta is determined by molecular weight, the degree to which the drug is bound to maternal plasma proteins, lipid solubility, and degree of ionization (Tranquilli et al., 2013; Wiebe , Howard, 2009). Drugs with high lipid solubility are permeable, due to the placental barrier being considered to be a lipoprotein. (Mathews, 2005). Once drugs have crossed the placental barrier, they pass through the fetal liver, then on to the fetal vena cava via the ductus venosus, where there is a dilutional effect occurring from blood from the caudal portion of the body. This provides some degree of protection to the fetus to high concentrations of drug. Some drugs such as propofol are metabolized and cleared rapidly from the maternal blood, limiting exposure to the fetu whilst others like neuromuscular blocking agents and glycopyrrolate (anticholinergic) do not cross the placenta (Proakis, 1978; Dugdale, 2010).

## PERIOPERATIVE MANAGEMENT

In order to minimize excitement, the dam must be handled in a calm and quiet manner, thus avoiding the release of catecholamine which leads to decreased blood flow to the uterus and fetus (Gilroy et al., 1986). After a quick but careful examination (measuring body weight, cardiac and respiratory frequencies, pulse, mucosae color, temperature), an intravenous catheter should be placed to allow fluid therapy and administration of the drug during the procedure and the recovery period (Smith, 2012). Any abnormalities in electrolyte, acid-base, calcium or glucose levels will need to be corrected prior to the start of the surgery (Biddle, Macintire, 2000; Pascoe, Moon, 2001; Kushnir, Epstein, 2012). The rate of fluids

administered can be between 5-10 ml/kg/hour and it can be increased when the gravid uterus is manipulated or the dam requires (hemorrhage, hypotension, low pulse) (Dugdale, 2010). Before induction, oxygen should be given by mask to prevent arterial desaturation if apnea occurs (Wiebe, Howard, 2009). Regarding medication that can be administered before the premedication of anesthesia, Seymour (1999), recommends a dose of metoclopramide (0.2–0.4 mg/kg i.v. or i.m), cimetidine or anticholinergics due to the risk of regurgitation and vomit. The clinical parameters of the dam need to be assessed during the entire procedure. (Simoes et al., 2016).

## TYPES OF ANESTHESIA

### Loco-regional techniques

The disadvantages of local anesthesia are represented by the larger amounts of anesthetic agents used, which are absorbed and can create fetal depression, as well as the fact that muscle relaxation and analgesia are less profound or uniform (Tranquilli et al., 2013). Local anesthetics are divided in two groups represented by esters (procaine and tetracaine) and amides (lidocaine, mepivacaine, bupivacaine and ropivacaine) (Gaynor, Muir, 2009). The duration of ester local anesthetics is prolonged in pregnant patients due to plasma cholinesterase activity being reduced. Greater spread and depth of epidural or spinal local anesthetics have also been reported in pregnant patients. Therefore, it is generally recommended that a smaller dose of spinal or epidural local anesthetics is used (Gaynor, Muir, 2009). The use of local anesthesia reduces the required dose of general anesthetics. When the plasma concentration of the drug increases, local anesthetics produce a predictable pattern of neurological excitement and then depression that may lead to apnea and cardiovascular collapse (Gaynor, Muir, 2009). Using epidural anesthesia is recommended because it doesn't have a negative effect on the puppies, while at the same time allowing the mother to remain awake. This in turn will mean that the mother can take care of the newborns immediately after the procedure (Scarda, Tranquilli, 2007). Another advantage of an epidural is that it can be used without general anesthesia resulting in a reduced effect on neonatal vigor. However, the lack of intubation in the dam leads to an increased risk of hypoxemia, regurgitation, and aspiration pneumonia (Luna et al., 2004; Ryan, Wagner 2006). Epidural anesthesia is a simple, safe and effective way to administer anesthetics and analgesic drugs for caudal abdominal surgeries in canines (Pascal et al., 2015). Lidocaine 2% without epinephrine is the most common local anesthetic administered, and when given as an epidural, neonatal blood concentrations should be minor. Studies have shown that the use of epidural anesthesia with lidocaine, whether accompanied or not by an opioid, has resulted in vigorous newborns. Disadvantages of epidural anesthesia include failure of satisfactory analgesia at the cranial end of the midline incision, movement of the bitch in response to intra-abdominal manipulation and mesenteric traction (Clarke et al., 2014). Hypotension, bradycardia, hypothermia, cord laceration, spontaneous movements of the head and front limbs, and difficulty in epidural needle placement can also occur. In case of respiratory problems, it may not be possible to intubate the mother without the use of a general anesthetic (Wiebe, Howard, 2009).

Compared to a non-pregnant bitch, a 25–35% reduction in amount of anesthetic administered is required (Pascoe, Moon 2001). Epidural anesthesia is preferred to local infiltration or field block techniques.

### General anesthesia

Premedication of the dam can have adverse effects on the fetuses. This means that short-acting drugs are preferred. (Ryan, Wagner, 2006). All opioids cross the placenta and can cause significant central nervous system and respiratory depression in neonates. Elimination of opioids can take up to 2 to 6 days. Buprenorphine is not recommended due to the lack of an antagonizing agent. Recent studies investigating the transplacental transfer and metabolism of buprenorphine in the isolated placenta have shown that the use of a single 'dose' of buprenorphine has had a limited rate of transfer (Nanovskaya, 2002). Butorphanol can be administered during surgery to achieve mild to moderate levels of sedation and post-

surgery analgesia. If significant neurological and respiratory depression occur, naloxone (0.04 mg/kg SC) may be administered as an antagonizing agent (Murrell, 2007). The use of a low dose of morphine (0.1-0.2 mg/kg) or meperidine (1-2 mg/kg) as premedication, may provoke vomiting and ensure gastric emptying. Tranquilizers, sedatives, and analgesics should not be used until the newborns are delivered (Wiebe, Howard, 2009). Premedication with anticholinergic drugs can potentially cause tachyarrhythmia and the production of gastric stasis promoting reflux of gastric contents. For this reason, it has been regarded as a controversial drug (Hall et al., 2001). Glycopyrrolate is not recommended since very little will cross the placental barrier to prevent bradycardia in the fetuses (Ryan, Wagner, 2006). However, anticholinergics have the advantage of reducing salivation and unavoidable excessive vagal tone with uterine traction. (Hall et al., 2001). Phenothiazines are contraindicated in pregnancy because of significant hypotension and reduced blood flow, as well as severe fetal neurological depression which has been observed after premedication with acepromazine (Valerie, 2009). Although some studies have stated that ketamine can be used as an anesthetic, without having any teratogenic or other adverse fetal effects (Briggs, 1998), protocols that included the use of ketamine or xylazine, methoxyflurane were associated with increased risk of puppy deaths. Therefore it's advised that they be avoided for cesarean section (Navarro, Friedman, 1975; Moon , Erb, 2002). Diazepam has been associated with muscle weakness and decreased ability to nurse or maintain body heat in human babies for hours after delivery. Clinical impression is that diazepam administration to the dam has the same effect in puppies and, therefore, should be avoided (Clarke et al., 2014). Benzodiazepines can be used immediately prior to induction and can be counteracted in the fetuses with flumazenil (0.1 mg/kg i.v.) (Ryan, Wagner, 2006). Induction of anesthesia must be tailored to each patient. Fentanyl–droperidol or barbiturate have both been used in the early years as the only injectable induction agents. While fentanyl is still considered to be a useful option, thiopental has been replaced with propofol or alfaxalone in order to produce more vigorous newborns. There have been a lot of studies comparing the effects of alfaxalone and propofol on induction of anesthesia (Ambros, 2008; Metcalfe et al., 2014; Muir, 2008; Doebeli et al., 2013; Maney, 2013). Alfaxalone and propofol are non-barbiturate anesthetic agents characterized by a smooth, rapid onset and short duration of action. Alfaxalone is a synthetic neuroactive steroid that produces unconsciousness and muscle relaxation (Ferre et al., 2006). Propofol has a very similar effect: rapid and smooth induction, good muscle relaxation, and quiet recovery (Morgan, Legge 1989; Ferre et al. 2006). It produces rapid induction of basal narcosis for intubation and inhalation (4-6 mg/kg IV) and should be administered slowly (20 sec) to decrease the incidence of apnea. (Wiebe, Howard, 2009).Neither drug accumulates in tissues at clinical doses so both can be used for total intravenous anesthesia (Ambros et al. 2008). A study of the cardiopulmonary and anesthetic effects of an induction dose of alfaxalone or propofol has been done by Maney (2013) in eight adult female mixed-breed dogs. The results showed that there were no clinically significant differences in cardiopulmonary effects between propofol and alfaxalone. A single bolus of propofol resulted in shorter recovery time and fewer adverse events than a single bolus of alfaxalone (Maney, 2013). Doebeli et al. (2013) studied the effects on newborn puppies of anesthesia induction with propofol (2-6 mg/kg IV) versus alfaxalone (1-2 mg/Kg IV) during the cesarean section. Neonatal viability was assessed using a modified Apgar score that took into account heart rate, respiratory effort, reflex irritability, motility and mucous membrane color (Doebeli et al., 2013). The results indicated that alfaxalone is better suited for anesthesia induction, resulting in improved neonatal Apgar scores compared with propofol induction. Using alfaxalone induction, puppies recovered from anesthesia more quickly (Doebeli et al., 2013; Metcalfe et al. 2014). Compared with alfaxalone, propofol is reported to cause more cardiorespiratory depression and to increase $PaCO_2$ which may negatively influence the puppy viability (Muir, 2008; Ambros, 2008). Anesthetic recovery of the

dams was smooth and rapid in both anesthetic agents (Doebeli et al., 2013), compared to Jimenez et al. results (2012), who described poorer recovery quality after alfaxalone induction compared with propofol. However this last study was not made on bitches undergoing a cesarean section. A more recent study, by Metcalfe et al. (2014), also compared the clinical safety and efficacy of alfaxalone and propofol as induction agents in canines. The maintenance was performed with isoflurane and oxygen. A number of 74 bitches were divided in two groups, alfaxalone group and propofol group respectively. They were monitored during the anesthesia and all the variables were recorded, as well the puppy viability. Premedication was not permitted in this study to prevent confounding of premedicant effects on the variables being measured (Metcalfe et al., 2014). After induction and delivery of the puppies, local anesthetics, analgesic, anti-emetic, antibiotic, procoagulant and tocomimetic drugs were administered. NAIN-S drugs were also administered subsequent to delivery (Metcalfe et al., 2014). This study showed that induction of anesthesia in canines undergoing cesarean section with either drug gave equivalent results (Metcalfe et al., 2014).

Propofol followed by isoflurane anesthesia has been proven to be superior to anesthesia with thiopental (Funkquist et al., 1997). Administration of intravenous propofol followed with isoflurane was also found to have increased survival among pups, increased vigor, and increased vocalization (Moon et al., 2000). In cases of severe maternal compromise or maternal cardiac disease, etomidate (1–2 mg/kg i.v.) is used for induction of general anesthesia, and can be used with midazolam to reduce any excitatory side effect (Robertson 1992; Pablo, Bailey 1999). Published reports describe the use of propofol, thiopental, ketamine, thiamylal, xylazine and alfaxalone as injectable induction agents, followed by halothane, methoxyflurane and isoflurane with and without nitrous oxide as inhalational induction agents for caesarean section in bitches (Moon et al., 2000; Moon et al., 2002; Luna et al., 2004; Doebeli et al., 2013). Luna et al. (2004) divided a number of 24 bitches undergoing cesarean section in 4 groups of 6. After a clinical examination the bitches were sedated with 0.5 mg/kg of chlorpromazine intravenously, followed 15 minutes later by either 8 mg/kg of thiopentone, intravenously (group 1), 0.5 mg/kg of midazolam combined with 2 mg/kg of ketamine, intravenously (group 2), or 5 mg/kg of propofol intravenously (group 3). The bitched were intubated immediately after the induction, and anesthesia was maintained with enflurane in 100 ml/kg of oxygen. The bitches of group 4 underwent epidural anesthesia at the lumbosacral space, using 2-5 mg/kg body weight of 2% lidocaine with adrenaline and 0-625 mg/kg of0-5 % bupivacaine with adrenaline (Luna et al., 2004). While the heart rate remained the same for every group of puppies, the respiratory rate was increased in the puppies delivered after epidural anaesthesia, compared to the ones delivered after anaesthesia with propofol/enflurane or midazolam/ketamine /enflurane. Overall, they were less depressed after epidural anaesthesia, followed by propofol/enflurane thiopentone/enflurane and midazolam/ ketamine/enflurane anaesthesia. Epidural anaesthesia produced the least respiratory and neurological depression in the puppies. The use of midazolam or ketamine before enflurane anesthesia induced the most severe neurological depression and also 10 per cent mortality rate. The results of this study suggest that as far as the neurological and respiratory functions of the puppies were concerned, the best anesthetic technique for caesarean section appeared to be epidural anesthesia. In cases for which epidural anesthesia is unsuitable, propofol appeared to be the safest induction agent in terms of puppies neurological reflexes, followed by thiopentone and midazolam/ketamine. It is recommended that the time from induction to delivery of pups be minimized to reduce respiratory depression as a result of their exposure to inhalant anesthetics. Inhalation agents may be used to induce or maintain anesthesia in calm and or depressed dams, resulting in rapid fetal and maternal equilibration (Tranquilli et al., 2013). Deep maternal anesthesia can lead to hypotension, reduced uterine blood flow, and fetal acidosis. Anesthetic substances such as isoflurane, sevoflurane, or desflurane are preferred over

halothane because of the faster induction and recovery, but are highly dependent on other agents used for premedication (Valerie, 2009). Isoflurane and other gas anesthetics should be given at the lowest concentration to maintain maternal consciousness (1%-2%). The minimum alveolar concentration of most volatile anesthetics is reduced by approximately 25% during pregnancy. In general, anesthetics can be administered with 100% oxygen. Isoflurane commonly is used to maintain anesthesia for caesarean section in several species, including canines. Its use had been positively associated with puppies' vocalization, a sign of vigour and good Apgar score (Moon-Massat, Erb 2002). Depression of the newborns by inhalation anesthetic is related to the concentration and duration of administration. Additional anesthetics can also be administered once the newborns have been delivered, depending on the anesthetic protocol chosen for the cesarean section. Its purpose would be the closure of the uterus and abdomen. For example, start or increase inhalant administration or injection of an opioid such as butorphanol if none has already been given (Clarke et al., 2014). Sevoflurane has been shown not to reduce puppy vigor (Gendler et al., 2007). Using alfaxalone CRI as a maintaining agent has resulted in a rapid recovery and a good muscle relaxation (Ambros et al. 2008; Suarez et al. 2012). It has a high margin of safety and minimal cardiovas-cular effects (Rodriguez et al., 2012). Ruiz et al. (2016), compared isoflurane (2%) and alfaxalone on bitches undergoing cesarean section, evaluating the maintenance of anesthesia, recovery from anesthesia and the effects on puppies. All dogs were induced with alfaxalone intravenous, mechanically ventilated, analgesia being administered after the delivery of puppies. The results of this study suggest that maintenance of anesthesia with an alfaxalone CRI during the cesarean section has similar cardiopulmonary effects to isoflurane but induces longer recoveries in the mothers, while puppies are associated with lower Apgar scores. However, survival and mortality were similar to those obtained with isoflurane (Ruiz et al., 2016).

## RECOVERY

Following the closure of the skin, pain management is obtained with local infiltration of anesthetics at the surgical incision in the dam, and often lidocaine (2 mg/kg) or bupivacaine (2 mg/kg in combination with lidocaine) are administered (Baltzer, 2013). It is recommended that after having a cesarean section, the administration of NSAIDs be restricted to only one dose, (Mathews, 2005) and be administered only if the dam is normotensive and normovolemic (Costea, 2016). Oxytocin can be administered to aid uterine involution (Dugdale, 2010). Puppy survival and acceptance are directly linked to the length of the period between first breath and first contact with the mother. For this purpose it is preferred that the recovery from caesarean sections be as short as possible (Ruiz et al., 2016).

## CARE AND EVALUATION OF NEWBORNS

Loses to neonatal diseases can be managed by identifying the neonates at risk as soon as possible. The development of a protocol for neonatal assessment is therefore fundamental to this identification as well as allowing a greater knowledge of neonatal physiology (Vassalo et al., 2015). More authors evaluated the newborns viability using the Apgar score, which consisted in assessing puppy`s heart rate, respiratory rate, reflex irritability, motility and mucus color (Silva et al., 2009; Veronesi et al., 2009; Groppetti et al., 2010; Batista et al., 2014). Besides this, blood samples were taken and handed for laboratory analysis. There are many factors to consider during evaluation of the newborn. The most important immediate factors are respiration and body temperature. Immediately after delivery, the fetal membranes should be removed from the neonate's face and the umbilical cord is clamped and cut distal to the clamp (Johnson, Casal, 2012). The neonate is cleaned and dried, and stimulated by rubbing with a warm, clean, dry towel. Also, holding the neonate with its head and neck slightly lower than its body will allow fluid in its mouth and pharynx to flow out (Waldemar et al., 2010). The risk of

hypothermia is increased in neonates, meaning that maintaining warmth is of great importance. Last but not least, in case the newborn does not breathe spontaneously, it has to be stimulated and oxygenated. The pups can be intubated using a flexible 14 or 18G intravenous catheter or 2.0-3.0 OD endotracheal tube (Kraus, 2016). An aspiration/resuscitation device can be used to clear respiratory tract and stimulates the respiratory reflex. Another method of respiratory stimulation is acupuncture, involving the use of a 25G needle which is inserted into the nasal philtrum until it contacts bone and then twisted. As a final measure, doxapram in a dosage of 1 to 5 mg (approximately 1 to 5 drops from a 20- to 22-gauge needle) can be topically administered to the oral mucosa or injected intramuscularly or subcutaneously. If the neonatal respiratory depression is thought to be related to the dam`s anesthesia or sedation, then naloxone and atipamezole may be used as antagonizing agents (Dugdale, 2010). During fluid therapy, neonates are more predisposed to dehydration or fluid retention (Johnson, Casal, 2012). Fluids may be administered via an IV, IO, intraperitoneal, SC, or oral route. Depending on the circumstances, Ringers`s solution, lactated Ringer's, dextrose-containing solutions, or blood products may all be used (Moon et al., 2001; Lopate, 2009). Newborns are also exposed to hypoglycemia due to more factors, like: poor maternal nutritional status, dystocia, low birth weight, hypothermia, infection, hypoxia, inadequate nutrition, and congenital metabolic disorders (Casal, 1995; Lawler, 2008; Haskins, Casal, 1996; Davidson, 2003). Hypoglycemia is treated by administration of dextrose, to effect (PO, IV, IO, or SC). After improving the newborns' vital signs, suckling and bounding with the dam is essential.

## CONCLUSIONS

It is highly important that the anesthetic protocol be tailored to each patient. Therefore, various anesthetics protocols during cesarean section have been studied by a multitude of authors. Due to its limited effects on the fetuses and the short recovery time of the mother, the epidural anesthesia has been proven to be the most successful procedure (Moon et al., 2004; Scarda, Tranquilli, 2007). The downside is represented by the fact that the dam is not intubated, which leads to an increased risk of hypoxemia, regurgitation and aspiration pneumonia (Luna et al., 2004; Ryan, Wagner, 2006). However, most of the authors prefer general anesthesia, with good results being registered after using alfaxalone or propofol as an induction agent. The use of either of the two induction agents has been shown to result in viable and healthy newborns with an increased Apgar score (Doebeli et al., 2013; Metcalfe et al., 2014). Tranquilizers, sedatives and analgesics should ideally be avoided until the newborns have been delivered (Wiebe, Howard, 2009). During the maintenance phase of the anesthetic procedure, isoflurane has been positively associated with the newborns being vocal, which again is a sign of vigour and good survival score (Moon, Erb, 2002). Regardless of the anesthetic protocol used, the main aim is to minimise the length of the entire procedure, and thus reducing the cardiovascular and neurological depression in the newborns. It is also equally important to ensure that the mother has a speedy recovery, allowing her to care for the newborns as soon as possible.

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# A CASE OF HEPATIC CYST AND HEPATIC LOBE TORSION IN A CHOW-CHOW MALE

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***Abstract***

*The paper aims to describe a clinical case of a dog with a hepatic cyst and a hepatic lobe torsion. Abdominal ultrasound of the dog presented for anorexia and vomiting revealed an anechoic mass in the hepatic parenchyma and a hyperechoic area on the left lateral liver lobe. The biochemical findings in this case showed an elevated glutamic pyruvic transaminase (GPT) and alkaline phosphatase (ALP). The surgery consisted of a partial lobectomy of both affected lobes.*

***Keywords:*** *hepatic cyst, hepatic lobe torsion, surgery.*

## INTRODUCTION

Hepatic cysts can be congenital or can be acquired in time (Van Den Ingh, 1985). Hepatic cystic lesions predominantly remain asymptomatic and are found as a mere coincidence on abdominal imaging techniques, such as ultrasonography (USG), computed tomography (CT) and magnetic resonance imaging (MRI) (Lantinga, 2013).

Although patients usually don't show any sign of illness, the cysts can become large and cause abdominal distention or clinical signs such as lethargy and vomiting. Liver lobe torsion is a rare condition in dogs. Mostly, the cause is unknown but can be explained in case of congenital absence of the hepatic ligaments or traumatic rupture (Fossum, 2012; Scheck, 2007; Bhandal, 2008).

A double partial lobectomy was performed on a 13 y old male

Chow-chow, diagnosed on ultrasound exam with hepatic solitary cyst and hepatic lobe torsion. The outcome was good, the patient had full recovery in two weeks post-surgery.

## MATERIALS AND METHODS

A 13-year-old male Chow-chow was presented with signs of vomiting and lack of appetite. Physical examination of the patient revealed an enlarged liver upon abdominal palpation. When pressure was applied, it grunted.

The dog had a history of time to time vomiting for the past 6 months but its condition got worse and was brought into the clinic. It also had a scrotal fistula.

The CBC was normal but there were changes in the biochemical parameters as shown in table 1.

Table 1. Biochemical parameters

| Parameter | Value | Reference range |
|---|---|---|
| GPT | 85,9 UI/L | 8,2-57 UI/L |
| GOT | 16,9 UI/L | 8,9-49 UI/L |
| CRE | 2 mg/dL | 0,5-1,6 mg/dL |
| UREA | 34,3 mg/dl | 8,8-26 mg/dl |
| GLU | 74,5 mg/dL | 62-108 mg/dL |
| ALP | 289,4 UI/L | 10,6-101 UI/L |
| TBIL | 0,3 mg/dL | 0,1-0,3 mg/dL |

## RESULTS AND DISCUSSION

An ultrasound exam was performed to correlate the biochemical findings.

Ultrasound examination of the hepatic parenchyma revealed an anechoic structure that was associated with far acoustic enhancement (Figure 1). It also exposed a hyperechoic area on another lobe which was later confirmed as a liver lobe torsion (Figure 2).

The testes were also examined. The epididymis of the right testis was larger than an usual one and had mixed echogenicity (Figure 3).

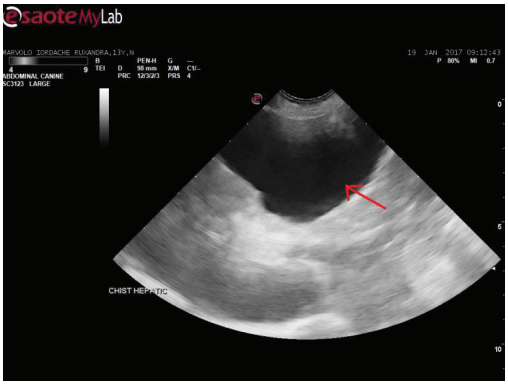


Figure 1. Hepatic cyst

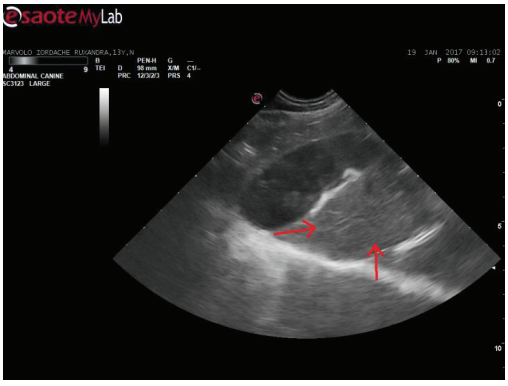


Figure 2. Left lateral liver lobe torsion

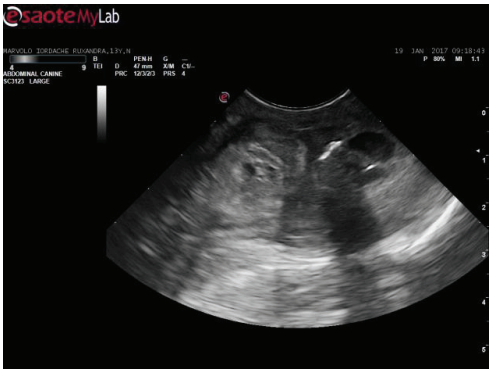


Figure 3. Modified structure of the right testicle

As a result of minimal change in the condition of the dog after a week of treatment with antibiotics, biliary drainers and antispasmodics, the patient was scheduled for surgery.

The patient was premedicated with Diazepam 0.2 mg/kg and butorphanol 0.2 mg/kg, induced with propofol and maintained with isoflurane gas. Analgesia was continued after surgery with Tramadol 2 mg/kg t.i.d.

Ventro-median retroxiphoydian laparotomy was performed and the liver was located.

A hepatic cyst was identified on the left medial liver lobe (Figure 4), along with the torsion of the left lateral liver lobe.

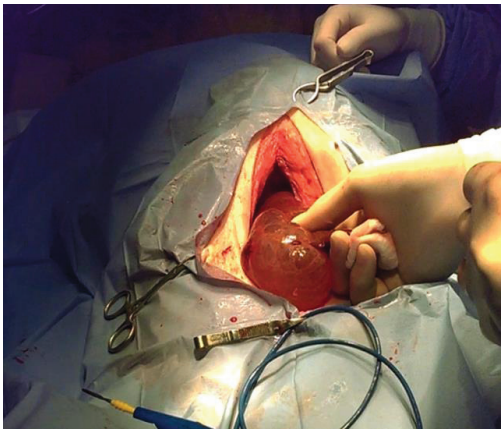

Figure 4. Hepatic cyst

The great omentum was pexed on the torsioned lobe so, some ligatures were applied and then the great omentum was transsected (Figure 5, 6).

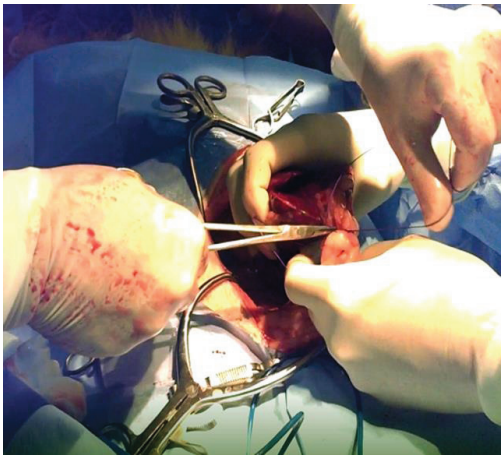

Figure 5. The application of ligatures on the pexed omentum

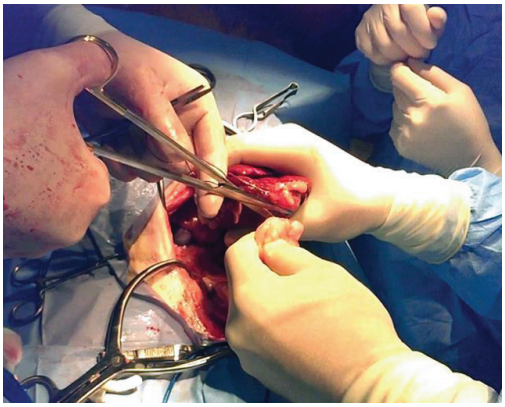

Figure 6. Cutting of the pexed omentum

Partial lobectomy was performed on both affected lobes (Figures 7, 8).

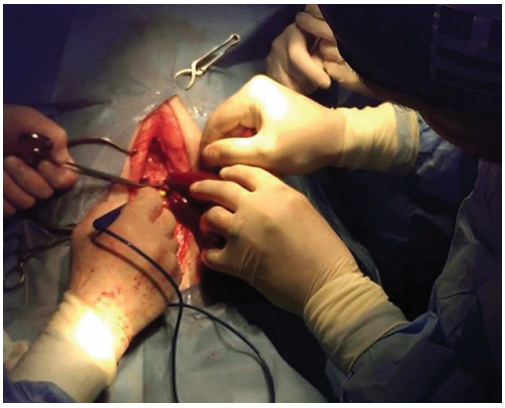

Figure 7. Hepatic parenchyma incised with the electrocautery scalpel

For the liver lobe with torsion, the line of separation between normal hepatic parenchyma and that to be removed was determined.
Separate ligations were applied along the normal parenchyma to isolate the damaged tissue (Figure 9).
The selected site of the liver parenchyma was incised with the electrocauter resulting in the partial removal of the liver lobe (Figures 10, 11).

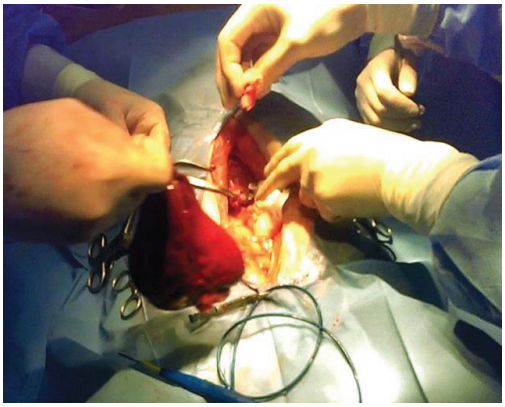

Figure 8. Partial lobectomy of the torsioned hepatic lobe

The same technique was applied for the hepatic cyst lobe.

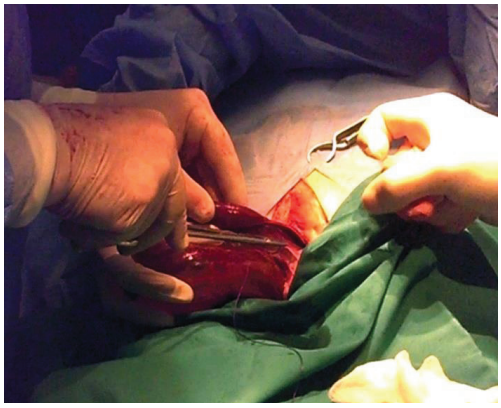

Figure 9. Separated ligatures were applied on the lobe with the cyst

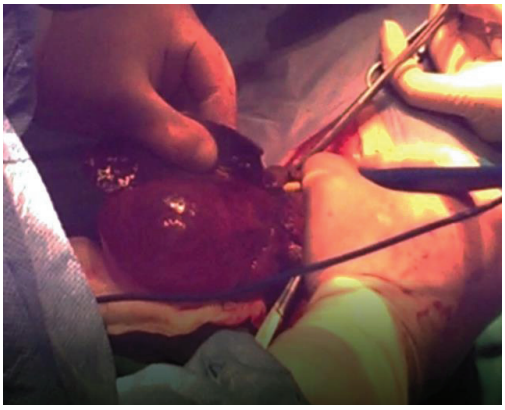

Figure 10. Partial lobectomy performed with the electrocautery scalpel

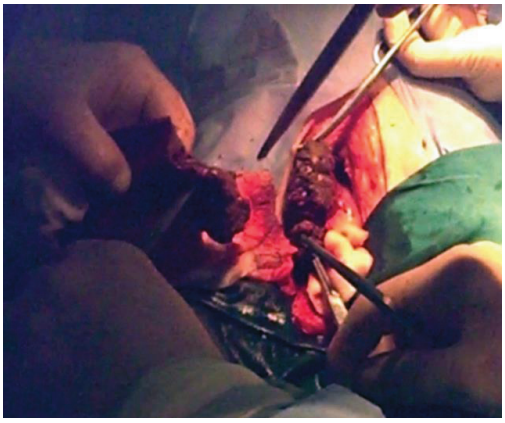

Figure 11. Partial lobectomy of the liver lobe

The abdominal cavity was closed in two layers: simple continuous suture of the *linea alba* with polydioxanone (PDS) 2/0 followed by a continuous "U" suture of the skin with 2/0 Nylon.
A bilateral orchiectomy was also performed for the removal of the modified testes.
The partially removed liver lobes and the testes were sent for a histopathological diagnostic.
The suspicion of liver cyst was confirmed (Figure 12).

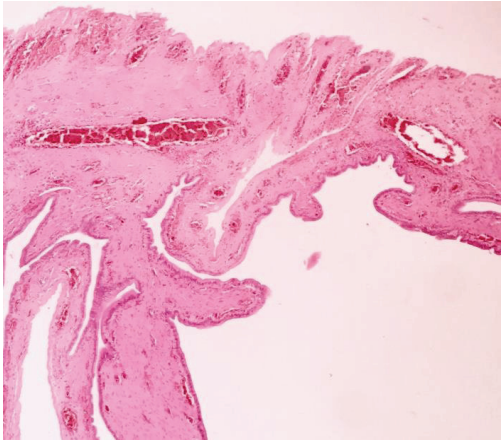

Figure 12. Bile duct (cross section- detail) - marked dilatation with several intraluminal prolongations of attenuated bile duct epithelium, suggesting compressive atrophy (bile duct dilatation/Polycystic liver disease).

The histopathological analysis showed a chronic hepatitis in thetorsioned liver lobe (Figure 13) and a chronic severe epididymitis of the testes (Figure 14).

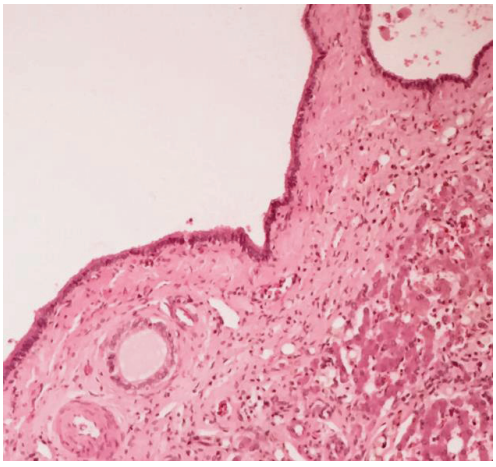

Figure 13. Liver - interstitial fibrosis dissecting groups of hepatocytes; the collagen fibers are arranged in layers surrounding markedly dilated bile ducts; the hepatocellular cords are compressed and occasionally dissociated (chronic hepatitis)

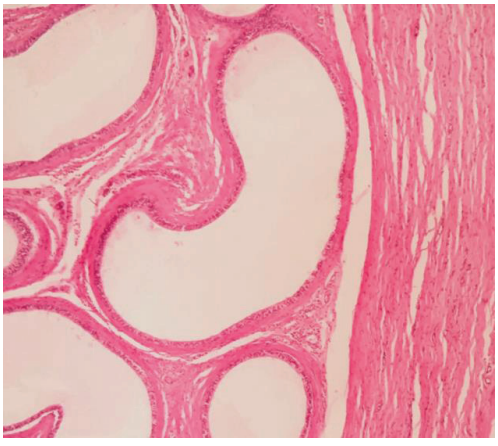

Figure 14. Epididymis (cross section) - severe thickness of tunica serosa, evident interstitial connective tissue proliferation; diffuse ectasia of ductus deferens(chronic severe epididymitis)

As in the clinical case of liver lobe torsion studied by Scheck (2007), the Chow-chow also had episodes of vomiting and obvious pain when abdominal pressure was applied on physical examination.
The study conducted by Bhandal et al. (2008) also revealed that the clinical signs were unspecific, with vomiting and a history of anorexia.
The Rottweiler showed no signs of pain at abdominal palpation. As in the case of the Golden Retriever, it also had peritoneal

effusion. Despite the long period of illness of the Chow-chow, no peritoneal effusion was found. In all three dogs, the major biochemical changes included elevated liver enzymes.
In most cases, the liver cystic lesions described by Van den Ingh and Rothuizen (1985) were coincidental findings.
A direct relation between the cystic lesions and clinical disease existed in one dog, a Pekingese which had been vomiting for a week until it was brought to the vet. As in our case, physical examination revealed hepatomegaly.

## CONCLUSIONS

The clinical signs of the patient along with the biochemical findings, attested a liver dysfunction. The ultrasound examination revealed an existing mass in the liver which could only be treated by surgery. The differential diagnostic of the hepatic cyst from hepatic abscess or neoplasia was confirmed through histopathological analysis.

The clinical findings of all authors cited in the paper revealed that all patients were presented with a history of anorexia and episodes of vomiting. Abdominal pain was present only in some cases, though the existing hepatomegaly. Elevated liver enzymes were present in all cases with liver lobe torsion.

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# EPIDEMIOLOGICAL AND MORPHOLOGICAL CHARACTERISTICS OF CUTANEOUS ROUND CELL TUMORS DIAGNOSED USING ASPIRATIVE CYTOLOGY IN DOGS

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***Abstract***

*This paper's purpose is to identify the epidemiologic and morphologic characteristics of cutaneous round cell tumors diagnosed using aspirative cytology in dogs. The study was conducted over a period of five years on a total of 225 dogs, aiming predisposition to gender, age and lesion location, as well as identifying the cytomorphologic characteristics of the lesions. Cytology was performed using FNA, smears being displayed by spreading, air dried and Romanowsky stained. Of the 225 studied cases of cutaneous round cell tumors, 110 (49%) were histiocytic tumors, 96 (43%) were diagnosed as mast cell tumors, 10 (4%) were plasma cell tumors, 2 (1%) cutaneous lymphomas and 7 (3%) were extragenital transmissible venereal tumors (cutaneous). The difference between sexes was not significant, 51% of the affected animals being males and 49% females. Most tumors were localized on the limbs (46%), followed by the trunk (38%) and head (20%).The relevance of the cytological examination was maximum for the mast cell tumors, differential diagnostic problems being faced between histiocytic tumors and plasma cell tumors or transmissible venereal tumors. Proper evaluation of cell populations, identifying the specific elements and morphological features of each cell type are essential, increasing the value of cytopathological diagnosis in veterinary medicine practice.*

***Key words****: round cell tumors, cutaneous, dogs, cytological diagnosis.*

## INTRODUCTION

Cutaneous round cell tumors are common in dogs, this category including mast cell tumors, histiocytic tumors, plasma cell tumors, cutaneous lymphoma and transmissible venereal tumors – extragenital type (Moore, 2007; Raskin, 2010).

## MATERIALS AND METHODS

This paper is a retrospective, epidemiologic and cytomorphological study conducted over a period of 5 years (2010 – 2014) on a total of 225 dogs diagnosed with cutaneous round cell tumors. The epidemiological study followed breed and gender predisposition, age and lesion location, and the morphological study aimed to identify the cytomorphological characteristics of the lesions, as well as the relevance of FNA (fine needle aspiration) in the diagnosis of cutaneous round cell tumors. The cytological examination was performed using FNA, the smears being displayed by spreading, air dried and Romanowsky stained.

## RESULTS AND DISCUSSIONS

Of the 225 studied cases of cutaneous round cell tumors, 110 (49%) were histiocytic tumors, 96 (43%) were diagnosed as mast cell tumors, 10 (4%) were plasma cell tumors, 2 (1%) cutaneous lymphomas and 7 (3%) were extragenital transmissible venereal tumors (cutaneous) (Figure 1).

The epidemiological and morphological data obtained on the studied cases are presented in the tables and charts below (Table 1).

It should be noted that in some cases, especially those diagnosed as mast cell tumors or histiocytic tumors, the lesions were multicentric.

Regarding the sex of affected animals, it was found that out of 225 dogs diagnosed with cutaneous round cell tumors, 115 were males (51%) and 110 were females (49%), gender differences not being significant (Figure 2).

As is evident from the data presented in the chart below, most tumors were located on the limbs (46%), followed by the trunk (38%) and head (20%) (Figure 3).

Table 1. Presentation of the studied cases

| Cutaneous round cell tumors | Total number of cases | Sex | | Age limits | Location | | | |
|---|---|---|---|---|---|---|---|---|
| | | M | F | | H | N | T | L |
| Mast cell tumor | 96 | 46 | 50 | 6 months – 15 years | 14 | 3 | 33 | 48 |
| Histiocytic tumor | 110 | 55 | 55 | 2 months – 14 years | 27 | 15 | 41 | 54 |
| Plasma cell tumor | 10 | 7 | 3 | 2-16 years | 2 | 1 | 5 | 2 |
| Limphoma | 2 | 2 | 0 | 2-7 years | 0 | 0 | 2 | 0 |
| TVT | 7 | 5 | 2 | 2-13 years | 2 | 0 | 5 | 0 |
| **TOTAL** | **225** | **115** | **110** | **2 months – 16 years** | **45** | **19** | **86** | **104** |

M = male, F = female, H = head, N = neck, T = trunk, L = limbs

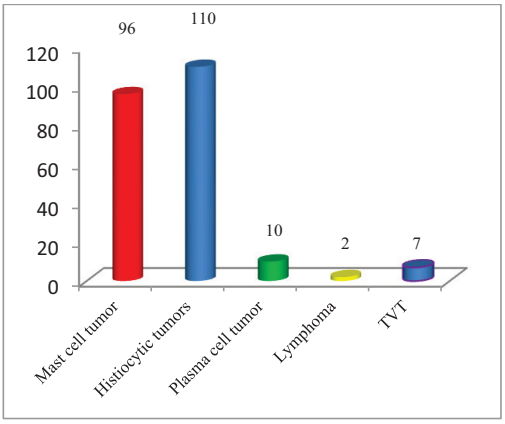


Figure 1. Cases distribution according to the lesion type

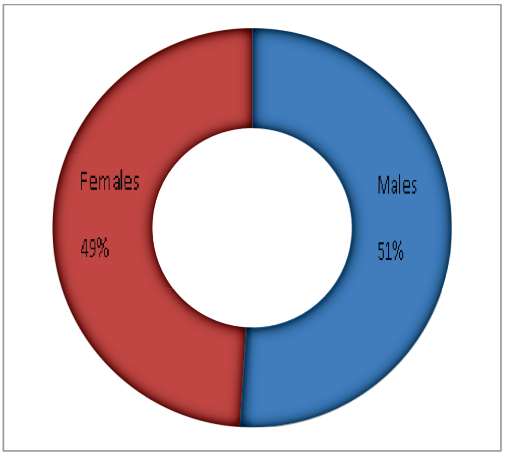


Figure 2. Cases distribution according to gender

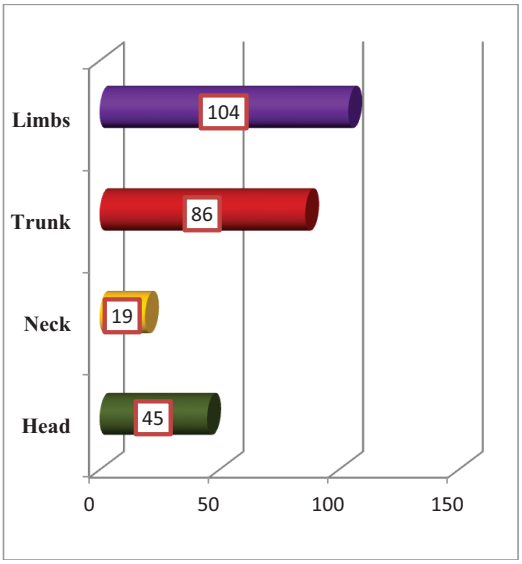


Figure 3. Distribution of cutaneous round cell tumors according to location

A special mention is required to be made concerning histiocytic tumors, which are divided into several distinct entities, each with its epidemiological, morphology and prognosis characteristics (Dinescu, 2011). Chart 4 exhibits the pathological entities which are included in the histiocytic skin tumors category in dogs, along with the incidence of each lesion among the studied cases.

Of the 110 cases diagnosed with histiocytic tumors, 48% were represented by canine cutaneous histiocytoma, 21% by reactive histiocytosis, and 31% were malignant histiocytic tumors (Figure 4).

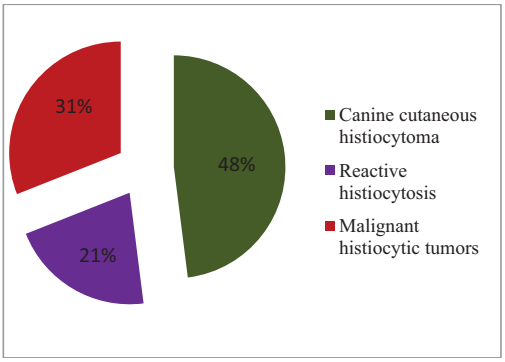


Figure 4. The incidence of different types of diagnosed histiocytic cutaneous tumors

A particular aspect was represented by the cutaneous form of the transmissible venereal tumor, metastasis of its genital form.
It was diagnosed in both males (n=5) and females (n=2), locations being the trunk (n=5) and head (n=2).
Considering the increased incidence of transmissible venereal tumor in our country, the increased frequency of extra genital location of this tumor is easily explained.
Thus, during the 5 years of this study, 43 dogs were diagnosed with transmissible venereal tumors.
Of these, 26 (60%) have presented genital localization and 17 (40%) extra genital localization, 7 of them having cutaneous localization and 10 other localizations (oral, nasal).
Gross aspects were equally diverse and uncharacteristic.
The majority had a nodular character, alopecic or covered with hair (Fig. 5, 6, 7), of elastic or increased consistency, while the larger ones often presented ulcerated areas (Fig. 6).

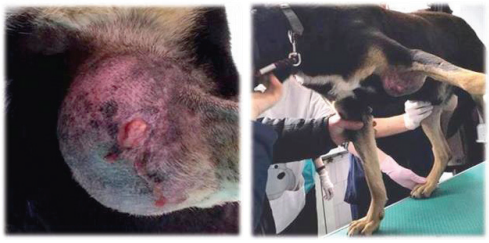

Figure 5 and 6. German Shepherd, 9 years old, with tumor localized at the medial humero-radio-ulnar joint (axilar region), left anterior limb, approx. 15 cm in diameter, elastic-hard consistency, adherent to the underlying tissues, ulcerated surface.

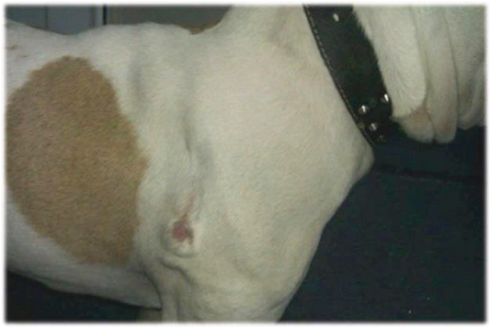

Figure 7. Amstaff, M, 8 years old. Nodular, partially depilated tumor, 3-4 cm in diameter, located in the olecranon region, right anterior limb, adhering to the skin, non-adhering to the substrate.

In case of round cell tumors, the cytomorphological pattern has a high degree of specificity, which allows a definitive diagnosis in a very high proportion (Curtseit, 2012).
The relevance of the cytological examination in the case of mast cell tumors is at the highest level, the cytopathologic diagnosis usually being more relevant than the histopathological, since it allows identification of mast cell specific granules (Fig. 8).

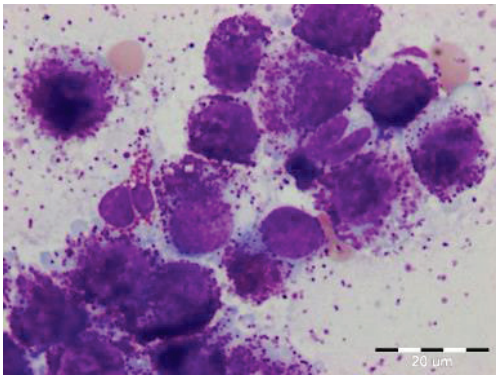


Figure 8. Mast cell tumor. Monomorphic population of round cells with tumoral mast cell morphology, prominent centrally or eccentric located nuclei, cells with intracytoplasmic metachromatic granules and moderate degree of degranulation. M-G.G. stained, x1000

Cytopathologic diagnosis of histiocytic tumors is based on the presence of a monomorphic population of round, small and medium sized cells, often with an indented nucleus, with a variable amount of cytoplasm, very often vacuolated (Fig. 9 and 10). Sometimes cytopathologic differential diagnosis problems occur between different types of histiocytic

tumors, epidemiological elements playing an important role in their differentiation.

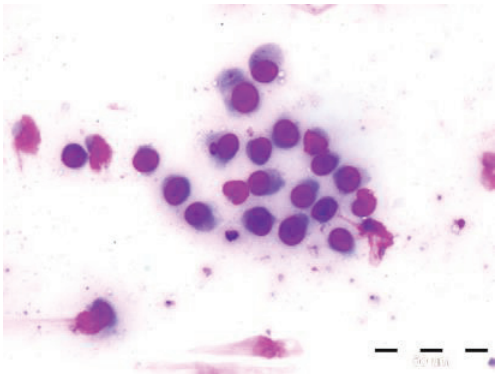

Figure 9. Benign histiocytic tumor - Canine cutaneous histiocytoma. Monomorphic population of round cells with histiocyte morphology, with variable amount of cytoplasm and eccentric nucleus. M-G.G. stained, x400

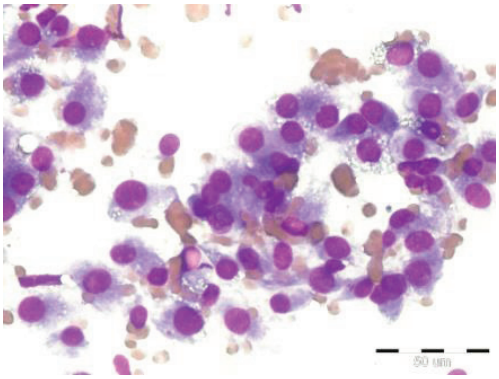

Figure 10. Malignant histiocytic tumor - Hystocytic sarcoma. Monomorphic population of round and spindle-shaped cells with round or oval, central or eccentric nuclei, with anisocytosis, anisocariosis, binucleation, basophilic cytoplasm with intracytoplasmic vacuoles. M-G.G. stained, x400

Plasma cell tumors also offer the cytological image of a round cell smear, the nucleus often having an eccentric position, showing a specific perinuclear blank halo. Bi- and multinucleation are common (Fig. 11).

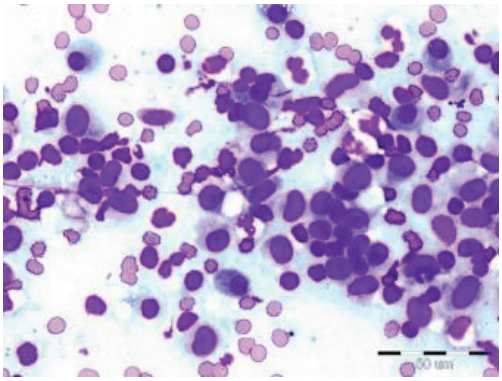

Figure 11. Plasma cell tumor. Monomorphic population of round cells with plasma cell morphology, with round or oval nucleus, finely granular chromatin, small unique nucleolus, basophilic eccentrically arranged cytoplasm. Sometimes a perinuclear bright halo is observed, specific to plasma cells. M-G.G. stained, x400

Cutaneous lymphomas are characterized by a monomorphic population of tumoral lymphoblasts, with eucromatic nuclei and numerous nucleoli. Mitoses can be frequent and atypical (Fig. 12).

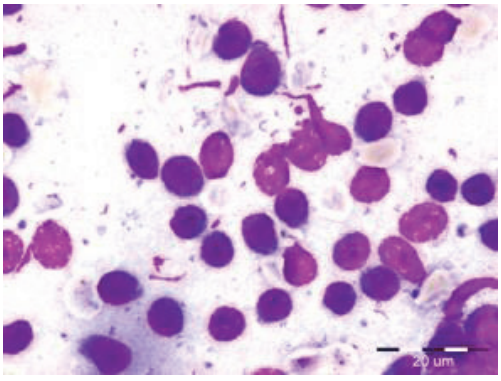

Figure 12. Cutaneous lymphoma. Monomorphic population of round cells with tumoral lymphocyte morphology, round nucleus, with anisocariosis, numerous nucleoli and scarce, slightly basophilic cytoplasm. M-G.G. stained, x400

Venereal transmissible tumor is also characterized by a monomorphic population of round cells, round nuclei with coarse chromatin and often vacuolated cytoplasm. Atypical mitoses are common (Fig. 13).

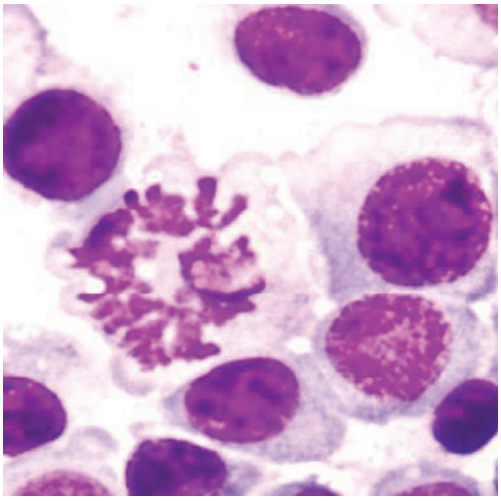

Figure 13. Cutaneous transmissible venerian tumor. Monomorphic round cell population specific to TVT, with abundant basophilic cytoplasm, with intracytoplasmic vacuoles, round nuclei, numerous nucleoli, coarse chromatin and atypical mitoses. M-G.G. stained, x1000

Differential diagnosis problems may arise in aspiration cytology between histiocytic tumors and plasma cell tumors or transmissible venereal tumors. Proper evaluation of cell populations, identifying the specific elements and morphological features of each cell type are essential, increasing the value of cytopathology diagnosis in veterinary medical practice.

## CONCLUSIONS

1. Of the 225 cases diagnosed with cutaneous round cell tumors, 96 (43%) were mast cell tumors, 110 (49%) histiocytic tumors, 10 (4%) plasma cell tumors, 2 (1%) cutaneous lymphomas and 7cases (3%) were extragenital transmissible venereal tumors - cutaneous.

2. Most tumors were localized on the limbs (46%), followed by the trunk (38%) and head (20%).

3. No gender predisposition has been observed, of the 225 dogs diagnosed with cutaneous round cell tumors 115 were males (51%) and 110 were females (49%).

4. Out of 110 cases of cutaneous histiocytic lesions, 48% (n=53) were diagnosed as canine cutaneous histiocytoma, 21% (n=23) as reactive histiocytosis and 31% as malignant histiocytic tumors.

5. Cytopathologic differential diagnostic problems have occurred with histiocytic tumors, but epidemiological elements have allowed their elucidation.

6. High specificity cytomorphological characteristics of round cell tumors allowed an accurate and definitive diagnosis in over 90% of cases.

7. Aspirative cytology is an option with a high diagnostic value in cutaneous round cell tumors in dogs.

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# PRELIMINARY RESULTS OF MVV AND CAEV SEROPREVALENCE IN ROMANIAN SHEEP AND GOATS

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***Abstract***

*Maedi-Visna (MV) and Caprine Arthritis Encephalitis (CAE) are diseases of sheep and goats. They are caused by lentiviruses which belong to Retroviridae family. The usual way of contamination is the cohabitation of animals. Diseases are widespread in many countries as: Norway, France, Italy, Spain, USA, Panama, Cyprus, Greece, and Japan. The present paper aimed to present the MVV and CAEV antibodies seroprevalence in samples collected in different sheep and goats farms from Romania. There were collected blood samples from the following counties: Cluj-Napoca, Ilfov, Constanta, Galati, Giurgiu, Braila, Arges, Bacau, Dâmbovita, Ialomita, Suceava, Calarasi, Buzau, Vrancea, and Vaslui. In order to determine the presence of antibodies, the samples were analysed by indirect ELISA, using commercial kits. There were registered negative results in only four counties and the possible existence of viruses in farms cannot be excluded. In order to confirm and strengthen the preliminary results, we recommend to analyses the samples by molecular biology techniques. Also, national authorities could establish a program of surveillance and diagnosis at national level, able to provide a more complete picture of the SRLVs prevalence in each county.*

***Key words****: small ruminant's diseases, maedi-visna, caprine arthritis encephalitis, SRLVs.*

## INTRODUCTION

The lentiviral pathology of small ruminants is caused by Caprine Arthritis Encephalitis Virus (CAEV) and Maedi-Visna Virus (MVV), viruses which caused persistent infections all over the world (Gufler et al., 2007; Stonos et al., 2014). CAEV and MVV are included in the group of small ruminant lentiviruses (SRLVs) belonging to *Retroviridae* family, *Lentivirus* genera (Junkuszew et al., 2016).

During the last decades, several wild species of ruminants have been introduced in Europe, and therefore new SRLV isolates have been reported. It is already known that wild ruminants could host emerging or re-emerging pathogens and the spread of them to domestic populations of sheep and goats can be done. Recent studies revealed several SRLV strains in Alpine ibexes (*Capra ibex*) from French Alps and in domestic hybrids, Rocky Mountain goats (*Oreamnos americanus*) or Mouflon (*Ovis orientalis*) (Sanjose et al., 2016).

The target cells of SRLVs are lymphocytes, mainly monocytes (Stonos et al., 2014), and the infections can evolve subclinical (latent infection) or clinical (Gufler et al., 2007; Junkuszew et al., 2016) with a long period of incubation (Sigurdsson et al., 1957; Haase, 1986). Bjorn Sigurdsson estimated that the specific viral diseases of small ruminants have spread rapidly in Iceland, through imported sheep from Germany (Sigurdsson et al., 1957; Haase, 1986).

Peterhans et al. (2004) concluded that the specific lentiviruses of small ruminants could affect sheep and goats and the most common routes which are incriminated are the direct contact and the lactogenic.

The presence of viruses as divergent genetic variants called quasispecies, may favor cross-species transmission (Sanjose et al., 2016).

An efficient vaccine is still a concern for science but, the high mutation rate of SRLVs daunt every attempt (Stonos et al., 2014).

The most common way of horizontal transmitssion is the close contact between small ruminants, even at pasture (Gufler, 2004, Gufler et al., 2007). Vertical transmission is not fully understood (Gufler et al., 2007). According with Straub (2004), the primary mode of infection is by the dam's milk, especially the colostrum.

The morbidity rate among individuals easily rise when infected colostrum is ingested, or if sick animals are in close contact with the susceptible ones (Junkuszew et al., 2016).

Nevertheless, Houwers and Van der Molen, (1987), Arsenault et al., (2003) and Berriatua et al. (2003) agree to consider the direct contact as less important in the economy of the disease.

There are five SRLV genotype (A-E) which have been discovered to date. Up to Gjerset et al. (2009), VMV-like and CAEV-like strains belong to genotypes A and B, while strain variants isolated from sheep and goats in Norway belong to genotype C. The strains isolated in Switzerland and Spain have been genotype D, and the ones isolated in Italian goats genotype E (Reina et al., 2010).

The control of SRLV infection could be realized by several procedures. One approach is based on the early detection of the infected animals (adult animals and their offspring) by serological methods (ELISA or AGID). The clinical picture of those infections register the decrease of the milk production and the poor quality of the milk, as a result of the increased somatic cells and shortened period of lactation. (Turin et al., 2005; Martinez-Navalon et al., 2013; Sanjose et al., 2015). The SRLV diseases are deeply harming the production of wool, milk and lamb. In that sense, the direct losses caused by death or premature culling can be consider on the second plan (Houwers, 1990; Brodie et al., 1998; Benavides et al., 2013).

The most convenient way to diagnose SRLV infections is to perform serology. A variety of laboratory techniques are available for this purpose. These include the agar gel immune-diffusion, enzyme-linked immunosorbent assay (ELISA), radioimmunoprecipitation (RIPA), radioimmunoassay (RIA) and western blotting (WB) (Minguijon et al., 2015; de Andres et al., 2005).

The researches cited by Perez et al. (2013) revealed that "*VMV and CAEV seroprevalence control methods may involve a combination of the following practices: culling of the flock and substitution by uninfected sheep, selective culling of seropositive animals, sheep replacement only with offspring from seronegative ewes, early culling of seropositive animals showing initial clinical symptoms, artificial rearing of lambs separated from the seropositive mother immediately after birth and segregation of the flock into two flocks based on serological status followed by separate management of the resulting flocks to avoid horizontal transmission*." (Perez et al., 2013).

The present paper aimed to present the preliminary results of MVV and CAEV antibodies seroprevalence in sheep and goats flocks in Romania, in a prospective serological study during the year 2016.

## MATERIALS AND METHODS

There are analyzed 1380 serum samples collected from 1145 sheep and 235 goats. The animals belong to the next counties: Cluj, Sibiu, Ilfov, Constanta, Galati, Giurgiu, Braila, Arges, Bacau, Dambovita, Ialomita, Suceava, Calarasi, Buzau, Vrancea and Vaslui (fig.1).


(Map source: http://d-maps.com/m/europa/roumanie/roumanie25.gif)

Figure 1. Assay-sampling Romanian Counties. The four Romanian regions are marked with colors: Blue – Moldavia, Purple – Dobrogea, Red – Muntenia, and Green – Transylvania

In order to evaluate the presence of SRLVs in flocks, serum samples collected have been pooled on each farm, as follow: 100µl of serum blood obtained from five animals (sheep or goats from the same flock) have been mixed into Eppendorf tube and used in one reaction. The serological examination has been done for

276 pooled sera (229 for sheep and 47 for goats).
The pooled sera have been tested using an ELISA commercial kit (IDEXX CAEV/MVV Total Ab Test, Switzerland) according to the manufacturer's instructions previously described (Gurau et al., 2015).
The serological results were edited and statistically analyzed with Anova: Single Factor data analysis tool. The variation of the serological results obtained in Muntenia, Moldavia, Transylvania and Dobrogea has been statistically validated ($p < 0.05$).
The geospatial analysis was designed in Microsoft Power Map in Excel.

## RESULTS AND DISCUSSIONS

The distribution of the results obtained in 16 counties is presented in table 1. Eleven counties provided positive pooled sera: Braila, Dambovita, Galati, Constanta, Vrancea, Vaslui, Suceava, Giurgiu, Ilfov, Sibiu, and Cluj. Serological prevalence of SRLVs in Romanian regions (Muntenia, Moldavia, Transylvania and Dobrogea) is variable ($p < 0.05$), with significant higher number of positive pooled sera in Moldavia than in the other three (fig. 2-5).
The distribution of the results obtained for each species are presented in tables 2 and 3. The pooled sera of sheep, from ten of the sixteen counties, respectively from Braila, Galati, Constanta, Vrancea, Vaslui, Suceava, Giurgiu, Ilfov, Sibiu, and Cluj, provided positive results.
In goats, only the pooled sera from four counties of the nine - Braila, Ilfov, Dambovita and Constanta, provided positive results.
Although the number of goat pooled sera is considerably lower compared to the sheep ones and thereby makes irrelevant a comparative analysis over the infection seroprevalence in the four Romanian regions, the comparative analysis of the seroprevalence in each county where it have been analyzed samples from both species, keep open the issue of the interspecies spread of the SRLVs.
The results provided on sheep and goats samples from Dambovita county, are suggesting the absence of transmission of goat SRLVs to sheep.
To the opposite, the prevalence of positive sera pools, quite uniformly distributed in goats and sheep in Ilfov county, equally suggests that the transmission between sheep and goats, the selection of a variant-specific host SRLVs limited or the presence of multiple variants. Of course, these assumptions will suffer a significant correction when overlapping the results to a rigorous epidemiological surveys, GIS related to the location of the holdings and phylogenetic analysis of samples.
The serological prevalence of SRLVs in sheep pooled samples from Muntenia, Moldavia, Transylvania and Dobrogea regions is variable, with higher prevalence of the positive pooled sera in Transylvania and Moldavia than in Muntenia and Dobrogea ($p < 0.05$) (fig. 6).
The positive results obtained in goat pooled samples have been twice more in Moldavia than in Muntenia and Dobrogea (fig. 7). In this study, we missed goat serum samples from Transylvania.

Table 1. Distribution of the pooled samples according to the flock origin

| **Results** | Braila | Bacau | Buzau | Arges | Dambovita | Galati | Constanta | Calarasi | Vrancea | Vaslui | Ialomita | Suceava | Giurgiu | Ilfov | Sibiu | Cluj | **Total** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| + | 11 | 0 | 0 | 0 | 5 | 26 | 2 | 0 | 1 | 1 | 0 | 1 | 3 | 5 | 11 | 68 | **134** |
| – | 54 | 7 | 4 | 3 | 7 | 14 | 7 | 3 | 4 | 3 | 3 | 8 | 3 | 4 | 3 | 10 | **137** |
| ± | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | **5** |
| **Total** | **65** | **7** | **4** | **5** | **12** | **40** | **9** | **3** | **5** | **4** | **3** | **9** | **6** | **9** | **15** | **80** | **276** |

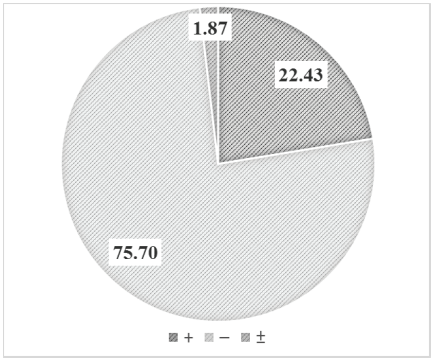


Figure 2. Serological prevalence of SRLVs in Muntenia region

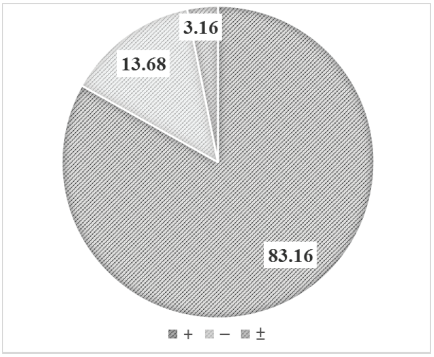


Figure 4. Serological prevalence of SRLVs in Transylvania region

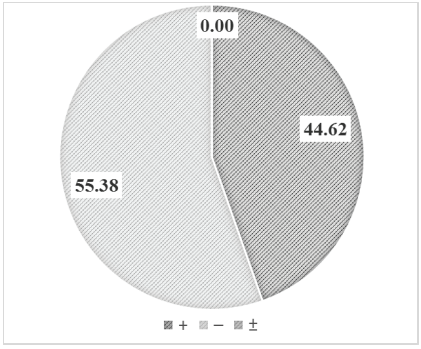


Figure 3. Serological prevalence of SRLVs in in Moldavia region

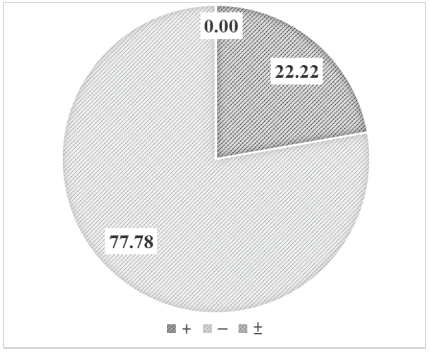


Figure 5. Serological prevalence of SRLVs in Dobrogea region

Table 2. Distribution of the sheep pooled samples according to the flock origin

| Result | Muntenia | | | | | | | | Moldavia | | | | | Transylvania | | Dobrogea | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Braila | Buzau | Ialomita | Giurgiu | Arges | Ilfov | Calarasi | Dambovita | Bacau | Galati | Vaslui | Suceava | Vrancea | Cluj | Sibiu | Constanta | |
| + | 8 | 0 | 0 | 2 | 0 | 3 | 0 | 0 | 0 | 26 | 1 | 1 | 1 | 68 | 11 | 1 | 122 |
| – | 38 | 4 | 2 | 2 | 3 | 0 | 3 | 4 | 3 | 14 | 3 | 6 | 4 | 10 | 3 | 3 | 102 |
| ± | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 0 | 5 |
| Total | 46 | 4 | 2 | 5 | 3 | 3 | 3 | 5 | 3 | 40 | 4 | 7 | 5 | 80 | 15 | 4 | **229** |

Table 3. Distribution of the goat pooled samples according to the flock origin

| Result | Muntenia | | | | | | | | Moldavia | | | | | Transylvania | | Dobrogea | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Braila | Buzau | Ialomita | Giurgiu | Arges | Ilfov | Calarasi | Dambovita | Bacau | Galati | Vaslui | Suceava | Vrancea | Cluj | Sibiu | Constanta | |
| + | 3 | 0 | 0 | 0 | 0 | 2 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 10 |
| – | 16 | 0 | 1 | 1 | 0 | 4 | 0 | 3 | 4 | 0 | 0 | 2 | 0 | 0 | 0 | 4 | 35 |
| ± | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 |
| Total | 19 | 0 | 1 | 1 | 2 | 6 | 0 | 7 | 4 | 0 | 0 | 2 | 0 | 0 | 0 | 5 | **47** |

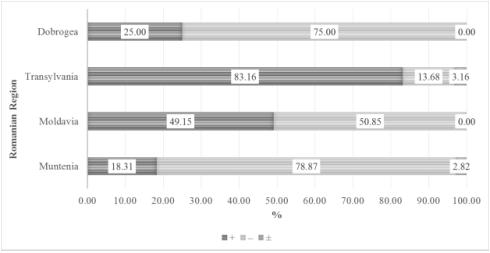


Figure 6. Serological prevalence of SRLVs in Romanian sheep ($p < 0.05$)

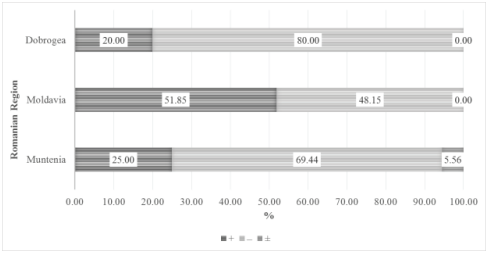


Figure 7. Serological prevalence of SRLVs in Romanian goats ($p < 0.05$)

The geospatial distribution of the prevalence of positive pooled sera in Romanian small ruminants is presented in figure 8.

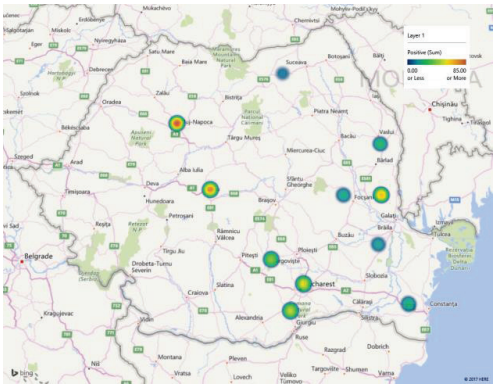


Figure 8. Geospatial distribution of seropositive results of ELISA CAEV/MVV Total Ab (IDEXX, Switzerland). The geospatial distribution of the positive results between Romanian regions: hotspots in the middle of Transylvania and in the south-east of Moldavia ($p < 0.05$)

At national level, the prevalence of goat positive results (40.00%) are quite similar to those reported by Gurau et al. (2015) in only one goat flock located in Braila County (38.46%). However, the prevalence in Moldavian counties is significantly higher. In both studies, serological positive results have been associated with few clinical cases of CAE (Gurau et al., 2015), but more clinical outbreaks could emerge in the next years, and therefore it is necessary to establish preventive measures. In similar circumstances, Gufler et al. (2007) recommended the introduction of a control or eradication program up to the prevalence of virus on the field, the structure of small ruminant population and the economic aspects (Gufler et al., 2007).

Moreover, if we take in consideration the studies of De Andres et al. (2013), our results could be underestimated; the SRLV strains circulating in different areas can be heterogeneous, and the performance of ELISA tests will vary accordingly. To solve the problem, it was proposed several ELISA-based strategies (De Andres et al., 2013). An alternative to ELISA could be PCR-based strategies and in the near future we are focused in testing with this method.

## CONCLUSION

SRLV infections among Romanian small ruminants should be considered. National authorities could establish a program of surveillance and diagnosis at national level, able to provide a more complete picture of the SRLVs prevalence in each county.

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# HAIR MINERAL CONTENT ANALYSIS IN CATS WITH DIFFERENT LIVER DISORDERS

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***Abstract***

*The main objective of this study was to evaluate the levels of 20 potentially toxic metals and essential minerals in hair samples from cats with different liver disorders, compared to control samples. Analysis of the hair elemental content of the cats with liver failure (n=5), cats with liver abscess (n=4), cats with chronic hepatitis (n=6), and clinically healthy cats as control (n=15), were performed by inductively coupled plasma-optic emission spectrometry (ICP-OES). In this study, Ca and Mg levels registered very significant differences ($p<0.001$), and Cu, Na, and Zn concentrations registered significant differences ($p<0.05$) between their levels in hair samples from cats with different liver disorders and control samples. No significant differences have been registered for heavy metals present in hair samples from cats with different liver disorders and clinically healthy cats. Independent of the significant differences, the highest values were registered by all the elements in hair samples from cats with chronic hepatitis, excepting Ca, Mg, and Se levels, which were higher in clinically healthy cats. The current study presents one of the first investigations of the suitability of hair as an indicator for mineral status of cats with different liver disorders in an urban area of Romania. Hair mineral levels determined in the present research may be considered as a contribution to a base of reference concentrations of minerals in female cats in Romania.*

***Key words****: hair, cats, heavy metals, minerals, liver disorders.*

## INTRODUCTION

The numerous functions of the liver, including but not limited to metabolism, storage, synthesis, and its implication in hematopoiesis, immunologic responses, digestion, and detoxification makes it one of the most important organs in the organism. Also, because of its capacity of regeneration, the hepatic injury has to be important or chronic in order to determine observable hepatic dysfunction or failure (Center, 2016a).

The liver role in xenobiotics excretion exposes it to high levels of toxic substances and their metabolites (Osweiler, 1996a).

Different toxins (e.g. mycotoxins, phytotoxins, phycotoxins) or prescription drugs (Goran and Crivineanu, 2016) or other toxic substances like heavy metals, certain herbicides, fungicides, insecticides, and rodenticides have been reported to be hepatotoxic (Center, 2016b).

Heavy metals are considered systemic toxicants, which induce multiple organ injuries, even at lower levels of exposure (Tchounwou et al., 2012).

Generally, after heavy metal absorption in the organism, they accumulate in one or more of the organs (liver, kidney, bone, and brain) (Goran and Crivineanu, 2016), metabolize, and are excreted via feces and urine. They are also excreted in sweat and accumulated in keratin-rich tissues, like hair and nails (Oostdam et al., 1999; Poon et al., 2004).

Hair or other keratinized skin structures samples were used for evaluating the mineral status of animals or humans, because of their easy and non-stressful sampling way. Also, unlike blood and tissue samples, the levels of most minerals in hair are higher (Combs et al., 1982; Combs, 1987; Batool et al., 2015). Hair mineral analysis as a screening and diagnostic tool has started to become routine since the early 1970s (Campbell, 1985; Foo, 1993, Kosla et al., 2011; Skibniewska et al., 2011). Many researchers have reported correlations between hair mineral content and blood or tissue mineral concentrations (Goran and Crivineanu, 2007; Crivineanu et al., 2008; Roug et al., 2015), and the use of hair samples in order to evaluate

heavy metals pollution (Patra et al., 2006; Crivineanu et al., 2010; Filistowicz et al., 2011; de Almeida Curi et al., 2012; Baran and Wieczorek, 2013; Petukhova, 2013; Skibniewski et al., 2013).

Also, in recent years, more researchers have reported correlations between hair mineral content and different health conditions both in humans and animals, which makes mineral hair content evaluation a good option to both patients and veterinarians or physicians (Poon et al., 2004; Adams et al., 2006; Michalak et al., 2012; Hernández-Moreno et al., 2013; Badea et al., 2016a, Badea et al., 2016b).

Mineral elements have essential roles in organisms' biological processes, as enzyme cofactors, nervous system functions, and in redox processes, but both essential and nonessential minerals can be toxic in certain doses (Osweiler, 1996b). Some of them are implicated in etiology of hepatotoxic disorders (Center, 2016b), after their liver accumulation, which is influenced by problems of metal elimination from the liver. Copper and iron as transition metals play an important role in oxidative stress, and if they accumulate, they are known to lead to necroinflammatory liver disorders, where they enhance liver oxidant damage (Labuc, 2012).

The main objective of this study was to evaluate the levels of some potentially toxic metals and essential minerals in hair samples from cats with different liver disorders, compared to control samples, using inductively coupled plasma-optic emission spectrometry (ICP-OES).

## MATERIALS AND METHODS

*Sampling and samples preparation*

Analysis of hair elemental content of cats with liver failure (n=5), cats with liver abscess (n=4), cats with chronic hepatitis (n=6), and clinically healthy cats as control (n=15), were performed by ICP-OES.

All cats with liver disorders in this study were above 8 years of age, and in the control group, 7 were above 8 years of age (5 females and 2 males). In this study, all cats with liver disorders were females except one (a male diagnosed with liver abscess), and in the control group, 11 cats were females and 4 were males.

The cats with liver disorders exhibited symptoms which led to the suspicion of a liver injury, the diseases being confirmed by ultrasound examination and biochemical blood tests. The male with liver abscess died, and the diagnostic was also confirmed by necropsy.

The hair samples were collected from all studied animals from the flank region, placed in plastic recipients, labeled, and transported to the laboratory. In the laboratory, the hair samples were stored in dark, dry places, with constant temperature. The samples were initially degreased, washed, rinsed, and then disaggregated. All hair samples were weighed and then digested using a Speedwave MWS-2 Berghof microwave oven as following: Step 1: 120$^{o}$C, power 50%; Step 2: 180$^{o}$C, power 75%; Step 3: 100$^{o}$C, power 40%. The samples were then analyzed to assay the presence of 20 mineral elements by ICP-OES.

*Spectrometric analysis*

Digested samples were treated with 5 mL $HNO_3$, 0.8 mL HCl and 1 mL $H_2O_2$, then diluted to 10 mL with ultrapure water and analyzed by a Thermo iCAP ICP-OES spectrometer (RF1100 W; reading time 30 s, washing time 30 s, nebulizer gas flow 0.5 L/min; auxiliary gas flow 0.5 L/min; sample injection pump flow 50 rpm). Calibration curves were developed using standard solutions of 0.001 ppm, 0.01 ppm, 0.1 ppm, 1 ppm, 5 ppm, 10 ppm, 50 ppm obtained by dilution from a multi-element ICP MERCK standard containing 1000 mL/L of Al, Ba, Be, Bi, Ca, Cd, Co, Cr, Cu, Fe, K, Li, Mg, Mn, Na, Ni, Pb, Se, Sr, and Zn.

*Statistical analysis*

Statistical analysis was performed using the software of VassarStats: Website for Statistical Computation (http://vassarstats.net/). One-Way ANOVA was performed for all samples' mineral concentrations, and when ANOVA generated $p \leq 0.05$, means comparison was carried out by all-pair Tukey HSD Test.

## RESULTS AND DISCUSSIONS

Because in this study cats with liver disorders were represented by a majority of females, the male with liver abscess was excluded from the study. Also, in order to make a correct

interpretation of the results, all males and females below 8 years were excluded from the control group.

In this regard, this study evaluated total mineral content of hair samples in female cats over 8 years old with liver failure (n=5), liver abscess (n=3), and chronic hepatitis (n=6), compared to mineral levels in hair samples from healthy female cats over 8 years old (n=5), which represented the control samples.

Of the 20 elements that could be determined (Al, Ba, Be, Bi, Ca, Cd, Co, Cr, Cu, Fe, K, Li, Mg, Mn, Na, Ni, Pb, Se, Sr, and Zn), Ba, Be, Bi, Co, Cr, Li, Mn, and Sr levels were below the analysis method detection limit, and Al, Ca, Cd, Cu, Fe, K, Mg, Na, Ni, Pb, Se, and Zn levels were over the analysis method detection limit.

The mean mineral contents of hair samples from clinically healthy cats and those with different liver disorders are presented in Table 1 and expressed as *parts per million* (ppm).

Table 1. Mean heavy metal and mineral levels in cat hair samples (ppm)

| Element | Health status | | | | *p*-value |
|---|---|---|---|---|---|
| | LF | LA | CH | HA | |
| Al | 10.75[a] | 13.3[a] | 40.95[a] | 15.1[a] | 0.16 |
| **Ca** | **169.8[a]** | **135.3[a]** | **582.95[b]** | **811.2[b]** | **0.001** |
| **Cu** | **0.465[a]** | **0.38[a]** | **1.85[b]** | **0.78[b]** | **0.05** |
| Cd | 0.028[a] | 0.049[a] | 0.343[a] | 0.032[a] | 0.41 |
| Fe | 170.3[a] | 88.3[a] | 417.7[a] | 15.0[a] | 0.50 |
| K | 26.95[a] | 9.2[a] | 104.6[a] | 54.0[a] | 0.24 |
| **Mg** | **15.65[a]** | **11.4[a]** | **51.25[b]** | **72.3[b]** | **0.001** |
| **Na** | **252.25[a]** | **79.4[a]** | **1272.05[b]** | **414.9[a]** | **0.03** |
| Ni | 0.35[a] | 0.68[a] | 1.785[a] | 0.12[a] | 0.24 |
| Pb | 0.092[a] | 0.13[a] | 0.42[a] | 0.028[a] | 0.33 |
| Se | 0.054[a] | BDL | 0.062[a] | 0.103[a] | 0.36 |
| **Zn** | **4.95[a]** | **2.7[a]** | **18.4[b]** | **10.1[ab]** | **0.05** |

*Levels not connected by the same letter are significantly different. The comparison can be made only between health statuses for the concentration of one element and not between different elements concentrations.
**LF – liver failure; LA – liver abscess; CH – chronic hepatitis; HA – healthy animals.
***BDL – below method detection limit.

In all samples that registered levels over the method detection limits, the highest values were observed in hair samples from cats with chronic hepatitis, with the exception of Ca, Mg, and Se levels, which were elevated in clinically healthy cats.

Another study on hair mineral content also reported that in the hair of clinically healthy cats above 5 years of age levels of Ca, Fe, K, and Mg were higher than in the study group (Badea et al., 2016a).

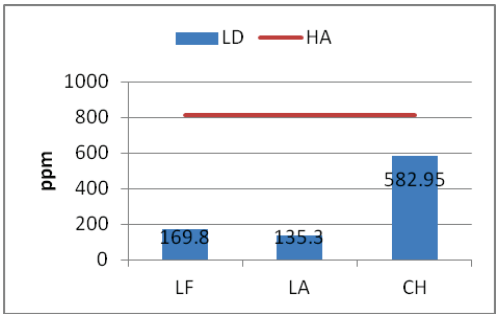
In this study, Ca and Mg levels registered very significant differences ($p<0.001$), and Cu, Na, and Zn concentrations registered significant differences ($p<0.05$) between their levels in hair samples from cats with different liver disorders and control samples.

Ca levels registered the highest concentrations in healthy animals hair samples (811.2 ppm, $p=0.001$), and very significant differences between its levels in hair samples from cats with liver failure and liver abscess, and those from cats with chronic hepatitis and clinically healthy cats ($p<.01$). The differences were not significant between Ca levels in cats with liver failure and liver abscess, and also between its levels in cats with chronic hepatitis and control group (figure 1).

Combs et al. (1982) have reported that Ca hair levels should not be able to influence dietary changes for calcium, as there is a constant homeostasis of blood calcium, and Ca levels increase or decrease only for short time after dietary changes.

Other researchers have found that Cd effects on Ca metabolism develop gradually; as cadmium

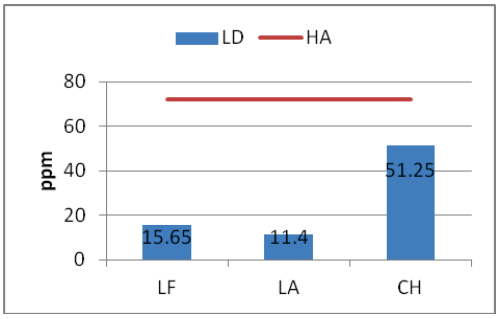
accumulates in the organism, Ca level decreases (Staessen et al., 1991).




*LF – liver failure, LA – liver abscess, CH – chronic hepatitis

Figure 1. Mean Ca levels in hair samples from cats with different liver disorders (LD) compared to clinically healthy animals (HA)

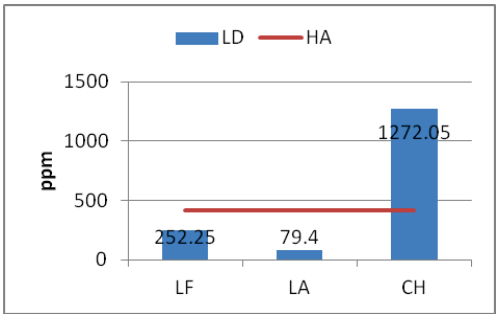
In the case of Mg, the differences registered were also very significant ($p$=0.001), with the same correlations pattern as that observed in the case of Ca (figure 2). The highest Mg level was found in hair samples from clinically healthy cats (72.3 ppm), followed by that in hair from cats with chronic hepatitis (51.25 ppm).




*LF – liver failure, LA – liver abscess, CH – chronic hepatitis

Figure 2. Mean Mg levels in hair samples from cats with different liver disorders (LD) compared to clinically healthy animals (HA)

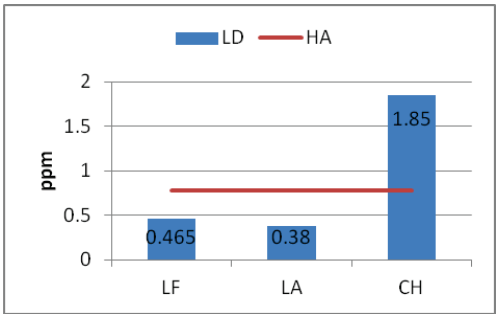
Na levels registered significant differences in hair samples from cats with different liver disorders compared to the control samples ($p$=0.03). The highest Na levels were observed in hair samples from cats with chronic hepatitis (1272.05 ppm), followed by a mean level almost 3 times lower registered in hair samples from clinically healthy cats (414.9 ppm) (figure 3). The differences were significant between its levels in hair samples from cats with chronic hepatitis and those in hair samples from cats with the other liver disorders and control samples ($p$<.05), and there were no differences between Na levels in hair from cats with liver failure, liver abscess, and in healthy animals, respectively.




*LF – liver failure, LA – liver abscess, CH – chronic hepatitis

Figure 3. Mean Na levels in hair samples from cats with different liver disorders (LD) compared to clinically healthy animals (HA)

Another element that registered significant differences between its levels, was the trace mineral Cu ($p$=0.05). Cu levels registered the highest concentrations in cats with chronic hepatitis hair samples (1.85 ppm), followed by those in clinically healthy cats (figure 4).




*LF – liver failure, LA – liver abscess, CH – chronic hepatitis

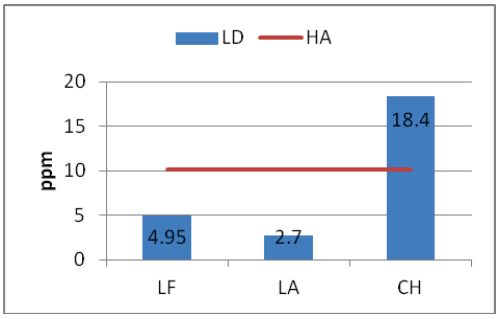
Figure 4. Mean Cu levels in hair samples from cats with different liver disorders (LD) compared to clinically healthy animals (HA)

The correlations of the significant differences between its levels could be made between hair samples from cats with liver failure and liver abscess vs. those from cats with chronic hepatitis and clinically healthy cats ($p$<.05). The differences were not significant between the levels in cats with liver failure and liver abscess, and also between the levels in cats

with chronic hepatitis and those in healthy animals. Haynes and Wade (1995) have reported that liver degeneration in Siamese cats was due to excessive copper accumulation, which could also influence its levels in keratin-rich tissues. Other researchers have found that Cu levels in hair samples from dogs with atopic dermatitis were significantly decreased ($p$=0.01) in the study group compared to control samples (Badea et al., 2016b).

As it was reported by other researchers, that Cu accumulation lead to necroinflammatory liver disorders (Haynes and Wade, 1995; Labuc, 2012), the significant higher Cu mean levels in hair samples from cats with chronic hepatitis could also be positively correlated, in the present study, to this chronic disorder.

Zn also registered significant differences between its levels ($p$=0.05), the highest concentrations being found in cats with chronic hepatitis hair samples (18.4 ppm), followed by those in clinically healthy cats, which were almost 2 times lower (figure 5). The correlations of the significant differences between its levels could be made between hair samples from cats with liver failure and liver abscess vs. those from cats with chronic hepatitis ($p$<.05). The differences were not significant between the levels in cats with liver failure, liver abscess, and clinically healthy cats, and also between the levels in cats with chronic hepatitis and control group.




*LF – liver failure, LA – liver abscess, CH – chronic hepatitis

Figure 5. Mean Zn levels in hair samples from cats with different liver disorders (LD) compared to clinically healthy animals (HA)

Skibniewska et al. (2011) have reported that Zn registered higher levels in female feral cats (268.09 ppm), and lower in pet female cats (214.49 ppm), levels that were much higher than those registered in the presented study, independent of health status.

All the other determined elements registered no significant differences ($p$>0.05) between the levels in hair from cats with different liver disorders and clinically healthy cats.

In the case of K, even if significant differences were not determined between hair samples from the healthy cats and those with liver disorders, its levels registered the same pattern as that found in Na. K levels were the highest in hair samples from cats with chronic hepatitis (104.6 ppm), and the lowest in hair samples from cats with liver abscess (9.2 ppm), which was almost 6 times lower than in the case of clinically healthy cats hair samples.

Fe also registered no significant differences between its levels in study groups and control hair samples ($p$=0.50). The highest mean level was observed in cats with chronic hepatitis hair samples (417.7 ppm), followed by liver failure and liver abscess cats hair samples, and the lowest mean levels in clinically healthy cats (15 ppm). Horiguchi et al. (2011) found that cadmium causes hemolysis, which could determine iron accumulation. Also, Fe accumulation as transition metal plays an important role in oxidative stress, leading to necroinflammatory liver disorders (Labuc, 2012).

The highest Se mean level was observed in hair samples from clinically healthy cats (0.103 ppm), followed by hair samples from cats with chronic hepatitis and liver failure. Se levels in hair samples from cats with liver abscess were below the method detection limit. Se also registered no significant differences between its levels in study groups and control hair samples ($p$=0.36).

The potential toxic metals have registered no significant differences between their levels, but almost the same pattern was observed in all these elements' mean levels. The highest levels of Al, Cd, Ni, and Pb were found in hair samples from cats with chronic hepatitis (40.95, 0.343, 1.785, and 0.42 ppm, respectively), followed by Ni and Pb levels in hair samples from cats with liver abscess and liver failure, by Al levels in healthy cats and cats with liver abscess, and Cd levels in liver abscess and clinically healthy cats.

Hyder et al. (2013) found that environmental

cadmium exposure was associated with different liver disorders in humans.

Markiewicz-Górka et al. (2015) reported that exposure to low levels of Cd and Pb through food, water, and air is common in industrial and urban areas and is a real threat to the health of the general population, and could be evaluated using animal models.

Badea et al. (2016a) have reported that, in hair from clinically healthy cats over 5 years of age, Al, Cd, Ni, and Pb registered higher values than those in hair samples from the cats with renal failure, and, in the present study, the same elements registered levels almost 2 times lower in control hair samples.

Kosla et al. (2011) reported that higher Ni content in the coat of cats was found in hair samples from cats above 2 years of age (above 2 years - 0.87; below 2 years - 0.58 ppm). Another research reported that, in female cats with renal failure, Ni (study group - 0.22; control group - 0.19 ppm) and Pb (study group - 0.05; control group - 0.03 ppm) registered higher values compared to clinically healthy females. Filistowicz et al. (2011) have reported that farm fox hair registered higher levels for both Ni (farm foxes - 0.48; wild foxes - 0.3 ppm) and Pb (farm foxes - 0.64; wild foxes - 0.63 ppm). de Almeida Curi et al. (2012) have found traces of Pb in all species analyzed (maned wolf - 2.34 ppm; crab-eating fox - 2.45 ppm; hoary fox - 1.5 ppm), but Cd was not detected.

Skibniewski et al. (2013) have found that Pb content in hair samples from pet female cats registered lower levels (0.98 ppm) than feral female cats (3.58 ppm), which are much higher compared to the highest Pb levels determined in hair samples of both healthy animals and cats with different liver disorders analyzed in the present study.

Another research showed that in cats with renal failure, females registered higher levels of toxic metals like Al (10.56 ppm) and Ni (0.21 ppm) (Badea et al., 2016a), which could also be observed in the present study for the same elements, and also for Cd, Cu, Pb, and Zn in hair samples of cats with chronic hepatitis.

López Alonso et al. (2004) have reported that interactions between trace and toxic elements indicate that toxic elements compete with the essential metals, even at low levels of metal exposure, but in the same time the mineral status evaluation needs to be realized choosing a specific tissue or organ, also indicated by Elsenhans et al. (1987), who have reported that potential target organs for the evaluation of metal exposure need to be carefully analyzed for interfering metal-metal interactions.

## CONCLUSIONS

The current study presents one of the first investigations of the suitability of hair as an indicator for the mineral status of cats with different liver disorders in an urban area of Romania.

Moreover, hair mineral levels determined in the present research may be considered as a contribution to a base of reference levels of minerals in female cats in Romania.

Ca, Cu, Mg, Na, and Zn registered significant differences between their levels in hair samples from cats with different liver disorders and clinically healthy cats.

In this research, hair samples from cats with chronic hepatitis registered higher levels of Cu, Zn, and Na, and Ca and Mg registered higher levels in control samples.

Cu mean levels in hair samples from cats with chronic hepatitis could be positively correlated to this chronic disorder.

No significant differences have been registered for heavy metals present in hair samples from cats with different liver disorders and clinically healthy cats.

Independent of the significant differences, the highest values were registered by all the elements in hair samples from cats with chronic hepatitis, excepting Ca, Mg and Se levels, which were higher in clinically healthy cats.

Mean hair concentrations of all toxic elements, Al, Cd, Ni, and Pb, were situated below the determined levels for cats reported by other authors.

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# A SURVEY ON ECTO- AND ENDOPARASITES IN SOME MIGRATORY BIRDS IN THE DANUBE DELTA, ROMANIA

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***Abstract***

*Migratory birds are important carriers and reservoirs for a variety of pathogens, with a great potential of their spreading. The Danube Delta Biosphere Reserve is one of the most important migration stopover for a great diversity of migratory birds where they feed and recover the energy supplies towards the African wintering grounds. Knowledge about the circulation of pathogens, parasites included, in different areas and different bird gatherings contributes to a better understanding of the epidemiology of some parasitic diseases which are responsible for changing in host population dynamics and the potential risks for vector-borne diseases, including zoonoses. Therefore, the present study aimed to investigate the occurrence of ecto- and endoparasites in some migratory birds at the hotstop in the Danube Delta (Southeastern Romania). For this, a total number of 260 birds (Passeriformes and Pelecaniformes), belonging to five families (Sylviidae, Turdidae, Laniidae, Paridae, Ardeidae) and 12 species were investigated, during of a ringing session, in August 2016. All birds were examined for external parasites. Additional, 23 birds were also subjected for endoparasite infections using flotation method and microscopic examination. Overall, out of the total birds 22.30% (58/260) were found positive for ectoparasites (feather mites, chewing lice), while 12 of the 23 investigated (52.17%) were positive for internal parasites. As ectoparasites, were detected feather mites in 21.53% (56/260), belonging to Trouessartiidae and Proctophyllodidae families; chweing lice in 0.38% (1/260), belonging to the genus Menachantus (Phtiraptera), and fowl mite Ornithonyssus spp. (Mesostigmata) (0.38%; 1/260). The most common endoparasite infection was with coccidia (Apicomplexa) (39.13%; 9/23), represented by Eimeria spp. and Isospora spp. The prevalence of internal and external parasites found in the present study highlight the need for further investigation of parasitofauna in wildbirds considering the fact that, parasites might have a negative effects on population dynamics of birds.*

***Key words****: parasites, migratory birds, Passeriformes, Danube Delta, Romania.*

## INTRODUCTION

The Danube Delta Biosphere Reserve (DDBR) is one of the most important migration stopover for a great diversity of migratory birds (more than 250 different species) where they feed and recover the energy supplies towards the African wintering grounds. Around 900 million birds fly annually in this place during the two migratory sessions (spring and autumn) (Zehtindjiev and Liechti 2003).

The Danube Delta has a greater diversity of habitats and food resources than other passage areas from Europe, and for this reason, migratory birds make a longer stopover in the Danube Delta wetlands (Ion, 2009).

Migratory birds are important carriers and reservoirs for a variety of pathogens, with a great potential of their spreading, including pathogens with zoonotic risk. In Europe, different migratory bird species migrate to survive seasonal climate changes (Hahn et al., 2009).

This migration provides the right path for circulations of different pathogens, including parasites (Fuller et al., 2012).

Among bird ectoparasites, feather mites (Acari: Astigmata) are the most diverse arthropods found on different orders of birds; they are living permanently on the feathers of birds (Proctor, 2003; Clayton et al., 2010).

Other parasites, suchs as insects, chewing lice (Phthiraptera: Ischnocera, Amblycera) are also permanent ectoparasites of different domestic and wild birds species, that feed on feathers and skin scales. They can deteriorate the quality of the plumage, provoke small holes on

feathers increasing the feather brakes and chronic dermatosis (Vas et al., 2008; Mitrea, 2011).

Different endoparasitic species such as, protozoan, nematodes, cestodes can cause clinical diseases and mortality in wild birds (Rossi et al., 1977; Schoenaer et al., 2012).

Even though, not all parasites shows lethal effects, some parasite emergence can change host population dynamics and modify coevolution relationships between hosts and their parasites (Best et al. 2010).

Improving the knowledge of ocurrence and circulation of parasites in migratory birds can contribute to a better understand of the distribution and epidemiology of some diseases and the role of birds as carriers and spreading of some vector-born pathogens. Little information is available on parasite species encountered in migratory birds in the Danube Delta Bosphere Reserve. Therefore, the present study aimed to investigate the presence of ecto- and endoparasites in some migratory birds at the hotstop in the Danube Delta.

## MATERIALS AND METHODS

*Animals and study area*

The study was carried out in the Danube Delta Biosphere Reserve, near Maliuc village (45° 10′ 31.02″ N, 29° 4′ 35.72″ E). DDBR (added in 1991 on UNESCO World Natural Heritage list), has a significant ecological diversity (30 types of ecosystems) and the existence of many areas where human impact is still absent (Goriup et al., 2007).

The study was performed in August 2016, during a ringing session, within an ornithological camp. A total of 260 birds were parasitological investigated; the birds were captured using the ornithological mist-nets and ringing. Nests were arranged in reed bands and were checked at every 30 minutes. All birds were extracted and identified by species (and whenever possible aged) and ringed with individually numbered metal rings. All the birds have been subjected for parasitological investigations, as follows:

(i) for ectoparasites, prior to their release, birds were carefully inspected for the presence of ectoparasites; when were present, they were collected from the body of birds with a fine forceps and preserved in absolute ethanol for later examination using a separate vial for each bird. Additional, when feather mites were observed, one feather were collected and preserved in separate vial, for further mite identification. The ectoparasites collected were identified under microscopic examination to genus and/or species level, based on morphological features (Mironov, 2012; Mironov and González-Acuña, 2013).

(ii) for endoparasites, fecal samples were collected only if there were present in the bag where the bird was kept; samples were placed in tubes and stored at 4°C and transported to the laboratory and examined in the next two days, by the flotation method (saline solution), for detection of protozoan oocysts and worm eggs (Ionita and Mitrea, 2013).

## RESULTS AND DISCUSSIONS

During the ringing session performed in August 2016, in the DDR, a total number of 354 birds were ringed and 260 of them were investigated for external parasites; out of these, from 23 birds, faecal sample were also collected for parasitological examination.

Overall, the total number of 260 birds captured and direct examined during the ringing session belonged to 12 species and four families of Passerines: Sylviidae (seven species), Laniidae (one species), Turdidae (one species), Paridae (two species), and one family of Pelecaniformes: Ardeidae (one species), (Table 1).

Out of the total examined birds, 22.30% (58/260) were found positive for ectoparasites. Of the ectoparasites, were detected as follows: feather mites, in 21.53% (56/260), belonging to *Trouessartiidae* (Fig. 1. A, B) and *Proctophylloidae* families (Fig. 2); chweing lice, in 0.38% (1/260), belonging to the genus *Menachantus* (Phtiraptera) (Fig. 3), and fowl mite, *Ornithonyssus* spp. (Mesostigmata) (0.38%; 1/260).

Of the 23 birds from which fecal samples were collected and coproparasitological investigated, 12 (52.17%) of them were found positive for internal parasites. The endoparasites found in the feces of wild birds in the present study, belonged to protozoa, nematodes and cestodes.

The most common endoparasite infection were represented by protozan coccidia, *Eimeria* spp. and *Isospora* spp. 39.13% (9/23) (Fig. 4. A, B). However, even though some eggs have been identified to major groups (coccidia, cestodes, nematodes), they could not be precisely identified to species leavel.

In table 1 are detailed the recorded parasitological data.

Table 1. Birds classification and the number of positive for ecto- and/or endoparasites of some wild birds in the Danube Delta Biosphere Reserve

| Nr. crt. | Birds species | Family/ migrating statues/ type of diet | No. of positive birds with ectoparasites | | | | No. of positive birds with endoparasites | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Total | Feather mites | Chew-ing lices | Fowl mite | Total | Eimeria/ Isospora spp. | Cesto-des | Nema-tode |
| 1. | *Acrocephalus arundinaceus* | Sylviidae/ migratory/ insectivorous | 16 | 16 | 0 | 1 | 3 | 3 | 0 | 0 |
| 2. | *Acrocephalus schoenobaenus* | | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| 3. | *Acrocephalus melanopogon* | | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4. | *Acrocephalus palustris* | | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| 5. | *Acrocephalus scirpaceus* | | 27 | 27 | 0 | 0 | 6 | 4 | 1 | 0 |
| 6. | *Ixobrychus minutus* | Ardeidae/ migratory/ carnivorous | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7. | *Luscinia luscinia* | Turdidae/ migratory/ insectivorous | 2 | 2 | 0 | 0 | 1 | 0 | 0 | 1 |
| 8. | *Locustella luscinioides* | Sylviidae/ Migratory/ insectivorous | 3 | 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| 9. | *Lanius collurio* | Laniidae/ migratory/ insectivorous | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 |
| 10. | *Parus major* | Paridae/ partial migratory/ insectivorous | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| 11. | *Parus caeruleus* | | 6 | 6 | 0 | 0 | 0 | 0 | 0 | 0 |
| 12. | *Sylvia borin* | Sylviidae/ migratory/ insectivorous | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 13. | **Total (n)** | | **58** | **56** | **1** | **1** | **12** | **9** | **1** | **2** |
| 14. | **Prevalence (%)** | | **22.30** | **21.53** | **0.38** | **0.38** | **52.17** | **39.13** | **4.34** | **8.69** |


Fig. 1. *Trouessartia* spp.: A) dorsal view of male; B) dorsal view of female

Fig. 2. *Proctophillodes* spp. ventral view (10X)

Fig. 3. *Menachantus* spp. dorsal view (stereomicroscop)

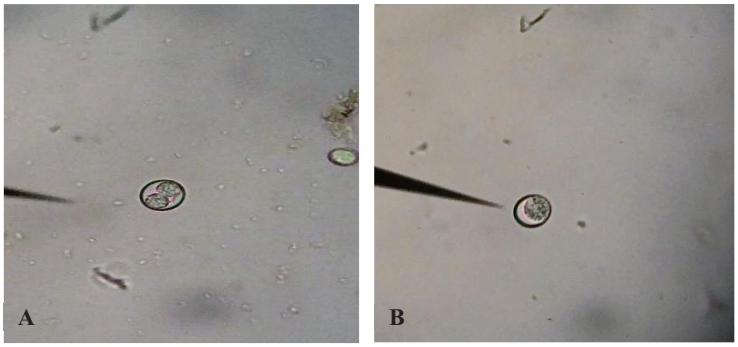


Fig. 4. Coccidial oocyst in fecal samples from passerines birds (*Acrocephalus scirpaceus*): A) *Isospora* spp. oocyst (20X); B) *Eimeria* spp. oocysts (20X)

In the present study, feather mites belonged to *Proctophyllodida*e and *Troussartiidae* families, similar to the data obtained by other authors (Lyra-Neves et al., 2003; Kolarova and Mitov, 2008), where the most frequently species of feather mites found, belonged to *Proctophyllodes*, *Analges, Pterodectes* and *Trouessartia* genera. The families *Proctophyllodidae* and *Trouessartiidae* are predominately associated with wild birds passerines, while species of the families *Analgidae* are known from various orders of birds (Proctor, 2003).

Regarding the ectoparasites in wild birds, in Romania, more than 25 species of feather mites were studied (Constantinescu et al., 2013) and from the chewing lices, suborder Ischnocera (91.91%) and Amblycera (8.09%) were recorded by some authors (Adam and Sandor, 2005).

Ectoparasites, including chewing lice, may play a role in bird migration, and especially in small transcontinental passerine birds (Sychara et al. 2011). The study performed in cliff swallows showed that chewing lices together with other ectoparasites may influence the return rate of birds from wintering sites to their nests (Brown et al. 1995).

In the present study, the most common endoparasites found in investigated birds were represented by Coccidia. Similar data were reported by Bandelj et al. (2015), in Slovenia, where the most frequently detected parasite infection in wild birds was with coccidians, and the prevalence of internal parasites was 15%. In the present study, intrenal parasites were more found in insectivorous passerines (39.13%; 9/23). It has been reported that the prevalence of internal parasites in European passerine birds is not associated with migration but with the type of diet, and insectivorous and omnivorous passerines birds were more prone to be infested with a variety of parasite during their feeding (Bandelj et al., 2015).

## CONCLUSIONS

The present study provides data on the presence and prevalence of some ecto- and endoparasites in wild birds. To our knowledge, endoparasite infections with *Eimeria* and *Isospora* found in wild birds in the DDBR are described for the first time in the present study.

The findings emphasize a high occurrence of feather mites in migratory birds from Danube Delta Biospfere Reserve.

Due to intense migration of wild birds, follow-up studies of prevalence of parasites species and other epidemiological factors that might have a negative effect on wild bird populations are useful and necessary.

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# THE MONITORING AND RESPONSE OF TRANSFUSION REACTIONS TO GLUCOCORTICOID THERAPY

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***Abstract***

*The transfusion reactions represents one of the major risks of transfusion therapy, regardless of the blood product used, whole blood, erythro-concentrate or frozen plasma.*
*This paper presents the evolution of transfusion reactions and treatment of 60 dogs during the period 2014-2016 monitored in the Clinics of the Faculty of Veterinary Medicine in Bucharest. The clinical signs, CBC, biochemistry were observed for all dogs as the animals came to the clinics.*
*The treatment provided has a base content of glucocorticoid mixture of drugs and complementary symptomatic therapy. The glucocorticoid therapy refers to the usage of hemi-succinate hydrocortisone at the beginning of each transfusion and at the administration of Prednisolone at the end.*
*The glucocorticoid combo therapy was used in all transfusions, regardless of the product or sub-product of blood being used. For 70% of the cases was used whole blood, for 20% erythro-concentrate and for 10% plasma.*
*The data processed using the parameters above shows a decreased number of transfusion reactions when used the glucocorticoid drug combo on the 50% of the case load in comparison to the other 50% which did not receive the glucocorticoid therapy prior to transfusion. This study shows that the glucocorticoid combo therapy might be helpful in a variety of cases in which the pathology presented allows the usage of glucocorticoid therapy.*

***Key words: transfusion, reactions, blood, glucocorticoid.***

## INTRODUCTION

Considering the risk and the necessity of the transfusion therapy, studying the transfusion reactions should be done more often covering as much details possible as well as monitoring the transfused patients.

According to BSAVA manual of Canine and Feline Haematology and Transfusion Medicine, transfusion reactions are divided in 2 large categories: immunological and non-immunological. The immunological reactions are of the most concern, especially the acute hemolytic reaction, as well as other sensitized allow-antibody's mediated incompatibilities. (Day et al., 2012). The acute hemolytic reaction refers to antigen-antibody, a type II, hypersensitivity reaction that usually manifests with a variety of clinical signs, fever, tachycardia, dyspnea, tremors, vomiting, shock, collapse and hemoglobinuria. (Day et al., 2012)

Non-immunological reaction is an anaphylactic type reaction with a various clinical signs such as edema, urticaria, pruritus, dyspnea, pulmonary edema. On this type of reaction, if not intervened, could express in the super-acute phase shock, hemolysis, even death, or they can evolve to 2-21 days and develop hemolytic syndromes. (Day et al., 2012).

This type of reactions are usually results of various modifications due to the patient's status, type of disease, infusion rate, blood quantity, hypokalemia, polycythemia, hyperproteinemia, hyperammonemia and hyperphosphatemia (Day et al., 2012). This type of reaction could be minimized in some cases with preventive measures such as the glucocorticoid combo (hydrocortisone hemi-succinate and prednisolone) and could ensure more efficacy and safety on the patient's transfusion.

According to the National Medicines Agency in Romania, hydrocortisone hemi-succinate is a systemic glucocorticoid being the ester group in the 21-position oxidril; it is a fast acting glucocorticoid with an half time of 90 minutes to intravenous administration and a therapeutic effect that could reach 8-12 hours.

The second glucocorticoid is prednisolone, also a systemic glucocorticoid, with an half time up to 3.6 hours to intravenous administration, and

prolonged to intramuscular or subcutaneous administration.
The glucocorticoid combo therapy was used prior and after the transfusion, hydrocortisone hemi-succinate pre-medicated at 15 to 30 minutes prior to transfusion and prednisolone at 4-6 hours from the start of the transfusion.

## MATERIALS AND METHODS

In order to characterize the evolution of the transfusion reactions, the study was performed on 60 dogs, 30 received glucocorticoid combo therapy and 30 did not received (control group), and were monitored during the transfusion at 15, 30, 45 minutes, 1, 2, 4, 12, 24 and 48 hours.
All the dogs were measured for clinical indicators (temperature, blood pressure, heart rate, respiratory rate, mucous membrane color, capillary refill time, attitude, response to transfusion therapy) and para-clinical indicators: CBC (pre-transfusion, 24, 48 hour, 7 to 21 days), biochemistry (urea, creatinine, aspartate transaminase, alanine transaminase, total protein, plasma color).
All subjects of this study were tested for D.E.A. 1.1 group, with various manufacturer group test.
The cases that received the glucocorticoid combo therapy were not suffering from any condition that was incompatible with this type of treatment.
The results of these patients were cross-matched to the type of blood product received, glucocorticoid medication received and intensity of the transfusion reactions: minor (fever, tachycardia, dyspnea, tremors, vomiting, hypokalemia, polycythemia, hyperproteinemia, hyperammonemia, hyper-phosphatemia), medium (collapse, hemoglobinuria, edema, urticaria, pruritus, pulmonary edema) and severe (shock, hemolytic syndromes and all immunological reactions).
All the data collected had been processed and interpreted to evaluate the efficiency of the glucocorticoid combo therapy in reducing the transfusion side effects.

## RESULTS AND DISCUSSIONS

The usage of the glucocorticoid combo therapy on the 30 dogs used in the study shows that the number of transfusion reactions diminished considerably. From these dogs, 70% of them (21 dogs) had no transfusion reaction, as to 30% (9 dogs) of these that had a type of transfusion reaction, 8 dogs had a minor reaction, one medium reaction, but no severe reaction observed (Figure 1).

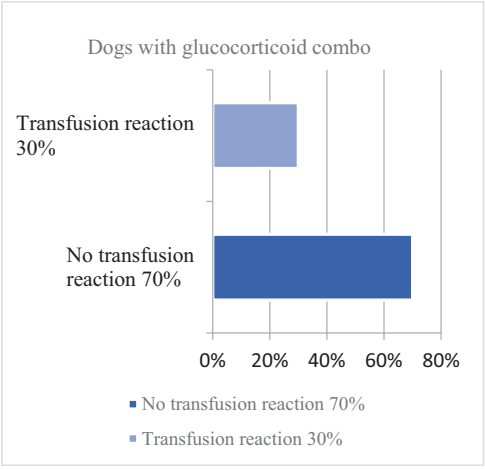


Figure 1. Percentage of transfusion reactions on dogs with glucocorticoid combo therapy

The other 30 dogs, the control group, showed a different balance 46.66% ( 14 dogs) had no transfusion reaction, as to 53.33% ( 16 dogs) that showed all of the intensity grades.
From the 16 dogs that had reactions, 10 of them had minor reactions, 5 of them with medium reaction and one severe reaction (Figure 2).

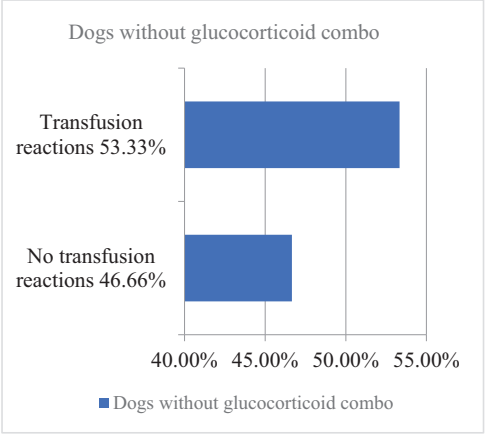


Figure 2. Percentage of transfusion reactions in dogs without glucocorticoid combo therapy

Another important factor in this study is the blood product used. The cross-match between the blood product and the transfusion reactions showed that from the total of 60 dogs, 58.3% (35 dogs) were non-reactive, and 41.6% (25 dogs) had a type of transfusion reaction.
The cross-match between transfusion reactions intensity and the blood product showed that 40% (10 dogs) had a minor reaction when whole blood was transfused, 8% (2 dogs) to frozen plasma and 24% (6 dogs) to erythro-concentrate.
The medium intensity reactions showed a diminished number, 12% (3dogs) to whole blood, 4% (1 dog) to frozen plasma and 8% (2 dogs) to erythro-concentrate.
There was only one dog (4%) with severe reaction to whole blood.
This cross-match of the reactions shows that an enlarged number of transfusion reactions were to whole blood 56% (14) dogs, 32% (8 dogs) to erythro-concentrate and 12% (3 dogs) to frozen plasma (Figure 3).

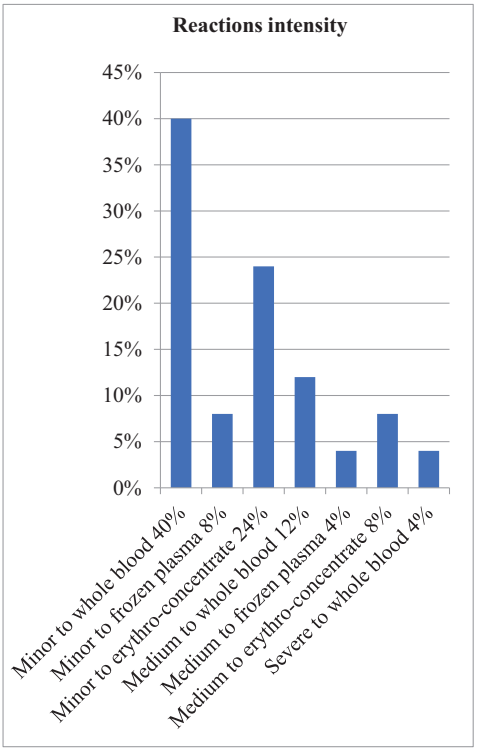


Figure 3. Percentage of transfusion reactions cross-matched to blood product

## CONCLUSIONS

The percentage of transfusion reactions in dogs that received the glucocorticoid combo therapy decreased by 24% in comparison to the control group.
Due to the glucocorticoid combo therapy, the transfusion reactions were less severe, the dogs that received it had only minor or medium reactions compared to the control group, which also presented severe reactions presented.
In conclusion, the usage of the glucocorticoid combo therapy in the cases where the pathology allows it, Hydrocortisone Hemi-succinate and Prednisolone, could be easily used in transfusion therapy and could be a real advantage to the patient and his evolution after transfusion.

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# CORRELATIONS BETWEEN HEART RATE AND LACTIC ACID DURING SUBMAXIMAL EXERCISE IN DOG

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***Abstract***

*Five healthy Golden Retrievers aged from 1.5 to 3 years old, three males and two females, were studied during a normal "playing" time considered as submaximal exercise, under ordinary circumstances, preserving the usual routine (location, normal weather conditions, to the same group). From the medical history, it was excluded previous symptoms as fatigue or other cardiac related symptoms based on subjective observations. Each individual heart rate was measured ten minutes intervals, during 60 minutes exercises, using a heart rate monitor device. Blood lactate was measured with fast strip, at the beginning and at the end of the test. The results showed that heart rate was normal in 4 cases, ranging between 101 and 173 beats per minute( bpm), and abnormal in one dog reaching the maximal heart rate during a submaximal test, with four consecutive values between 215 and 245 bpm. The lactate values were normal for all the subjects, at the end of the test, none of the dogs achieving the superior lactate threshold. After performing a cardiac examination in all five subjects (including Doppler echocardiography and Holter electrocardiogram), a good correlation was observed between heart rate values of the dog that reached maximal heart rate and a suspicion of a cardiac problem (preclinical case of dilated cardiomyopathy), but no evidence of altered or a raise in blood lactate under this submaximal test.*

***Keywords:*** *dog, exercise, heart rate, lactic acid.*

## INTRODUCTION

Numerous studies have been conducted in order to establish a gradual scale of different types of exercise in dogs and horses (Dojana *et al*., 2008), using human medicine protocols adapted in veterinary medicine. These adapted protocols (for example Bruce's protocol) are based on the assumption that a normal and gradual cardiovascular response is obtained in otherwise healthy subjects. Instead, an abnormal response will be recorded in dogs affected by different cardiac conditions, such as decompensate heart diseases (degenerative mitral valve disease, cardiomyopathy etc.) or occult diseases (paroxysmal arrhythmia, occult stage of dilated cardiomyopathy etc.). The variety of breeds that are prone to develop heart disease raised the issue of intraoperatory variability when a cardiovascular stress test is performed, especially on the individual response. In dogs, we are confronting with a large variation in effort tolerance, both in fast running or endurance, starting from the top "athletes" like the Alaskan Sled Dogs to brachycephalic breeds, such as French Bulldogs, or "sprinters" as Greyhounds. Another important variable is the composition or type of muscle fiber, as an adaptive response to training (Toniolo *et al*., 2006).

The major difference between breeds, and their performances are due to prevalence of "fast twitch fiber" or Type II, and "slow twitch fiber", or Type I (Dojana *et al*., 2013), but integrity of cardiopulmonary or hepatic functions play an important role also. At this time, there are several protocols that can be used in veterinary medicine, but as is stated before, most of them are adapted from human medicine. The 6 M.W.T. - six minute walk test (Boddy *et al*., 2004; Swimmer *et al*., 2011) is used at this time to reduce the variability of data received from the patients, when they were asked about at which level of fatigability arrived the dogs during perform simple tasks. The 6 M.W.T. and submaximal exercise (Kittleson, 1996) are used at the moment for dogs that already have a cardiopulmonary pathology (for example decompensate heart disease, idiopathic pulmonary fibrosis etc.).

But this protocol is not reliable in Alaskan Sled Dogs (Stepien, 1998), or other breeds that are known to sustain maximal effort over a short period of time (for example breeds that are participating in Mondioring or Agility Competition).

The purpose of this study was to investigate the correlative evolution of heart rate and lactate during a normal session of "playing", in natural conditions or psychical stimulation. In order to obtain a reference and objective guiding values that can be used to define fatigability in Golden Retrievers, we aimed to observe the fluctuation of heart rate and blood lactate during 60 minutes of exercise.

## MATERIALS AND METHODS

Five healthy Golden Retrievers, 3 males and 2 females, ranging from 1.5 to 3 years old were included in this study. The owners were instructed answer a short list of questions, which are normally asked in the veterinary practice during anamnesis, and to estimate without any other data the "fitness" condition of each dog. Based on the recent medical history, and the absence of clinical signs, all five dogs were considered healthy, fit and in excellent condition. Four were stated also that signs of fatigue appear after 1-2 hours of intense playing, running or swimming. Just in one dog was stated that signs of moderate exercise intolerance appear after 30 minutes of playing. None of the dogs were used with the medical device, but they were used with ordinary restraining belt.

There were none specific exercises with the intended purpose to achieve a premature state of fatigue. The dogs were allowed to play as usually, with each other and they were free in open park (Figure 1), not restrained in the leash, except for the brief moment when we collected the blood sample. The Sports Watch device has the advantage of easy reading of heart rate in real time, during exercises. The stress test is safer, allowing the abrupt termination of the test if the heart rate rises at a level beyond maximal limit.

Heart rate was measured in real time using this adapted device, which consist in two units, a belt used to receive impulses from the precordial area and a watch with real time display of heart rate. The belt has two sensors that need to be lubricated with simple water, in order to assure a better electric communication between the device and the dog's chest (Figure 2). After the heartbeat was captured, the belt was secured with co-adhesive elastic bandages. The heart rate was measured from 10 to 10 minutes, in each dog, during 60 minutes of session. The measurements were made during running or walking or under 30 seconds from the beginning of the rest period in order to obtain a reliable value, with no other influences from physiological recovery heart rate, which is a component of balance between sympathetic and parasympathetic nervous stimulation.

Figure 1. 60 minutes of free exercises and playing

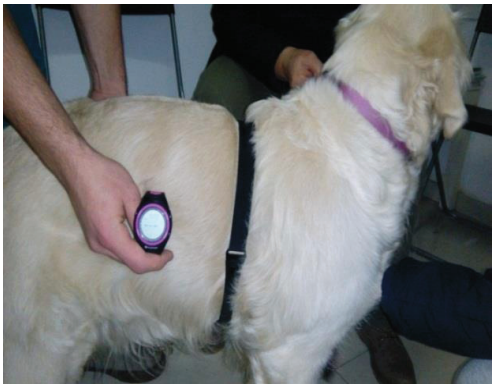

Figure 2. The "Sports watch" device

The blood lactate was measured using the fast strip "Accutrend Lactate and Glucose", by venous puncture, at the beginning and at the end of the program, just before the recovery period, in order to obtain the values of lactate before capillary transport and hepatic biotransformation.

## RESULTS AND DISCUSSION

Measurements of the heart rate, during the 60 minutes were taken for each of the dogs, in 4 cases the average value was 156 bpm, 115 bpm, 162 bpm, and respectively 134 bpm, and in one case 193 bpm, above the normal superior range, with a maximal heart rate of 245 bpm (Table 1).

Table 1- Heart rate values (bpm), during 60 minutes of sub maximal exercise, measured from 10 to 10 minutes

| Time (min) | dog 1 | dog 2 | dog 3 | dog 4 | dog 5 |
|---|---|---|---|---|---|
| 10 | 144 | 117 | 123 | 160 | 102 |
| 20 | 159 | 238 | 101 | 149 | 133 |
| 30 | 157 | 243 | 120 | 171 | 133 |
| 40 | 170 | 245 | 113 | 173 | 125 |
| 50 | 151 | 215 | 133 | 153 | 131 |
| 60 | 156 | 103 | 104 | 167 | 180 |
| | | | | | |
| Mean ± SEM | 165± 8.5 | 193.5± 7.6 | 115.6± 11.3 | 116.1± 10.4 | 134.0± 8.8 |

Four dogs had values between the expected range during submaximal exercises, and they not achieved a maximal heart rate as in incremental exercise test on treadmill observed in Labrador Retrievers (Ferasin *et al*., 2009). In one case, the heart rate was above the average with a mean value of 193 bpm, with 4 consecutive values above 200 bpm. Based on the fact that none of the dogs did a maximal test, observing them during one hour, was assumed that the dog that reached a higher heart rate, had a cardiac pathology, unobserved until then. In the individual chart completed before the test, the owner of this dog stated that after 30 minutes of normal playing, it showed signs of fatigue, adopting a sternal recumbence, presenting tachypnea and reluctance to walk. After performing a complete cardiac exam, measuring the blood pressure, the 5 minute 12 lead electrocardiography, the 24 hour Holter electrocardiography, the transthoracic 2D-two dimensional echocardiography, TM-time motion method, and Spectral Doppler (CWD-continuous wave Doppler, PWD- pulsed wave Doppler), it was concluded that this dog didn't had decompensate cardiac problem. Some small lesion were observed, at a valvular level, valve with a modified aspect of the ending parts of the leaflets and with minor regurgitate jet on the mitral with a central pattern, and a small regurgitate jet at the tricuspid valve. Calculating the score for DCM- dilated cardiomyopathy according to Dukes (2003), the patient was negative (normal sphericity index, normal ejection fraction measured by Biplane Simpson method, shortening fraction and ventricular volumes). The 12 EKG lead were normal, and the Holter exam also was negative for cardiac arrhythmia, with no ventricular extra systole noted.

Table 2- Lactic acid values (mmol/L) in the 5 dogs during the 60 minutes of submaximal exercise measured during resting and at the end of the normal maximal

| No. of the dog | dog 1 | dog 2 | dog 3 | dog 4 | dog 5 |
|---|---|---|---|---|---|
| Resting values | 1.2 | 1.3 | 1.0 | 1.5 | 0.9 |
| Values at the end of the exercise | 1.1 | 1.7 | 0.8 | 1.2 | 2 |

The blood lactate values obtained were in normal reference ranges for dogs, and the threshold was considered achieved when the resting values were above the maximal with 1 mmol/l (Yoshida *et al*., 1987). None of the five dogs reached the lactate threshold, after 60 minutes of normal play, and in all the cases the resting values were normal. The values of lactate did not correlate with the increased maximal heart rate observed in one of the dogs. Based on the amount of data from human medicine, normally is tend to apply the same protocols in dogs, (ex. 6 Minute Walk Test), but the numerous variable that we deal with regarding the peculiar physiology of dogs (ratio between types of muscular fiber contained in the muscle involved in locomotion, individual and breed endurance, normal hemodynamic etc.) (Toniolo, 2006), this test must be "adjusted" to every breed on a base protocol.

The opportunity of this test is suggested by the lack of specificity in case of routine practice, were the symptomatology described is very ambiguous. Fatigue or lack of energy varies from a subjective point of view in each case, most of the time the owners making a parallel with human endurance. In some cases, they describe the fatigue based on experience, and this could be applied in older dogs, but in young dogs (1-3 years), the data received are very confusing. Another aspect is the reduced psychological need to play in dogs when they reach the "adult" age, between 1.5 and 2 years, leaving a window of 12-24 months when we

are not able to quantify the measure of fatigue. At this time protocols exist for majority of the breeds used in competitions, like Alaskan Sled Dog or Greyhound, but with no real use in daily practice (Stepien *et al*., 1998).

The fact that none of the five dogs, reached the lactate threshold during 60 minutes of normal play, even if one dog achieved the maximal heart rate, suggested that Golden Retriever are less prone to show fatigue in a normal session or in a daily routine, obscuring in that way one of the first symptoms of heart disease. On the other hand, the signs of fatigue during 60 minutes or less, presented by this breed, on young and "healthy" dogs, should be considered as a sign of potential heart disease.

## CONCLUSIONS

The lactate threshold and maximal heart rate in these dogs performing a 60 minutes daily routine was not achieved under normal conditions, and the maximal heart rate observed in one case did not correlate with lactate threshold.

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# ASCOSPHEROSIS INCIDENCE IN BEES INVESTIGATED FOR MAJOR BACTERIOSIS IN THE BEEKEEPING YEAR 2016

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***Abstract***

*Ascospherosis is an invasive mycosis occurring in Apis melifera bees, caused by Ascosphera apis that affects the 1-5 days aged bee larvae of maximum receptiveness at the age of 1-2 days. From the total of 18 apiaries identified in the active-inactive season 2016 in which the evolution of mycotic diseases was diagnosed, the chalk brood was present as a morbid entity with unique evolution in 10 apiaries (55.55 %), stone brood evolved in 2 apiaries (11.11 %), and mycotic diseases of mixed evolution were registered in 3 apiaries (16.66 %), out of which suspicions of major bacterial diseases in one apiary (5.55% cases) and in 2 apiaries evolved together with internal and external parasitoses (11.11 %). Regional incidence of chalk brood places the south-east area on first place having over 2/3 of positive tests. Season incidence of the chalk brood shows that over 38.8 % cases presented it at the end of the beekeeping season, and in the inactive season months (January – February) the incidence is minimum (11.11 % cases). Complex laboratory tests in all the 18 apiaries diagnosed positively with mycotic diseases permitted identification of Ascosphera apis spores in the samples collected from live bees intestines, pollen, bee bread and brood combs. Bee colonies in the monitored apiaries (59.56 %), in which ascopherosis evolved and did not present clinical signs, may be deemed infestation sources.*

***Key words****: Apis mellifera carpatica, chalk brood, honey bee.*

## INTRODUCTION

Ascospherosis is an invasive mycosis found in *Apis melifera* bees, caused by *Ascosphera apis*, which accompanies most often the disease episodes in major bacteriosis in bees (Savu & al, 2013).

The history of the incidence and geographical spread of the parasitosis in *Romania* is based on research carried out in the Beekeeping Research and Production Institute in Bucharest, which after 1971 made available for beekeepers the product *Micocidin*, especially for prophylactic purposes and to stimulate bee colonies (*Ogradă, 1986*).

The causal agent *Ascosphera apis* affects larvae aged 1-5 days, of maximum receptivity when aged 1-2 days.

After spores' germination on bee brood larvae, these will invade their organism so that the vegetative forms will invade the entire larva under the form of a mycelium (Yoshiyama Mikio, 2011). Mummified larvar forms end up being dark brown to black in color and remain an infestation source (Sarah A. Maxfield-Taylor*, 2015*).

Taxonomic classification of the etiologic agent *Ascosphera apis* is the following: *Ascomycota; Pezizomycotina; Eurotiomycetes; Eurotiomycetidae; Onygenales; Ascosphaeraceae; Ascosphaera apis* (Annette Bruun Jensen et al*., 2011*).

## MATERIALS AND METHODS

Studies performed in the beekeeping year 2016, as part of the research project 157/2014, on a number of 287 apiaries belonging to all beekeeping regions of Romania (N, S, E, V), out of which 18 apiaries were selected for investigations regarding laboratory confirmation of the clinical evolution of mycotic diseases caused by *Ascosphera apis*.

The 18 apiaries diagnosed positively for mycoses totaled 1,625 bee colonies out of which 531 bee colonies were affected by the disease and 126 bee colonies presented mortality.

Laboratory tests were carried out on intestine samples collected from live bees, pollen and bee bread, from bee colonies diagnosed positively for mycoses. The methodology of

laboratory tests observed protocols O.I.E. 2008, for bee diseases diagnosis, also using an original method of investigating adult bee intestine content.

## RESULTS AND DISCUSSION

The results of clinical tests confirmed increased regional incidence of chalk brood in Romania in the S-E area of the country (66.66 % positive sample).
This may be justified also through the presence of a large number of samples from this area collected while monitoring major bacterioses in bees.
The regional incidence of chalk brood in Romania in the beekeeping year 2016 is presented in table 1 and figure 1

Table 1. Regional incidence of chalk brood in Romania

| Area of investigated apiaries | Number of apiaries |
|---|---|
| South | 12 (66.66 %) |
| North | 3 (16.66 %) |
| Esst | 2 (11.11 %) |
| West | 1 (5.56 %) |

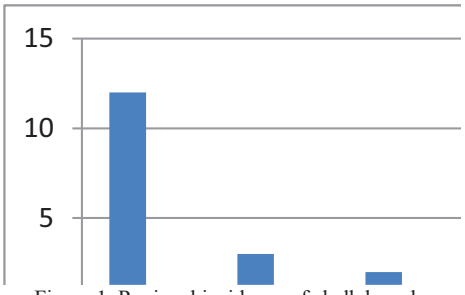


Figure 1. Regional incidence of chalk brood in Romania in the active-inactive season 2016

As regards the season incidence of this disease in bee brood, we noticed at the end of the active season an incidence in 38.88 % cases, as compared to the end of the inactive season (11.11 %), an indication of the dependence on the evolution of brood development in hives and, probably due to a protection effect of propolis with which hives are padded at the end of the active season, that has an antiseptic action during the inactivity period (November, December, January and February) (Vojvodic, S and al. 2011a., Vojvodic, S and al 2012.).

A slight increase in the number of cases occurs in sprin months (March, April, May) when the bee brood develops (22.22%) and progressively grows in the summer (June, July, August), at the flow peak. Season incidence of chalk brood throughout the entire beekeeping season 2016 is presented in table 2 and figure 2.

Table 2. Season incidence of chalk brood in the (active-inactive) beekeeping season 2016

| Season | Number of apiaries |
|---|---|
| Winter (January - February) | 2 (11.11 %) |
| Spring (March –April -May) | 4 (22.22 %) |
| Summer (June – July - August) | 5 (27.77 %) |
| Autumn (September - October) | 7 (38.88 %) |

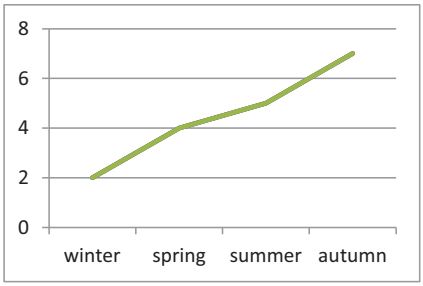


Figure 2. Status of season incidence of chalk brood in the 18 apiaries diagnosed positively in the (active-inactive) beekeeping season 2016

Of the 287 tested apiaries, 18 apiaries (6.27 %) were declared positive, having clinical signs typical of mycotic diseases.
The 18 apiaries diagnosed positively were made up of 1,625 bee colonies, out of which 531 bee colonies were affected by the disease, with mortality in 126 bee colonies) (Table 3, figure 3).

Table 3. Status of investigations in the apiaries examined in the beekeeping active-inactive season 2016

| Number of examined apiaries | Number of apiaries diagnosed with mycotic diseases | Total number of bee colonies affected by mycotic diseases | Bee colonies affected by chalk brood | Mortality |
|---|---|---|---|---|
| 287 (100 %) | 18 (6.27 %) | 1,625 (100 %) | 531 (35 %) | 126 (8.22 %) |

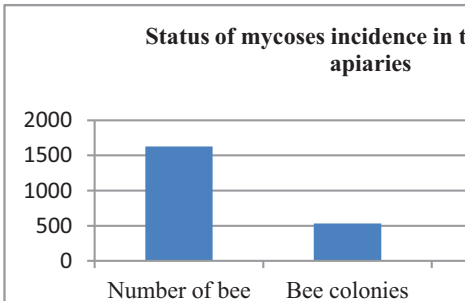


Figure 3. Status of investigations in the examined apiaries diagnosed with chalk brood in the beekeeping season 2016

Of the total of 18 apiaries in which mycotic diseases evolution was diagnosed, chalk brood was present as a unique morbid entity in 10 apiaries (55.55 %), stone brood evolved in 2 apiaries (11.11 %), and mycotic diseases with mixed evolution were registered in 3 apiaries (16.66 %).

The evolution of mycotic diseases with bacterial diseases was registered in one apiary (5.55%), and the evolution of mycotic diseases together with internal and/or external parasitoses was registered in 2 apiaries (11.11 %).

The evolution of mycoses alone or in association with other diseases is presented in table 4.

Table 4. Incidence of apiaries affected by mycotic diseases in the active season 2016

| Apiaries with | | | | | |
|---|---|---|---|---|---|
| mycotic diseases | chalk brood | stone brood | mixed mycotic diseases | mycotic diseases + suspicion of major bacterial diseases | mycotic diseases + parasitoses (internal, external) |
| 18 (100 %) | 10 (55.55 %) | 2 (11.11 %) | 3 (16.66% ) | 1 (5.55 %) | 2 (11.11 %) |

Following the laboratory tests, 287 test bulletins were issued (for each apiary), according to work protocols as part of project 157/2014 (stage 3/2016). Clinical tests allowed identifying in the monitored apiaries mycoses caused by *Ascosphera apis* in 18 apiaries, based on the aspect of brood combs in which the presence of the typical chalk brood was found (Fig. 5, 6, 7).

Complex laboratory testing of all the 18 apiaries positively diagnosed with mycotic diseases permitted the identification of *Ascosphera apis* spores in the samples collected from live bees intestine, pollen, bee bread and brood combs (Figure 8). According to the guidelines of good practice in beekeeping, easily accessible to beekeepers, the use of uncontrolled pollen for supplementary feeding of bees in Romania is avoided (Beekeepers' Association in Romania, 2011).

Figure 5. Diagnosed suspicion of major bacterial diseases accompanied by ascospherosis (Pathology Laboratory, ICDA, Nikon D 200)

Figure 6. Aspect of an affected chalk brood larva (Pathology Laboratory, ICDA, Nikon D 200)

Figure 7. Brood larva affected by ascospherosis (white color) compared to a normal larva (Pathology Laboratory, ICDA, Nikon D 200)

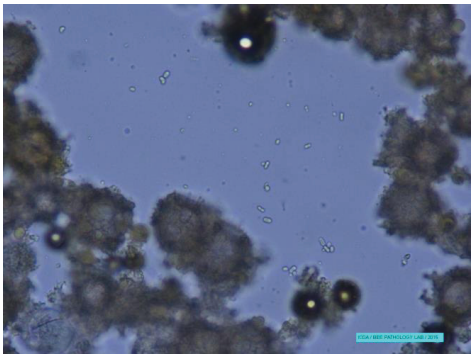

Figure 8. Identifying *Ascosphera apis* spores in the pollen from an apiary in which only ascospherosis developed (infestation source) (Pathology Laboratory, ICDA, Nikon ob x 40)

Microscopic examination of brood comb and samples of live bee intestine content showed various evolving forms of *Ascosphera apis* fungus, presented in fig. 9, 10, 11, 12, 13. (Aronstein K.A. and K.D. Murray, 2010.)

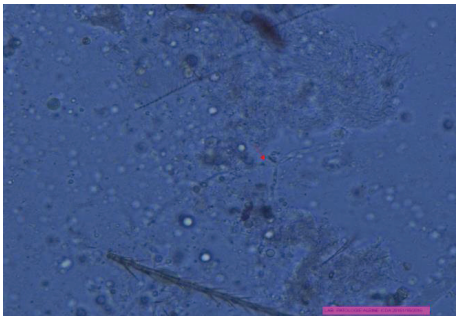

Figure 9. Development of *Ascosphera apis* ascogonia (Pathology Laboratory, ICDA, Nikon ob x 40)

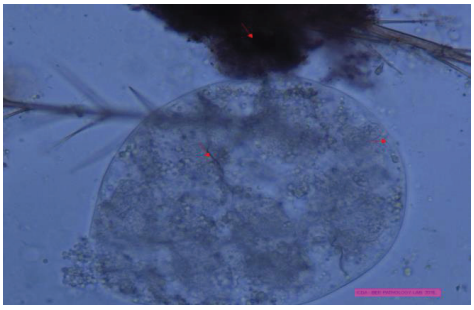

Figure 10. Green-brown ascocysts of *Ascosphera apis*. Detail of an ascocyst partially opened, containing spore-balls (Pathology Laboratory, ICDA, Nikon ob x 40)

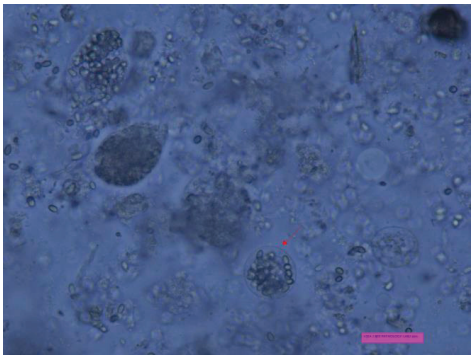

Figure 11. *Ascosphaera apis* spore cyst surrounded by fungal mycelia; B) inside the spore cyst there are numerous spore-balls; each spore-ball has millions of oval shaped spores. (Pathology Laboratory, ICDA, Nikon ob x 40)

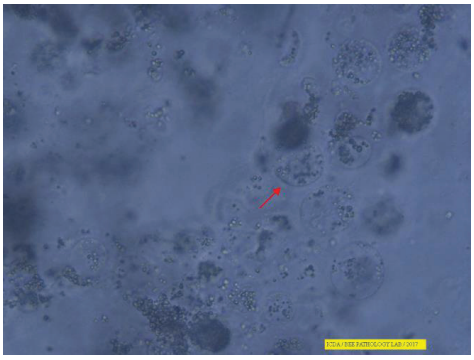

Figure 12. Asci resulting from the ascoma break that contain the fungal ascosphere identified in the intestine of a live bee collected from an apiary in which only ascospherosis evolved (Pathology Laboratory, ICDA, Nikon ob x 40)

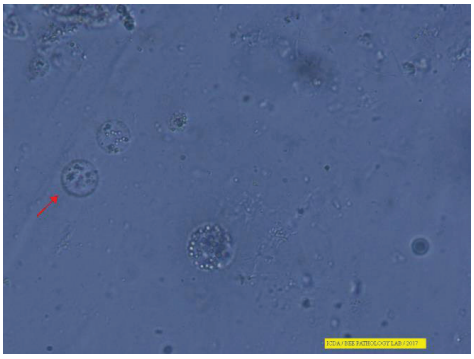

Figure 13. Showcasing ascospores identified in live bee intestine (Pathology Laboratory, ICDA, Nikon ob x 40)

*We mention that the research conducted in the project 157/2014 has no connection with the activity of official territorial or central laboratories nominated for the monitoring and control of bee diseases.*

## CONCLUSIONS

Monitoring bee colonies as regards the evolution of major bacterioses indicated the presence of chalk in 55.55 % of the tested samples, a unique infestation (5.55 % associated with suspicion of major bacterioses).

The regional incidence of chalk brood places the South-East area on the first place with over 2/3 of the positive tests.

Season incidence of chalk brood shows that in over 38.8 % of the cases are at the end of the beekeeping season, and in the inactive season months (January-February) the incidence was minimum in 11.11 % cases.

Food (additional and stimulation feeding), temperature, humidity, seem to play an important part in the season incidence of chalk brood, thus 66 % cases are found in the hot season.

The presence of chalk brood in 55.55% cases, together with major bacterial diseases, shows that the common element of morbid bacterial and fungal entities is in a deficit of the immune system.

In 2016, out of the 287 tested apiaries, 18 apiaries (6.27 %) were declared positive for brood mycoses, having clinical signs typical of mycotic diseseas.

Bee colonies from the monitored apiaries (59.56 %), in which ascospherosis evolved and did not manifest sings of clinical disease may be considered infestation sources.

It is recommended to test live bees and inspect the main infestation sources (pollen, honey).

## ACKNOWLEDGEMENTS

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# ASSESSING THE PREVALENCE OF *GIARDIA* INFECTION AND THE ASSOCIATED RISK FACTORS IN OWNED DOGS AND CATS, IN BUCHAREST'S URBAN AREA

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***Abstract***

*Giardia is a ubiquitous organism that affects humans and animals, with potential to contaminate the water and food, raising a concern in public health interest due to its zoonotic risk. In order to estimate the parasitic pressure for the both animal and human health, it was evaluated the prevalence of Giardia infection in pets (owned dogs and cats) originated from the urban area of Bucharest. Therefore, within 4 months (May-August, 2016), a total number of 188 faecal samples from dogs and 79 fecal samples from cats were investigated. Animals were of different breeds or mixed and different ages (from 1 month up to 16 years for cats, and up to 18 years, for dogs, respectively). Faecal samples were processed by zinc sulphate 33% solution flotation, Lügol stained and microscopically examined for identification of Giardia cysts. Additionally, other parasitic stages (oocysts, eggs) were also registered. Overall, out of the total faecal samples of dogs and cats, 41.49% (95%CI: 34.36-48.89), and 34.18% (95% CI: 23.87-45.71), respectively, were positive for parasite infections. Giardia cysts were recorded in quite similar prevalence in dogs, of 21.28% (40/188) and cats, of 22.78% (18/79). A higher prevalence of Giardia infection was found in puppies (23.89%) and older dogs (30.00%), and kittens (26.42%), respectively, compared to the adults (15.38% in dogs and 14.29% in cats). Additional, other parasite infections were found, as follows: in dogs, Isospora spp. (12.23%), Ancylostoma caninum (5.85%), Toxocara canis (4.26%), Uncinaria stenocephala (0.53%), Toxascaris leonina (0.53%) and Dipylidium caninum (0.53%); while in cats, Toxocara cati (10.13%) and Isospora spp. (8.86%) infections were registered. The findings of the present study are of relevance for the both animal and public health, emphasizing potential high risks for parasite infection, including parasites with zoonotic potential.*

***Key words****:Giardia infection, dogs, cats, zoonotic risk, Bucharest, Romania.*

## INTRODUCTION

Worldwide, dogs and cats as pets, or living around people play an important social role, contributing to the owners' wellbeing, to the emotional development of children, as well as for more complicated tasks such as utility animals for blind people, animal therapy, guard dogs, hunting dogs, military and police use (Nikolić et al., 2008; Martinez-Moreno et al., 2005; Mateus et al., 2014).

Parasites that infect companion animals are responsible for important zoonotic diseases worldwide. In humans, parasite species zoonotically transmitted can cause: larva migrans (*Toxocara* spp.), cutaneous larva migrans (*Ancylostoma* spp.), diarrhea and pruritus (*Dipylidium caninum*), hydatid disease (*Echinococcus* spp.), coenurosis (*Taeniidae*), mild to severe illness (fever, malaise, and lymphadenopathy to mental retardation, blindness, epilepsy in fetus) in case of *Toxoplasma gondii*, chronic malabsorptive and allergic manifestations (*Giardia duodenalis*), diarrhea (*Cryptosporidium* spp.) (Schantz, 2007; Mitrea, 2011).

From this point of view, infected animals can represent source of infection and potential risks for the public health due to their close proximity to humans. The risks are considered greater in urban areas, children communities (nurseries, kindergartens) or in urban areas with poor hygiene and ignorance (Mateus et al., 2014; P.A.H.O., 2003).

*Giardia duodenalis* (syn. *G. intestinalis* or *G. lamblia*) is a flagellate protozoan that can infect various mammalian hosts, including man, dogs and cats, and is considered a species complex.

Currently, based on genetic analyses, there are recognized at least seven assemblages (A-G). Out of these, dogs are infected primarily with Assemblages C and D, cats with Assemblage F, while humans are infected with Assemblages A and B (Monis et al., 2003; Thompson et al., 2008). However, assemblages A and B are considered to be of broad host specificity, as these have been reported also in several animal species, including cats and dogs, thus are potentially zoonotic (reviewed in Ballweber et al., 2010).

Oftenly, *Giardia* epidemics are transmitted through drinking water or recreational water, as well as directly from person to person, in communities and kindergartens (P.A.H.O., 2003; Cacciò et al., 2003; Carmena et al., 2007; Clayton, 2012).

Improving the knowledge of occurrence and prevalence of endoparasites in different areas and different communities contributes to improving animal health and to develop control measures, including for assessing the risks for the public health.

Therefore, the present study aimed to investigate the prevalence and the associated risk factors for *Giardia* infection in owned dogs and cats, as companion animals, originating from the urban area of Bucharest, in order to evaluate the potential risks for the both animal and public health.

## MATERIALS AND METHODS

During of May - August 2016, a coprological study was carried out in 44 veterinary clinics, located in the six districts of Bucharest (South Eastern Romania) [44°26′7″N, 26°6′10″E] (Figure 1).

In the study were included 267 pets – companion animals: 188 dogs and 79 cats. Animals were assigned into different breed, age, and gender groups. For age criteria there were considered four categories: puppies and kittens under 6 months old, puppies and kittens between 6 and 12 months old, adults (between 1 and 8 years old) and older than 8 years.

Individual faecal samples were randomly collected from dogs and cats of all ages with or without intestinal symptoms.

The fecal samples were transported to the laboratory where they were examined immediately or stored at 4°C and examined within the next two days.


Figure 1. Veterinary clinics, number of samples and positive *Giardia* samples distribution, stratified by Bucharest's districts

All faecal samples were subjected for parasitological investigations by the flotation method, using a solution of zinc sulphate (ZnSO4) 33% and Lügol staining, and microscopically examined for the presence of *Giardia* cysts but also for worm eggs and protozoan oocysts.

Briefly described, for each sample, an amount of 2-3 g of faeces were mixed with 15 ml of 33% zinc sulphate solution, poured into a test tube, topped off with the zinc sulphate solution to form a convex meniscus and placed a coverslip for about 15 minutes.

A drop of Lügol's iodine was added to the slide, before placing the coverslip and after that the sample was examined by an optical miscroscope, using a 40x objective (Ionita and Mitrea, 2013).

*Giardia* cysts (occasionally trophozoites) were identified based on the morphological characteristics (Figure 2, Figure 3).

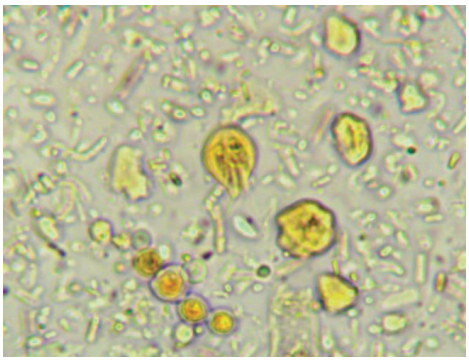

Figure2.*Giardia* trophozoite in dog faecal smear (Lügol staining; 40X)

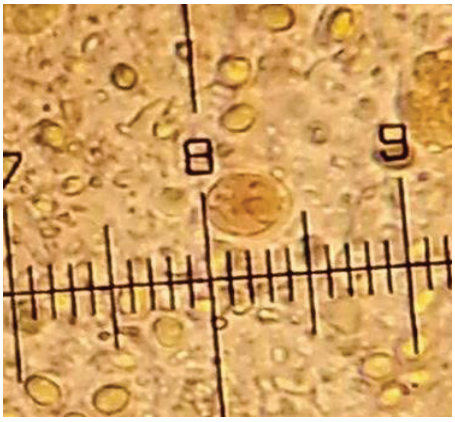

Figure 3.*Giardia* cyst in dog faecal smear (Lügol staining; 40X)

The statistical analysis was performed using Quantitative Parasitology 3.0 free software (Rozsa et al., 2000). 95% confidence interval (CI), *P*-values by chi-square test (for the differences of age class) were computed (Mitrea et al., 2013). Statistical significance was considered for $P \leq 0.05$.

## RESULTS

For assessing the prevalence and the associated risk factors for *Giardia* and other parasite infections, we conducted a parasitological survey on 44 veterinary clinics in the six districts of Bucharest. The study comprised a total number of 267 pets – companion animals, including owned dogs (n=188) and cats (n=79) originating from the urban area of Bucharest.
Overall, of the total dogs and cats, 41.49% and 34.18%, respectively, were positive for at least one parasite infection. Detailed recorded data will be presented by host species, dogs and cats, respectively.

### *Dogs*

In dogs, fecal samples were collected from 188 animals (107 males; 81 females), with age ranging from one month to 18 years (mean 2.23 years, SD=3.21), of which: 78 were puppies under 6 months old (43 males; 35 females), 35 were puppies between 6 months and 12 months (19 males; 16 females), 65 adults (40 males; 25 females) and 10 were old animals (5 males; 5 females). The majority of dogs (n=146) were pure-breed (36 different breeds) and the rest (n=42) were mixed-bred (Figure 4).

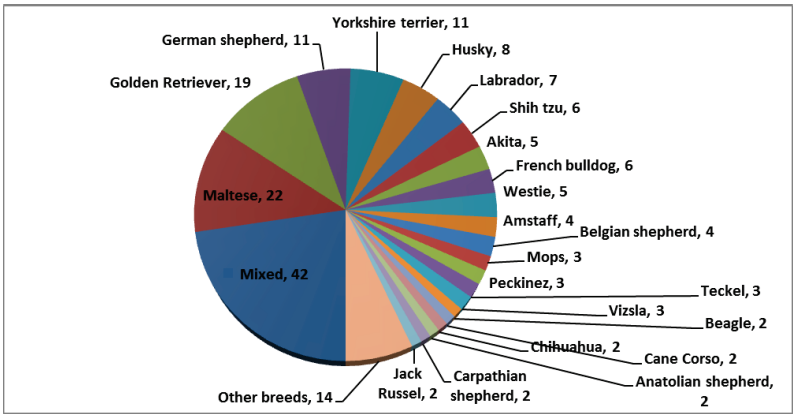


Figure 4. Dog breeds included in study

Of the total dogs, 105 (55.85%) displayed clinical signs such as vomiting, soft faeces, fetid diarrhea, and/or presence of mucus in stool. Eighty-three dogs (44.15%) were considered as clinically healthy on the time of presentation.

All dogs originated from the studied area and had access to urban green areas/parks in the municipality of Bucharest.

Overall, out of the total 188 dog faecal samples investigated, 78 (41.49%; 95%CI: 34.36 - 48.89) were found positive for at least one intestinal parasite species. 66 samples (35.11%) were positive for one parasite species and in 12 samples (6.38%), were detected mixed infections. Of the positive subjects, 60 dogs showed digestive clinical signs (60/78; 76.92%).

*Giardia* cysts were found in 40 of the 188 samples (21.28%) (Table 1).

Table 1. Prevalence of *Giardia* cysts in faecal samples of owned dogs, stratified by age and gender

| | Positive sample (%) / Samples by age and sex | | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|
| Age | 1month≤6months | | >6months ≤ 1year | | >1 year ≤ 8 years | | >8 years | | |
| Sex | M | F | M | F | M | F | M | F | |
| Dogs | 12/43 | 8/35 | 5/19 | 2/16 | 3/40 | 7/25 | 2/5 | 1/5 | 40/188 |
| Dogs Total | 20/78 25.64% | | 7/35 20.00% | | 10/65 15.38% | | 3/10 30.00 % | | 21.28 % |
| | 27/113 (23.89%) | | | | 13/75 (17.33%) | | | | |
| | *P* = 0.282 | | | | | | | | |

Six dogs were found co-infected with other parasites, as follows: *Isospora* spp. (in 2 samples), *Ancylostoma caninum* (in 3 samples) and *Trichuris vulpis* (in one sample) (Table 2).

The rate of infection, according to the age groups was higher in old dogs (30.00%), followed by puppies under one year old (23.89%), and adults (15.38%) (Figure 5).

When comparison was made between infected dogs <1 year old (and dogs >1 year old, a higher prevalence was found in the first age group (27/113; 23.89%) than for the second group (13/75; 17.33%), but no statistically significant (*P*=0.282).

Males (n=107) and females (n=81) had close value prevalences 20.56% and 22.22% (*P*=0.783).


Figure 5.Prevalence of*Giardia* cysts in faecal samples of owned dogs, according to age and gender category

Regarding the breeds, *Giardia* infection was found most frequently in Golden Retriever (n=8), mixed (n=8), Belgian Shepherd (n=3) and Maltese Bichon (n=3). Other affected breeds were Amstaff, Husky and Westie, with 2 samples each, and Akita, Beagle, Chihuahua, Anatolian Shepherd, German Shepherd, Corgi, Argentinian Mastiff, Jack Russel, Kangal, Labrador, Malinois and Yorkshire Terrier with one sample each.

Of the 40 positive subjects for *Giardia* infection, 35 displayed digestive clinical signs (ranging from soft faeces, diarrhea, flatulence to blood drops in faeces). It is noteworthy that five animals positive for *Giardia* showed noclinical digestive signs. They were from all age categories: 2 smaller than 6 months, one puppy between 6 and 1 year old, one adult and one old dog.

Additionally, were also found other parasites, as follows: *Isospora* spp. (12.23%), *Ancylostoma caninum* (5.85%), *Toxocara canis* (4.26%), *Uncinaria stenocephala* (0.53%), *Toxascaris leonina* (0.53%) and *Dipylidium caninum* (0.53%) (Table 2).

Among these, were found 4 types of associations: *Isospora* spp. and *Toxocara canis*, *Ancylostoma caninum* and *Toxocara canis* associations were found each one in 2 samples; *Isospora* spp. and *Ancylostoma caninum*, *Trichuris vulpis* and *Toxascaris leonina* associations were found in one sample each (Table 2).

Table 2. *Giardia* infection prevalence and other intestinal parasites, including co-infections, in owned dog originating from urban area of Bucharest; presence (+/-) of clinical signs

| Parasite species and/or associations | Puppies (≤1 year) | | Adult (>1 ≤8 yrs) | | Old (>8years) | | Total | | Clinical signs | |
|---|---|---|---|---|---|---|---|---|---|---|
| | No. | % | No. | % | No. | % | No. | % | + | - |
| *Giardia duodenalis* total | 27 | 23.89 | 10 | 15.38 | 3 | 30 | 40 | 21.28 | 35 | 5 |
| *G. duodenalis* as single parasite | 26 | 23.01 | 6 | 9.23 | 2 | 20 | 34 | 18.09 | 29 | 5 |
| *G. duodenalis* and *Isospora* spp. | 1 | 0.88 | 1 | 1.54 | 0 | 0 | 2 | 1.06 | 2 | 0 |
| *G. duodenalis* and *A. caninum* | 0 | 0 | 3 | 4.62 | 0 | 0 | 3 | 1.60 | 3 | 0 |
| *G. duodenalis* and *T. vulpis* | 0 | 0 | 0 | 0 | 1 | 10 | 1 | 0.53 | 1 | 0 |
| *Isospora* spp | 18 | 15.93 | 0 | 0 | 0 | 0 | 18 | 9.57 | 15 | 3 |
| *Isospora* spp. with *T. canis* | 2 | 1.77 | 0 | 0 | 0 | 0 | 2 | 1.06 | 2 | 0 |
| *Isospora* spp. with *A. caninum* | 1 | 0.88 | 0 | 0 | 0 | 0 | 1 | 0.53 | 1 | 0 |
| *Ancylostoma caninum* | 2 | 1.77 | 3 | 4.62 | 0 | 0 | 5 | 2.66 | 4 | 1 |
| *A. caninum* and *T. canis* | 2 | 1.77 | 0 | 0 | 0 | 0 | 2 | 1.06 | 0 | 2 |
| *Toxocara canis* | 3 | 2.65 | 1 | 1.54 | 0 | 0 | 4 | 2.13 | 2 | 2 |
| *Trichuris vulpis* | 1 | 0.88 | 2 | 3.08 | 0 | 0 | 3 | 1.60 | 0 | 3 |
| *T. vulpis* with *T. leonina* | 1 | 0.88 | 0 | 0 | 0 | 0 | 1 | 0.53 | 1 | 0 |
| *Uncinaria stenocephala* | 0 | 0 | 1 | 1.54 | 0 | 0 | 1 | 0.53 | 0 | 1 |
| *Dipylidium caninum* | 0 | 0 | 0 | 0 | 1 | 10 | 1 | 0.53 | 0 | 1 |

***Cats***:

Cats were predominantly of common breed cats (n=60), the rest 19 belonging to 8 breeds as shown in Figure 6. There were 47 males and 32 females, with age varying between one month and 16 years (mean 2.26 years; SD = 3.24). Digestive signs were registered only in 34 of the subjects and ranged from soft stools to vomit, vomit and stools with parasitic elements, stools with streaks of blood.

Out of the 79 investigated cats, 27 were positive for parasite infections (34.18%; 95% CI: 23.87 - 45.71). In 22 (27.85%) samples, one single parasite species was detected, while in five (6.33%) were found mixed infections: 4 (5.06%) with two species and 1 (1.27%) for three parasite species. Out of the 27 positive cats for at least one parasite, 17 (62.96%) manifested clinical signs.

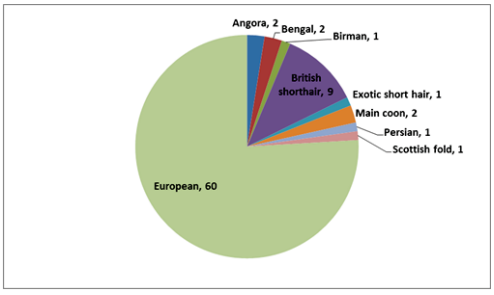


Figure 6.Cat breeds included in the study

*Giardia* cysts were found in 18 samples (22.78%) (Table 3).

Out of these, there were found mixed infections with *Isospora* spp. (in one sample), with *Toxocara cati* (in 2 samples) and with both (*Isospora* spp. and *T. cati*) in one sample (Table 4).

Higher prevalence was found in kittens under one year old (26.42%) compared to adults (14.29%) (Figure 7). No race susceptibility was noted. Females had higher prevalence (28.13%) than males (19.15%), but with no statistical significance $P = 0.35$.

It should be noted that 6 cats with positive samples for *G. duodenalis* showed no clinical digestive signs. They were 5 kittens under 6 months old and one cat of 2 years old.

Other parasite species were recorded, as follows: *Toxocara cati* (10.13%) and *Isospora* spp. (8.86%)

.

Table 3. Prevalence of *Giardia* cysts in faecal samples of owned cats, stratified by age and gender category

| | Positive sample (%) / Samples by age and gender | | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|
| Age | 1month ≤=6months | | >6months <= 1year | | >1 year ≤8 years | | >8 years | | |
| Sex | M | F | M | F | M | F | M | F | |
| Cats | 6/23 | 7/16 | 1/7 | 0/7 | 1/13 | 2/8 | 1/4 | 0/1 | |
| Cats Total | 13/39 (33.33%) | | 1/14 (7.14%) | | 3/21 (14.29%) | | 1/5 (20.00%) | | 18/79 22.78% |
| | 13/39 (33.33%) | | 5/40 (12.50%) | | | | | | |
| | *P* = 0.027 | | | | | | | | |

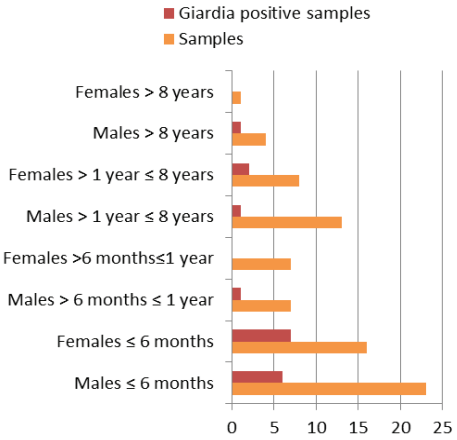


Figure 7. Prevalence of *Giardia* cysts in faecal samples of owned cats, according to age and gender category

Table 4. Prevalence of *Giardia* infections and other parasitic associations found in cats; presence (+/-) of clinical signs

| Parasite species and/or associations | Kittens ≤6 months | | Kittens >6mos≤1 yr. | | Adult cats >1 ≤8years | | Old cats >8 years | | Total | | Clinical signs | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No. | % | No. | % | No | % | No. | % | No. | % | + | - |
| *Giardia duodenalis* total | 13 | 33.33 | 1 | 7.14 | 3 | 14.29 | 1 | 20.00 | 18 | 22.78 | 12 | 6 |
| *G. duodenalis* as single parasite | 10 | 25.64 | 1 | 7.14 | 2 | 9.52 | 1 | 20.00 | 14 | 17.72 | 9 | 5 |
| *G. duodenalis* and *Toxocara cati* | 2 | 5.13 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 2.53 | 1 | 1 |
| *G. duodenalis* and *Isospora* spp. | 0 | 0 | 0 | 0 | 1 | 4.76 | 0 | 0 | 1 | 1.27 | 1 | 0 |
| *G. duodenalis*, *Isospora* spp. and *Toxocara cati* | 1 | 2.56 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1.27 | 1 | 0 |
| *Isospora* spp. | 3 | 7.69 | 1 | 7.14 | 0 | 0 | 0 | 0 | 4 | 5.06 | 2 | 2 |
| *Toxocara cati* | 4 | 10.26 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 5.06 | 3 | 1 |
| *Isospora* spp. *and Toxocara cati* | 1 | 2.56 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1.27 | 0 | 1 |

## DISCUSSION

Here we present a study assessing the prevalence of *Giardia duodenalis* in owned dogs and cats from Bucharest area. The rate of *Giardia* infection was recorded in quite similar prevalence for the both, dogs (21.28%) and cats (22.78%).

The resemblance is maintained even within age groups: 23.89% and 26.42% in puppies respectively kittens, 15.38% in adult dogs and respectively 14.29% in adult cats and 30.00% in old dogs and 20.00% old cats.

The age is considered a risk factor; in this study, subjects under 6 months old had registered higher prevalence values than average: puppies 25.64% (average was 21.28%), kittens 33.33% (average = 22.78%). In case of the kittens ≤ 6 months old (13/39; 33.33%) compared with cats > 6 months old (5/40; 12.5%) a statistical significance was noted (*P*= 0.027) highlighting that age is a risk factor for *Giardia* infection in kittens under 6 months.

Dogs under one year old were having a prevalence of 23.89%, and dogs older than one year having 17.33%. The same can be observed for cats aged under one year (26.42%) and those over one year old (15.38%), but not statistical significant (*P*=0.303; *P*=0.394, respectively).

The mean prevalence values found in this study (of approximately 22%) is higher when compare with results reported from similar studies in Romania, for instance of 4.8% in clinically healthy household dogs from urban

areas in Romania (Mircean et al., 2012). This can be explained by the fact that the subjects of this study were animals brought to the veterinary clinics with various health problems, which can result in a higher prevalence than a simple screening. However, these values were lower (36.1%, South-Eastern Romania; 51.08% in Satu Mare county) than reported in studies by imunoenzymatic test ELISA (Jarca et al., 2008; Sommer et al., 2015) and 42.62% by Lügol staining in stray dogs from Timis county (Sorescu et al., 2014).

All these studies report occurrence of *Giardia* infection, based on different size/types of studies (epidemiological, clinical, etc.), therefore, variations are expecting.

Based on anamnesis or reports of clinicians, pets are often dewormed using common products primarily against worms, but that do not act on *G. duodenalis* and *Isospora* spp., increasing the risks of subclinical infections. The absence of clinical signs and the proximity to humans of this category of animals, living in the same space can increase the risk of zoonotic transmission of infection. Therefore, the owner needs to be instructed by the veterinarian to perform regular checks for coproparasitological of their animals.

Similar values are found in many places, almost independent of geographical area. Therefore in Northern Italy (Zanzani et al., 2014) in metropolitan and micropolitan area of Milan, G. *duodenalis* was the most common of the detected parasites. In dogs from micropolitan areas the prevalence varied between 20.37% - 25.58% and in the metropolitan area was of 16.05%. In cats, the prevalence in two micropolitan areas varied between 25.00% - 36.84% and in the metropolitan areas was 24.7%. In Germany, a study conducted between 2003 and 2010 (Barutzki et al., 2011) highlighted the overall value of G. *duodenalis* prevalence of 18.6% for dogs and 12.6% for cats, as well as the distribution according to age. The highest values of prevalence were up to 3 months (37.5% for dogs and 19.5% for cats) and between 3 and 6 months (38.2% for dogs and 24% for cats) (Barutzki et al., 2011).

In the Parisian area (Beugnet et al., 2000) carried out a parasitological study on owned dogs and cats. It was found that 25% of dogs and 20% of cats were infected with parasites; age was the main prevalence factor: 56.5% of dogs under 6 months being infected with at least one parasite. G. *duodenalis* was the most widespread, 30.4% of the animals younger than 6 months were infected with this parasite.

Other studies, for Canada, provide data on the prevalence of G. *duodenalis* in dogs. Animals under one year old were infected at a rate of 17.36%, and those over two years old just at a rate of 4.15% (Joffe et al., 2011).

## CONCLUSIONS

This study provides data on the prevalence of *Giardia* infection in owned dogs (21.28%) and cats (22.78%) from urban area of Bucharest. Animals younger than one year old were more susceptible to infection. Mixed parasite infections were recorded in the both dogs (6.38%) and in cats (6.33%), including parasites with zoonotic potential. Altogether, the findings of the present study are of relevance for the both animal and public health, emphasizing potential high risks for infection.

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# CORRELATION BETWEEN DURATION OF GAS ANESTHESIA WITH ISOFLURANE AND THE REDUCTION OF TEAR PRODUCTION IN GERIATRIC PATIENTS

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***Abstract***

*This study was performed in order to investigate the correlation between duration of gas anaesthesia with isoflurane and the reduction of tear production in geriatric patients. The study was conducted on 15 dogs (8 males and 7 females, ages between 9 and 14 years old) that were presented at the Faculty of Veterinary Medicine of Bucharest between September – October 2016. Preanesthetic examination was performed according to ASA status (American Society of Anesthesiologists). Patients premedication was made with Midazolam 0.2 mg/kg and Butorphanol 0.2 mg/kg injected intramuscularly (IM) or intravenously (IV). All dogs were intubated, induction was made with Propofol 4-6 mg/kg IV and maintenance was performed with Isoflurane. All patients had Schirmer Tear Test (STT) readings taken prior to intubation and immediately after the isoflurane was turned off. All dogs that were under isoflurane anaesthesia for less than 30 minutes had a slight reduction of tear production compared with those that exceeded 30 minutes in which it was noticed a drastic decrease in tear production. From the total of 15 dogs: 3 dogs that were under isoflurane anaesthesia for less than 30 minutes had a final STT of 15 mm/min +/- 3 mm/min compared with those in which anaesthesia time exceeded 30-40 minutes where the final STT was 5 +/- 3 mm/min.*

***Key words:*** *Geriatric patients, Isoflurane, Schirmer Tear Test, tear production.*

## INTRODUCTION

The precorneal tear film (PTF) is extremely important for the maintenance of the ocular surface health.

Its functions include primary oxygen source to the avascular cornea, removal of debris and exfoliated cells through drainage, lubricant between the eye lids and a source of protective antimicrobial proteins.

The PTF is described as a three structurally and functionally unique layers consisting of lipid, aqueous and mucin components.

The cornea is extremely vulnerable to injury during general anesthesia when the palpebral reflex and the corneal reflex cannot protect the eye from drying, from corneal abrasions or from other corneal injuries.

Dry corneal epithelium may be easily desquamated and removed by the normal movement of the eyelids that can cause painful postanesthetic ulcers of the cornea (Gelatt, 2013).

The objective of this study was to investigate the correlation between the duration of gas anesthesia with isoflurane and the reduction of tear production in geriatric patients. The hypothesis was that longer the anesthetic duration would cause a lower postanesthetic tear production.

## MATERIALS AND METHODS

The study was conducted on 15 canine patients of different ages, belonging to different breeds.

The patients were anesthetized for different surgical procedures: ovariohysterectomy, castration, mammectomy, cistotomy (Table 1).

Physical examination, complete blood count and ophthalmic examination on both eyes were performed.

Patients did not receive any ocular medication and had no abnormalities during examination.

Aqueous tear production was measured in millimetres per minute by use of the Schirmer Tear Test (STT) by placing the tear test strip in the ventral conjunctival fornix approximately one-third of the distance from the lateral to medial canthus (Fig. 1).

Table 1. Breed, age and gender particularities of all geriatric dogs submitted to the study

| Crt. No. | BREED | AGE | GENDER ♂/♀ |
|---|---|---|---|
| **1** | Poodle | 14 years | ♂ |
| **2** | Pekinese | 13 years | ♂ |
| **3** | Half breed | 13 years | ♀ |
| **4** | Golden Retriever | 12 years | ♀ |
| **5** | Poodle | 12 years | ♂ |
| **6** | Maltese Bichon | 12 years | ♀ |
| **7** | Bichon | 11 years | ♀ |
| **8** | Great Dane | 11 years | ♂ |
| **9** | Cocker | 11 years | ♂ |
| **10** | Mini Schnautzer | 10 years | ♂ |
| **11** | Schitzu | 10 years | ♀ |
| **12** | Cross breed | 10 years | ♀ |
| **13** | Cross breed | 10 years | ♂ |
| **14** | Bichon | 9 years | ♀ |
| **15** | Cross breed | 9 years | ♂ |

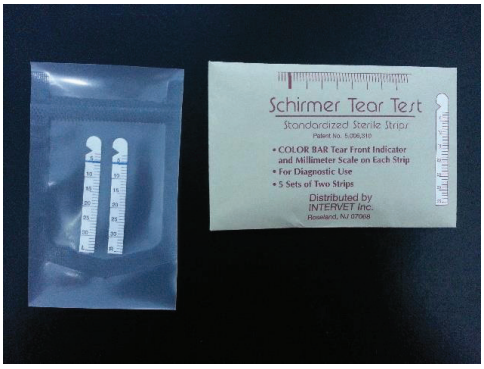


Figure 1. Schirmer Tear Test (original)

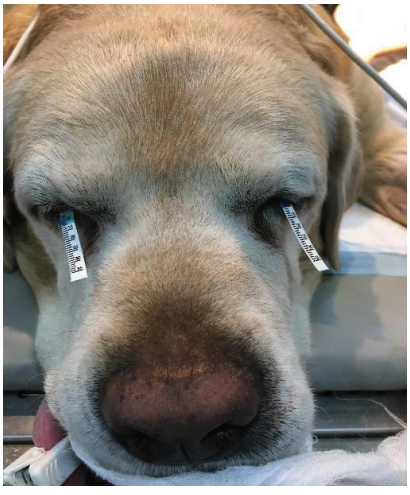

Figure 2. STT readings (original)

After the test strip was inserted, the eyelids were gently held closed for 1 minute, at which time the STT was read and recorded. Testing was performed bilateral (Fig. 2). Before each test was done, the inferior cul-de-sac was gently swabbed with a cotton- tipped applicator to remove accumulated tears and mucus.

Tear production was measured at baseline (before intubation) and immediately after the isoflurane was turned off.

Anesthesia was induced with Propofol (4-6 mg/kg IV). Intermittent positive-pressure ventilation (IPPV) was initiated by use of a volume-cycled ventilator delivering 12 breaths/minute to achieve a target end-tidal $CO_2$ of 35-45 mm/Hg. Oxygen flow was initially delivered at 2 L/minute with the vaporizer set to achieve an end-tidal concentration of 2.0% isoflurane within 10 minutes of induction (Costea, 2015).

After the target concentration was achieved, oxygen flow was decreased to (500+10/kg) L/minute. Crystalloid solutions were administered at 3-5 ml/kg/hour IV throughout anesthesia.

At the end of the surgery, the isoflurane was turned off and the Schirmer Tear Test was performed. Afterwards, the residual inhalant anesthetic was flushed from the breathing circuit.

IPPV was discontinued and patients were extubated when they began to breathe spontaneously and to reject the endotracheal tube (Costea, 2016). After STT was measured, an ocular lubricant was applied to each of the patient`s eyes to protect the cornea.

## RESULTS AND DISCUSSIONS

Mean tear production for Schirmer Tear Test measurement in all patients at baseline for the right and the left eyes were 20 mm/min +/- 5 mm/min (Figure 2). From the total of 15 dogs: 3 dogs that were under isoflurane anesthesia for less than 30 minutes had a final STT of 15 mm/min +/- 3 mm/min compared with those in which anesthesia time exceeded 30-40 minutes where the final STT was 5 +/- 3 mm/min (Table 2). Aqueous tear production was reduced in patients during anesthesia and returned to baseline values immediately in the recovery period, for all the cases.

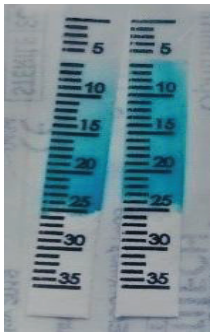


Figure 3. Schirmer Tear Test at baseline (original)

The decreased intra-anesthetic lacrimation observed in the present study may be attributable to vagolytic or sympathomimetic effects of the gas anesthetic (Ding, 2003). Because vagolytic and sympathetic activity were not measured in this study they cannot be definitively ruled out as factors.

Decreased intra-anesthetic lacrimation may be described as a blockade of trigeminal function associated with anesthetic depth. Lachrymal secretion is mostly dependent on afferent sensory function of the trigeminal nerve, followed by an efferent motor response by the facial nerve (Acosta et al., 2004).

The anesthetic depth may have abolished trigeminal sensory function in a way similar to the effect of inhalant anesthetics on other afferent input, thereby disabling a lachrymal response. Once the patient was able to stand, indicating the return of the afferent and efferent nerve function, sensory input from the trigeminal nerve was likely restored, resulting in normal tear production (Acosta et al., 2004).

Intra-anesthetic tear production may have also been caused by lagophthalmos. This is not the cause of decreased aqueous tear production but it is a condition that accelerates tear evaporation via decreased blinking.

Dogs with lagophthalmia may have a rapid evaporation of tear film from the corneal surface because of increased corneal exposure or decreased tear film quality (lipid or mucin). Tear evaporation was not evaluated during or immediately following anesthesia so, Schirmer Tear Test readings could not be correlated with tear film break-up time data (Tzubota, 1998).

Duration of anesthesia in the present study had a causal relationship with decreased postanesthetic tear production in geriatric dogs. This reveals that procedures longer than 30 minutes cause a decrease in tear production.

The hitch of lagophthalmos was not controlled in this present study; no effort was made to close the dogs' eyes during anesthesia. Future studies investigating the effect of lagophthalmos on intra-anesthetic aqueous tear production may include STT and tear film break-up time measurement in geriatric dogs with one eyelid taped closed and one eyelid left open during general anesthesia.

## CONCLUSIONS

All geriatric dogs in this study had a decreased intra-anesthetic tear production during isoflurane anesthesia. Ocular lubricant or tear replacement should be used as a corneal protectant for patients that are going to be under gas anesthesia with isoflurane for more than 30 minutes.

Table 2. Schirmer Tear Test values at baseline and after isoflurane was turned off; OD- right (oculus dexter) eye, OS – left (oculus sinister) eye

| Crt. No. | Anesthetic Duration (hour) | Baseline | STT after isoflurane was turned off |
|---|---|---|---|
| 1 | 25 minutes | OD 15 mm/min<br>OS 15 mm/min | OD 12 mm/min<br>OS 12 mm/min |
| 2 | 25 minutes | OD 20 mm/min<br>OS 18 mm/min | OD 18 mm/min<br>OS 15 mm/min |
| 3 | 30 minutes | OD 20 mm/min<br>OS 15 mm/min | OD 15 mm/min<br>OS 15 mm/min |
| 4 | 40 minutes | OD 15 mm/min<br>OS 15 mm/min | OD 5 mm/min<br>OS 5 mm/min |
| 5 | 45 minutes | OD 15 mm/min<br>OS 25 mm/min | OD 0 mm/min<br>OS 10 mm/min |
| 6 | 1 hour | OD 20 mm/min<br>OS 15 mm/min | OD 0 mm/min<br>OS 0 mm/min |
| 7 | 1 hour | OD 15 mm/min<br>OS 15 mm/min | OD 0 mm/min<br>OS 0 mm/min |
| 8 | 1 hour | OD 20 mm/min<br>OS 25 mm/min | OD 10 mm/min<br>OS 10 mm/min |
| 9 | 1 hour | OD 20 mm/min<br>OS 18 mm/min | OD 5 mm/min<br>OS 5 mm/min |
| 10 | 1 h 10 min | OD 18 mm/min<br>OS 15 mm/min | OD 0 mm/min<br>OS 0 mm/min |
| 11 | 1 h 15 min | OD 15 mm/min<br>OS 15 mm/min | OD 5 mm/min<br>OS 5 mm/min |
| 12 | 1 h 15 min | OD 15 mm/min<br>OS 10 mm/min | OD 0 mm/min<br>OS 0 mm/min |
| 13 | 2 hours | OD 15 mm/min<br>OS 15 mm/min | OD 5 mm/min<br>OS 5 mm/min |
| 14 | 2 hours | OD 15 mm/min<br>OS 12 mm/min | OD 7 mm/min<br>OS 5 mm/min |
| 15 | 2 h 15 min | OD 15 mm/min<br>OS 15 mm/min | OD 5 mm/min<br>OS 0 mm/min |

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# ANIMAL PRODUCTION, PUBLIC HEALTH AND FOOD QUALITY CONTROL

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# EVALUATION OF DIFFERENT TYPES OF BEER QUALITY AND CONSUMERS` SAFETY

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***Abstract***

*In the context of high consumption of different types of beer and given the consumer demand regarding the food safety, the purpose of this study was represented by the quality control of these products using physicochemical methods. The data revealed information regarding pH value, alcohol content, carbon dioxide content, value of the original, real and present extract, energetic value and foam quality determination. The results showed an uniformity of data from lots of the same sort, which proved a core in applying of the quality management system. In conclusion, it can be said that products obtained in the studied unit meet quality requirements imposed by applicable standards and consumption of these products presents no risk of physicochemical nature.*

***Key words****: beer, food safety, quality, physicochemical analyse.*

## INTRODUCTION

Beer was discovered thousands of years ago and became a very often consumed beverage for its refreshing and pleasant taste, but also as a reason to relax, meet with friend and social interaction (Walton, 2006).

A moderate beer consumption of around 330 millilitres per day for women and two beers for man brings a real benefit for health, reducing the risk of diabetes, osteoporosis and cardiovascular disease (Banu et al., 2011).

It is considered that consumption of beer and wine are more beneficial than drinking sparkling wine or distilled beverages (Tăpăloagă, 2012).

In this context, this paper presents an analysis of 3 different types of beer and quality parameters and legislative requirements.

## MATERIALS AND METHODS

The material was represented by 40 samples of two types of pale lager, divided into 2 groups for each type, depending on the time of sampling and 10 samples of flavoured beer who have undergone physical and chemical analyzes, respectively measuring of pH, alcohol content, carbon dioxide content, value of the original, real and present extract (Anton Paar method), energetic value and foam quality determination (Hartong method). The sampling scheme is shown in Table 1.

Table1. Sampling scheme

| Type | Group 1 | Group 1 |
|---|---|---|
| Type 1 pale lager | 10 samples | 10 samples |
| Type 2 pale lager | 10 samples | 10 samples |
| Type flavored beer | 10 samples | |

## RESULTS AND DISCUSSIONS

Taking into account the fact that for the 2 types of pale lager were obtained a large number of results, we presented the average for each type of analyse. For the flavoured type, the results were presented for each sample.

Regarding the quality of the type 1 pale lager for both groups (group 1.1 and 1.2), all results are within the limits imposed by the standard, the pH should be between 4.1 to 4.4, with average obtained 4.34 (figure 1), alcohol content had an average value of 4.94% compared to limits of 4.3 to 5.8% (figure 1) concentration of $CO_2$ varied between 5.1% and 5.5%, the average value being 5.5% (figure 1). The maximum value of original extract imposed by producer is 11,25˚P, which means that the analyzed beers were within the limits with an average of 11.04 ˚P, the real extract should be between 3.56 and 3.47˚P, the studied beers have an average of 3,46˚P, the present extract has a value of 1.65˚P, being the limits imposed by the manufacturer (figure 2).

The time of destruction of foam had an average value of 264 sec compared to 250 sec minimum value and the energy value was within the standard product, with an average of 165.5 kcal (figure 3).As regards quality assortment 2 for both groups of lager (group 2.1 and 2.2), all results were within the limits imposed by the standard, the pH had an with average of 4.32, alcohol an average value of 4.97% and $CO_2$ concentration was 5.5 (figure 1).

Original extract value should be maximum 11˚P which means that the analysed beers were within the limits set by legislation with an average of 10.95 ˚P, real extract should be between 3.56 and 3.47˚P, the studied beer fitted the standard with an average of 3.45, the present extract had a value of 1.67 ˚P, being the limits imposed by the producer (figure 2).

The time of destruction of foam had an average value of 265 sec reported to 250 sec minimum value, and energy value falled within the standard product, with an average of 166 kcal (figure 3).

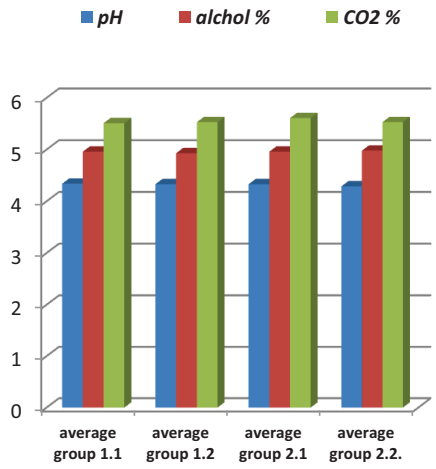


Figure 1. Average values for pH, alcohol and $CO_2$ concentration

For flavoured beer, all results are within the limits imposed by the standard, the pH should be between 2.85-3.15, with an obtained average value of 2.98, the alcohol concentration had an average of 4.97% between the limits of 4.3 to 5.8%, the concentration of $CO_2$ varied between 5.2 and 5.6%, with an average of 5.44% (figure 4).

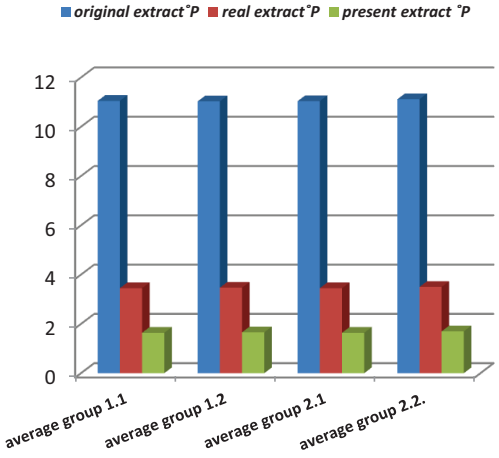


Figure 2. Average values for original extract, real extract, present extract

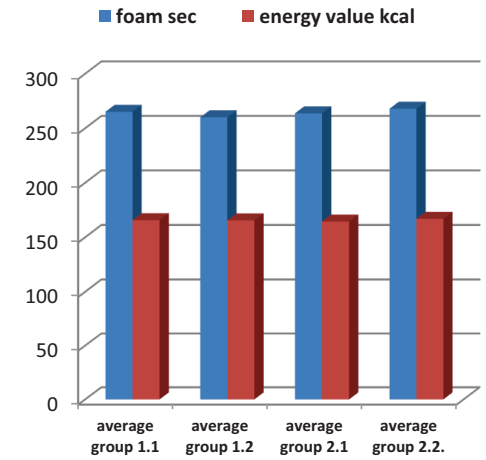


Figure 3. Average values for foam and energy value

The original extract value must be 1,75-12,05˚P which means that beers analysed within the limits set by an average of 11.38 ˚P, the real extract should be between 5.55 and 5.85˚P, the studied beer fitted the standard with an average of 5.59˚P, the present extract had a value of 4.25˚P, being within limits imposed by the producer (figure 5). The time of destruction of foam had an average value of 129 seconds compared to 125 seconds minimum value, and energy value was within the product standard, with an average of 155 kcal (figure 6).

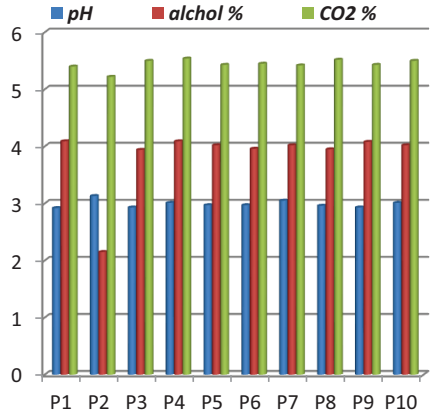


Figure 4. Average values for pH, alcohol and $CO_2$ concentration

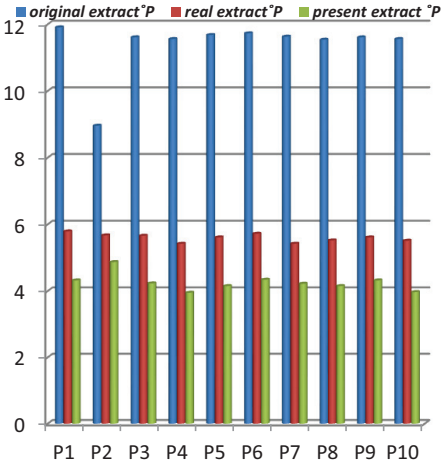


Figure 5. Average values for original extract, real extract, present extract

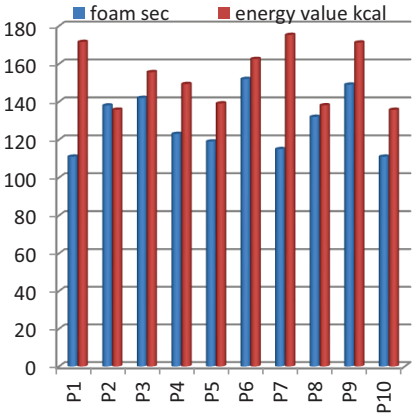


Figure 6. Average values for foam and energy value

## CONCLUSIONS

The technological flow for pale lager and flavoured beer comply with the majority of the studied literature, with minor variations that give the originality of the products (Banu, 2001, Hlatkly, 2013, Kunze,1996).

There is a uniformity of data from lots of the same sort, which proves the applying accordingly of the quality management system.

From data analysis we can observe that there are not significant differences between the two types of pale lager.

Also, there are fairly significant differences between pale lager and flavoured beers with lower values for all parameters for the second category.

In conclusion, it can be said that the products obtained in the studied unit meet the quality requirements imposed by legislative standards and consumption of these products presents no risk of physicochemical nature.

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# FORMULATION, PREPARATION AND CHEMICAL ANALYSIS OF PURIFIED DIETS FOR LABORATORY MICE AND RATS

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***Abstract***

*The aim of this study was the preparation and determination of the main chemical parameters for 5 purified diets for laboratory mice and rats: 2 diet for maintaining and growth/breeding animal colonies and 3 diets for inducing metabolic disorders (atherosclerosis, diabetes type II and obesity). Diet recipes for maintenance and growth are the classic recipes and diets that induce metabolic syndromes attempts to replicate human food behaviour with unidirectional nutrition (excess cholesterol, excess fructose and excess fat). For all chemical parameters were established limits values necessary to achieve the aims pursued by manufacturing these diets. Diets were prepared in our laboratory. To all diets were made the following measurements for determining the gross chemical composition: protein, fat, fibres, ash, dry matter and cholesterol for atherosclerosis induced diet. It was also calculated gross energy. For comparison purposes, similar diets were purchased from a specialized company, diets that were analysed for the same chemical parameters in the same specialized laboratory. The results showed that the values of the analysed parameters were within the limits set by recipes and compared to acquired diets the values of most parameters are close to having a coefficient of variation lower than 10. The results allow the transition to the next phase of that study, respectively the administration of purified diets in mice and rats and in achieving the induction of metabolic syndromes.*

***Key words****: purified diets, metabolic syndromes, gross chemical composition.*

## INTRODUCTION

Research of mechanisms for different morbid entities based on experimental models take increasingly higher in this research field. There are searched and established experimental models for understanding and treat some metabolic diseases, oncology diseases, toxicology, etc. The study of mechanisms of generation and development of pathologic entities such as diabetes or atherosclerosis concern broad categories of researchers from nutritionists to physiologists, pathologists, biochemists, etc.

At the same time, the expenses that mankind are assumed to understanding, preventing and combating these entities are very high. Along with human medicine, the veterinary one is increasingly involved in the study of these pathological entities providing appropriate experimental biological material more increasingly demanding by specialists, along with important contributions to the knowledge of their development mechanisms.

In procedures applied to animals, natural diets are the most used. They contain plant and animal various origin ingredients, have high palatability, are appreciated by animals, have high availability in the commercial market and are very cheap. Nevertheless due to the number of ingredients, different producers for each of these ingredients and higher variability in terms of nutritional value of each ingredient, successive batches of natural diets are very different regarding main chemical parameters (Savenije et al., 2010).

Studies on the induction of metabolic syndromes may not have the expected results because of the type of administered diet. Thus, the hypertensive effect of excessive NaCl dietary was diminished by the presence of genistein (soy phytoestrogens subclass) in rat diet trying to induce hypertension (Cho et al., 2007). Soy isoflavones have been shown also that can reduce serum cholesterol and triglycerides (Bakhit et al., 1994; Carroll et al. 1996) and can prevent the development of hepatosteatosis (Ascencio et al., 2004).

In studies on inducing obesity was observed that in rats fed with fat rich diets, but using soy as a protein source, the weight gained is less and body fat less than in rats fed with diets high

in fat but using casein as a source of protein (Torre-Villalvazo et al., 2008). Studies on the induction of diabetes may be compromised by the presence of soybeans in the diet by reducing the insulin resistance (Cederroth et al., 2008).

Purified diets have occurred in the early 1970s when the American Institute of Nutrition (AIN) created a committee that devised a diet consisting of purified ingredients (AIN-76A). The Committee acknowledged the need for a standardization of diets for laboratory mice and rats from a nutritional standpoint, but also eliminate all nutritional factors that can interfere with the study changing results.

Since then, AIN has changed diets receipts, currently standard purified diets for colonies of mice and rats being AIN-93 M and AIN-93 G (Reeves et al., 1993). Based on these diets were developed a variety of purified diets used for multiple experimental purposes. Because these ingredients are refined, (unlike cereals included in the composition of diets natural) diets purified allow researchers nutritionists to be able to define better the nutritional requirements of animals, but also through selective elimination or quantitative change at some point of a nutritional factor in diet can accurately assess the effect.

This means that there are virtually unlimited possibilities for modifications that can be made from a purified diet, making it a powerful research tool.

Purified diets to induce metabolic syndromes were manufactured in different forms with different amounts of added cholesterol for atherosclerosis induction (Xiangdong Li et al., 2011), fructose for diabetes induction (King et al. 2012) and fat for obesity induction (Buettner et al., 2007; Slavin et al., 2010).

Cantacuzino Institute (IC) is a manufacturer of laboratory animals and natural diets for laboratory animals, but Romanian research system requests also purified diets.

The long acquisition time, short validity time, long experiments and inconsistency of time between acquisition diets and animals purchase at optimal age and weight led to the idea of this project that aimed to standardizing purified diets manufactured at Cantacuzino Institute, induction of metabolic syndrome in rats and mice and standardizing animal models.

In this paper we describe the formulation, preparation of purified diets, their analysis and comparison with standard purified diets.

## MATERIALS AND METHODS

*Receipts*

The purified diets are diets wherein each nutrient is provided by one or more of purified ingredients. In these diets, protein requirements are provided by casein with added methionine (to meet the sulphur amino acids). Carbohydrates are provided by fructose corn starch and soybean oil satisfies the necessary fat and pure cellulose supply fibres needs. Vitamins and minerals ingredients are added in the form of specific mixtures for mouse and rats.

Recipes were calculated using EXCEL (Professional Edition, Microsoft) based on analytical components of each ingredient, component declared by each manufacturer.

The recipes for the maintenance and growth of colonies of animals are similar to AIN - 93 M (AIN - 93M. IC) and AIN - 93 G (AIN - 93 G. IC).

Other recipes diets for induction of atherosclerosis (IC – Ath), diabetes type II (IC – Db) and obesity (IC - Ob), have been established based on information from the specialized literature (Nishina et al., 1990; Getz et al. 2005; Buettner et al., 2006; Rossmeisl et al., 2003).

Where the certificate was not specified ingredients required parameter, it was placed ideal value.

The ingredients for recipes were: casein (Ca caseinate), L-Cystina, corn starch, maltodextrin, soybean oil, sucrose (sugar), fructose, cholesterol, cholic acid, cellulose (Arbocel), lard, antioxidant, anhydrous milkfat, vitamin and mineral premix, methionine, calcium carbonate, calcium phosphate.

All the ingredients for purified diets were purchased by company Nutristar Romania from the following manufacturers: CCPA France, JRS Germany, Dyets USA and Corman Belgium.

Estimated values of the main chemical parameters for each of the recipes are highlighted in Table 1.

Table1. Estimated values of the main chemical parameters (in percent)

| Parameter | AIN-93 G. IC | AIN-93 M. IC | IC - Ath | IC - Db | IC - Ob |
|---|---|---|---|---|---|
| Protein | 17 - 20 | 11 - 14 | 16 - 19 | 15 - 20 | 20 - 25 |
| Fat | 5-8 | 3-5 | 18-22 | 3-6 | 33-35 |
| Fibres | 3-6 | 3-6 | 3-6 | 3-6 | 7-10 |
| Dry substance | 87 – 90 | 87 – 90 | 93 – 96 | 90 – 95 | 92 – 95 |

*Preparation*

Preparation process was at Cantacuzino Institute, Baneasa Station. Cantacuzino Institute is a producer of natural diets for laboratory animals, manufacturing process being carried out in a factory with a capacity of 1000 kg / hour.

Because of the small amount of purified diets needed for this study, they were manufactured in the laboratory.

For all recipes, the ingredients of purified diets were weighed separately for each recipe and placed in a dish. The solid ingredients (fat, cholesterol and butter) were melted in a bain-marie and added in the same dish.

After all ingredients were added, they were homogenized for 45 minutes and made into dough by the addition of bi-distilled water in a ratio of 10%.

Then the slurry was granulated in a granulator GRAMILL GRM30 through a sieve of 8 mm. All of the recipes were dehydrated and dried by keeping for 3 days at room thermostat in a temperature of $37^0$ C. The diets were then stored in a refrigerator at 2-$8^0$ C to minimize oxidation.

Compared to manufacturing of natural diets, purified diets paste was made by adding water but do not by steaming as natural diets.

While the dehydration of natural diets is mechanically natural, purified diets was carried out by maintaining at thermostat. The storage is also different, natural diet was stored at room temperature, and purified diets at the refrigerator.

*Chemical analyses*

Chemical analyses were conducted at the Institute for Biology and Animal Nutrition Baloteşti, in the laboratory of chemistry and physiology of nutrition.

As a positive control, purified diets were purchased from the company Envigo Teklad Diets Italy: TD.94045 AIN 93-G; TD.94048 AIN-93 M; Adjusted TD.06414 Calories Diet (60 / Fat); TD.02028 Atherogenic Rodent Diet and TD.89247 60% fructose Diet.

For all the 10 diets were taken sample and counter sample which were sent to the laboratory.

In the chemistry laboratory were conducted chemical analyses following ISO standards and according to procedures recommended by FAO (FAO, 2011) (Table 2).

Table 2. Analysis method for chemical determination

| Determination | Analysis Method |
|---|---|
| Dry matter 103$^o$ C | Gravimetric method<br>Regulation (EC) no. 152/2009<br>SR ISO 6496: 2001 |
| Crude protein | Mineralization in the block and steam distillation method<br>Regulation (EC) no. 152/2009<br>SR EN ISO 5983-2: 2009 / AOAC 2001.11 |
| Crude fat | Extraction method with organic solvent<br>Regulation (EC) no. 152/2009<br>SR ISO 6492: 2001 |
| Crude fibres | Method with intermediate filtration<br>Regulation (EC) no. 152/2009<br>SR EN ISO 6865: 2002 |
| Crude ash | Gravimetric method<br>Regulation (EC) no. 152/2009<br>SR EN ISO 2171:2010 |
| Cholesterol (only for atherogenic diet) | Gas Chromatographic method<br>ISO 12228:1999 /<br>AOAC 994.10 |
| Gross energy | Calculation |

*Statistics*

To assess the comparative results of the chemical analyses at purified diets produced by Cantacuzino Institute and at purchased diets were calculated average, standard deviation and coefficient of variation using EXCEL software (Professional Edition, Microsoft) and GraphPad software (GraphPad 2016).

## RESULTS AND DISCUSSIONS

Table 3 and 4 shows the results of gross chemical analysis compared with estimated values by calculation (in percent).

Table 3. The results of gross chemical analysis for AIN recipes (in percent)

| Parameter | AIN-93 G. IC | | AIN-93 M. IC | |
|---|---|---|---|---|
| | estimated | obtained | estimated | obtained |
| Protein | 17 - 20 | 17.47 | 11 - 14 | 12.84 |
| Fat | 5-8 | 5.41 | 3-5 | 3.53 |
| Fibres | 3-6 | 3.24 | 3-6 | 3.64 |
| Dry matter | 87– 90 | 87.37 | 87– 90 | 89.58 |

Table 4. The results of gross chemical analysis for purified diets for metabolic syndrome induction

| Parameter | IC - Ath | | IC -Db | | IC - Ob | |
|---|---|---|---|---|---|---|
| | estimated | obtained | estimated | obtained | estimated | obtained |
| Protein | 16- 19 | 17.99 | 15- 20 | 18.45 | 20- 25 | 24.94 |
| Fat | 18-22 | 21.49 | 3-6 | 4.55 | 33-35 | 33.58 |
| Fibres | 3-6 | 4.14 | 3-6 | 5.69 | 7-10 | 9.69 |
| Dry matter | 93–96 | 95.9 | 90–95 | 93.77 | 92–95 | 96.52 |

A comparison between diets manufactured at Cantacuzino Institute and the standardized diets purchased from Envigo Teklad Diets shows close values, with one exception (Table5).

Table 5. The results of gross chemical analysis for produced and acquired purified diets

| Purified diets | Parameter | Envigo Teklad | IC |
|---|---|---|---|
| growth/breeding | Crude protein | 17.98 | 17.47 |
| | Crude fat | 6.07 | 5.41 |
| | Crude fibres | 1.92 | 3.24 |
| | Crude ash | 2.55 | 4.81 |
| | Dry matter | 90.12 | 87.37 |
| | Gross energy | 4217 | 3976 |
| maintaining | Crude protein | 13.12 | 12.84 |
| | Crude fat | 3.55 | 3.53 |
| | Crude fibres | 3.14 | 3.64 |
| | Crude ash | 2.71 | 4.3 |
| | Dry matter | 89.31 | 89.58 |
| | Gross energy | 3871 | 3825 |
| Diets for atherosclerosis induction | Crude protein | 17.46 | 17.99 |
| | Crude fat | 20.73 | 21.49 |
| | Crude fibres | 3.78 | 4.14 |
| | Crude ash | 2.86 | 4.97 |
| | Dry matter | 93.32 | 95.9 |
| | Gross energy | 5130 | 5070 |
| | Cholesterol | 0.6567 | 0.5731 |
| Diets for diabetes type II induction | Crude protein | 17.51 | 18.45 |
| | Crude fat | 0.43 | 4.55 |
| | Crude fibres | 5.49 | 5.69 |
| | Crude ash | 3.69 | 4.52 |
| | Dry matter | 87.86 | 93.77 |
| | Gross energy | 3787 | 4234 |
| Diets for obesity induction | Crude protein | 22.12 | 24.94 |
| | Crude fat | 29.07 | 33.58 |
| | Crude fibres | 8.42 | 9.69 |
| | Crude ash | 3.42 | 5.42 |
| | Dry matter | 87.86 | 96.52 |
| | Gross energy | 5425 | 6000 |

Statistical analysis in comparison of the two types of diets is presented in Table 6.

Table 6. Statistical results of comparison for produced and acquired purified diets

| Purified diets | Parameter | Average | SD | CV |
|---|---|---|---|---|
| growth/ breeding | Crude protein | 17.72 | 0.36 | 2.03 |
| | Crude fat | 5.74 | 0.46 | 8.13 |
| | Crude fibres | 2.58 | 0.93 | 36.17 |
| | Crude ash | 3.68 | 1.59 | 43.42 |
| | Dry matter | 88.74 | 1.94 | 2.19 |
| | Gross energy | 4096 | 170.41 | 4.15 |
| Maintaining | Crude protein | 12.98 | 0.19 | 1.52 |
| | Crude fat | 3.54 | 0.01 | 0.39 |
| | Crude fibres | 3.39 | 0.35 | 10.42 |
| | Crude ash | 3.50 | 1.12 | 32.07 |
| | Dry matter | 89.44 | 0.19 | 0.21 |
| | Gross energy | 3848 | 32.52 | 0.84 |
| Diets for atherosclerosis induction | Crude protein | 17.72 | 0.37 | 2.11 |
| | Crude fat | 21.11 | 0.53 | 2.54 |
| | Crude fibres | 3.96 | 0.25 | 6.42 |
| | Crude ash | 3.91 | 1.49 | 38.10 |
| | Dry matter | 94.61 | 1.82 | 1.92 |
| | Gross energy | 5100 | 42.42 | 0.83 |
| | Cholesterol | 0.61 | 0.05 | 9.61 |
| Diets for diabetes type II induction | Crude protein | 17.98 | 0.66 | 3.69 |
| | Crude fat | 2.49 | 2.91 | 116.99 |
| | Crude fibres | 5.59 | 0.14 | 2.52 |
| | Crude ash | 4.105 | 0.58 | 14.29 |
| | Dry matter | 90.81 | 4.17 | 4.60 |
| | Gross energy | 4010 | 316.07 | 7.88 |
| Diets for obesity induction | Crude protein | 23.53 | 1.99 | 8.47 |
| | Crude fat | 31.32 | 3.18 | 10.18 |
| | Crude fibres | 9.05 | 0.89 | 9.91 |
| | Crude ash | 4.42 | 1.41 | 31.99 |
| | Dry matter | 92.19 | 6.12 | 6.64 |
| | Gross energy | 5712 | 406.58 | 7.117 |

Purified diets are an important research tool for inducing several diseases, including metabolic syndromes that are most important. But making these diets and storage them poses some logistical problems what makes between finance, planning and execution of the procedure to pass a longer period of time. Therefore we felt that having experience of over 20 years in manufacturing natural diets and laboratory animals nutrition we can produce also purified diets.

Production of purified diets in laboratory conditions for small quantities not encountered problems; the technology is similar to that for manufacturing natural diets.

For purified diet formulas have been established and were calculated some values for crude chemical composition (protein, fat, cellulose and dry matter). These values were determined based on the values chemical

parameters diets to induce the desired metabolic syndrome (atherosclerosis, diabetes type II and obesity) and reproduce standard diets for maintaining and growth/ breeding colony of mice and rats. In calculating parameters was of special importance for the parameter values of each ingredient used in recipes diets, according to the analysis report.

The results obtained from chemical analyses have shown that all values obtained were within the limits anticipated, which confirms that when we know the nutritional values of each ingredient we can correctly predict the chemical composition of a diet.

Although purchased purified diets were accompanied by quality certificates and test reports, we found it necessary to do analyses of chemical composition because there are differences between laboratories, differences in method, apparatus, calculation, differences which can lead to variability in results (Bielohuby et al., 2010; Hristov et al., 2010). Analysed the diets, except for a few parameters, the variation in chemical composition values at the 5 diets of the two producers was low.

Standard deviation highlights the uniformity of data around the mean value and except for one parameter (gross energy), expected result because of the various ingredients used to produce diets. The coefficient of variation allows the comparison of statistical series in terms of standard deviation. It found a high coefficient of variation (CV = 14.29 - 43.42%) of all diets values of crude ash.

How values were higher in own diets than those purchased and reflects the total mineral raw ashes of a diet, we believe that the differences are due to vitamin and mineral premix used and its composition. A coefficient of variation was large and consisted of crude fibre on growth/breeding diet (CV = 36.17%), the percentage being higher on crude fibre to produced diet.

A paradoxical result was recorded of crude fat parameter for diet can induce type II diabetes. The coefficient of variation was 116.99, due to low percentage value of fat in the diet purchased. Although the analysis was repeated, because it was far from optimal and from received analysis reports, the result was maintained.

The gross chemical composition is different for the same feed, from one country to another, from one area to another, depending on the starting materials, technology of obtaining, the way of conservation. This makes the average data available in the literature to be insufficient to assess the nutritional value of a diet, which is why it were conducted extensive chemical gross tests for diets manufactured to be used in other experiments.

The results taken as a whole show similarity between diets produced and diets acquired. The values of chemical composition within the range expected, indicates that diets have been manufactured correctly and could go to the next stage of study, respectively the induction of metabolic syndromes by administering these diets. In the same time will continue to analyses for produced diets other nutritional parameters to determine sugars, amino acids, micro and macronutrients, vitamins.

## CONCLUSIONS

Purified diets are a powerful research tool for studying induced metabolism syndromes.

The recipes of purified diets that were produced at Cantacuzino Institute were calculated based on the values of nutrition that should induce metabolic syndrome (atherosclerosis, diabetes type II and obesity), setting margins of values.

Results of gross chemical composition analysis showed that the values obtained were within the margins established.

Comparative analysis of own diets and similar standardized purchased diets showed that unless crude ash value all other values are superimposable.

The results allow us to move on to the next phase of that study by administering diets to induce metabolic syndromes.

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# TESTING THE INFLUENCE OF THE ENVIRONMENTAL CLIMATIC FACTORS UPON DONKEY MILK QUALITY

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***Abstract***

*Our trial was conducted with the aim of emphasizing the influence of environmental factors acting as potential stressors upon donkey milk nutritional traits, within specific climatic conditions of North-West Romania. Milk from fifty jennies from four private farms, in different stages of lactation, from first up to the fifth, weekly collected during a three months period, February-April 2016, was qualitatively and quantitatively evaluated. In experimental areal, temperature, humidity, and wind velocity, were daily recorded. The average temperature of the experimental period (4.95°C±0.19°C) is with 0.8°C bigger compared to the average temperature of the experimental areal in 2010, suggesting the increase of the heat stress upon donkey milk production traits. Donkey milk content in lactose and fat, together with milk pH, increase from first to fifth lactation, while water and protein content decrease. We may consider the lack of correlations between donkey milk pH and fat content, and also between fat and lactose. Positive significant moderate correlations are identified between donkey milk lactose and protein, and also between lactose, protein, and donkey milk pH. Between fat and donkey milk water content, negative significant moderate correlation is identified. Temperature, humidity, and wind velocity have strong influence upon donkey milk fat, protein and water content, and moderate on donkey milk pH, and lactose. This study emphasizes the necessity of adopting suitable managerial practices in donkey rearing facilities, where the farmer is interested in valuating donkey milk. This assertion is based on the increasing of the temperature in the experimental areal, compared to the same period of the previous years, taking into account the negative influence of the heat stress on donkey milk quality.*

***Keywords:*** *protein, lactose, fat, water, pH, correlation, heat stress.*

## 1. INTRODUCTION

The donkey (*Equus africanus asinus* L.) is a member of the *Equidae* family. According to literature (Bökönyi 1991; Clutton-Brock, 1992; Eisenmann, 1995; Groves, 1986; Haltenorth and Diller, 1980; Kingdon, 1997), this specie originates from Africa, and it was domesticated in time, as result of a complex process (Bench, 2004).

From ancient times up to our days, traders and pastoralists use donkeys for transport, and/or pack animals, while sometimes farmers use donkeys for field and domestic works. Besides the small expenses needed of their maintaining, when we talk about their acquisition, we also speak of low cost (Marshall and Weissbrod, 2011).

The above mentioned low costs are the consequences of their perception as a single-use specie, compared to cattle, buffaloes, etc., which are used as multipurpose livestock, usually reared for meat, milk, but also for work and transport (Fernando and Starkey, 2004; Singh et al., 2005). Even they are not part of livestock species valuable due to their meat output, the jenny milk production become more and more interesting, in last decennials.

The donkey milk may be used instead of human milk, having better properties compared to cow milk (Fantuz et al., 2012; Salimei and Fantuz, 2012).

In Europe, the use of jenny milk as human milk replacer become a tradition, mostly because of its composition and physico-chemical traits (Aspri et al., 2016; Ragona et al., 2016; Trinchesea et al., 2015). The jenny milk composition is similar to human milk in terms of lactose, protein and fatty acids profiles, minerals (Bidasolo et al., 2012; Piovesana et al., 2015), and also other components, as shown in Table 1 (Guo et al., 2007).

Table 1. Composition of donkey milk compared to human milk (Guo et al., 2007)

| Issue | Donkey milk | Human milk |
|---|---|---|
| pH | 7.0-7.2 | 7.0-7.5 |
| Protein (%) | 1.5-1.8 | 0.9-1.7 |
| Fat (%) | 0.3-1.8 | 3.5-4.0 |
| Lactose (%) | 5.8-7.4 | 6.3-7.0 |
| Ash (%) | 0.3-0.5 | 0.2-0.3 |
| Total solids (%) | 8.08-11.7 | 11.7-12.9 |
| Caseins (%) | 0.64-1.03 | 0.32-0.42 |
| Whey proteins (%) | 0.49-0.80 | 0.68-0.83 |

Besides the properties already emphasized, the jenny milk has good palatability, valuable nutritional properties, reduced allergenicity, and clinical tolerability (Trinchesea et al., 2015). When adverse (Polidori and Vincenzetti, 2013).

Literature also mentions the utility of using donkey milk when allergy to cow milk, considered as one of the ”Big 8” allergens from food (Host, 2002), is recorded, because its proteins seem to be safer for infants’ consumption (D’Alessandro and Martemucci, 2012; Polidori and Vincenzetti, 2013). Another use of jenny milk is in cosmetics (Consentino et al., 2014)

Even donkey maintaining seems to be easy, because they have no special maintaining and welfare requirements (Consentino et al., 2010; Consentino et al., 2012; Zakari et al., 2015), the environmental issues may affect their behavior and wellbeing (Lagat and Nyangena, 2016).

According to Smith and Pearson (2005), Dey et al. (2010), Zakari et al. (2015), Kumar et al. (2011), and Pandey et al. (2012) temperature is one of the most important environmental stressor upon livestock animals, generally speaking, and on donkey behavior and productions, particularly.

Thus, heat stress, together with solar radiation, both direct and indirect, accompanied by high humidity proofed to be environmental stressors, which have high potential of affecting donkey welfare and productions. The amount of this influence depends on both intensity of climatic inputs, and intrinsic thermoregulation mechanisms specific for each donkey breed.

In Romania, few studies were conducted upon the donkey milk composition and quality (Marchis et al., 2015a; Marchis et al., 2015b), and also upon the influence of climatic on donkey maintaining, and their dairy production (Coroian et al., 2016).

In order to contribute to the knowledge concerning the composition of the donkey milk, obtained from individuals traditionally reared in Romania, in specific climatic conditions, besides laboratory quantifications of milk traits (fat, protein, lactose, water pH), our study also shows the results of testing the intensity of the interaction between donkey milk composition, and environmental stress factors represented by temperature, humidity, and wind velocity, respectively.

## 2. MATERIAL AND METHODS

### 2.1. The experimental areal

The trial was carried out in four donkey private farms, located nearby one to each other, in North-West Romania, in the vicinity of the town of Huedin (46˚52’00’’N, and 23˚02’00’’E), County of Cluj, during February – April 2016.

### 2.2. The environmental monitoring

The experimental area is characterized by a temperate continental clime, with historic annual average temperature of 7˚C – 8˚C, which recorded an alarming increase of almost 1 ˚C, in last decade, from 7.9˚C, to 8.8˚C (Romanian National Administration of Meteorology. Climatic monitoring). Climatic data (temperature, humidity, and wind velocity) were daily recorded with an automatic meteorological data station, WE900 from WTW, placed in experimental area.

### 2.3. Sample collection

Milk samples were collected from the morning milking of 10 primiparous, and 40 multiparous jennies. The animals are maintained within the same conditions, and receiving the same feeding level and composition. The milk samples of an average volume of 300 mL were collected, weekly, in standard containers, and transported to the laboratory, maintained at 4°C.

### 2.4. Laboratory analysis

The physicochemical analysis was performed in the same day. Lactoscan MCC device, from Milkotronic Ltd. was used for laboratory determinations. The principle of pH, fat, protein, lactose, and water determinations is based on the direct measurement of the speed of the ultrasound in milk.

### 2.5. Statistical analysis

Data were statistically processed with IBM SPSS Statistics v. 20. Descriptive statistics was used for characterizing the milk traits. The Pearson simple

correlations and multiregression analyze was implemented in order to emphasize the intensity of the relationships between milk traits, and the interrelations between the climatic factors characterizing the environment (temperature, ˚C; humidity, %; wind velocity, m/s) and each of the analyzed traits of the donkey milk (fat, protein, lactose, water, and pH), respectively.

## 3. RESULTS AND DISCUSSIONS

The monitoring of the environmental parameters characterizing the experimental field, emphasizes their means, calculated by the experimental period of three months, respectively. Thus, resulted a three months mean temperature of 4.95˚C, humidity of 67.92%, and wind velocity of 7.45 m/s (Table 2). The statistical analyze emphasizes a good representativeness of the humidity and wind velocity means, confirmed by the values of the coefficients of variation, CV = 16.44%, and CV = 25.77%, respectively (Merce and Merce, 2009). Not the same thing may be observed when we analyze the mean of the temperature in experimental field, when the variability expressed by the value of CV = 37.91%, over 30% (Merce and Merce, 2009), shows that the mean has no representativeness. This may be explained by the large variation of temperature, from a minimum of -5°C, to a maximum of +7°C (Table 2), because the experimental interval covers the end of winter and first two months of spring, when weather become warmer.

Temperature, which is one of the most important environmental parameter for donkey wellbeing, and milk production, because it may produce the heat stress that induces physiological disorders (Ayo et al., 2014; Zakari et al., 2015) recorded a mean increase of 0.8°C from 4.15°C in 2010 to 4.95°C in 2016, by a three months period, February – April, respectively (Fig. 1).

These results may be considered as part of the alarming phenomenon of climatic changes, which may be also encountered in the studied area.

Our trial shows different evolutions of donkey milk traits by lactation (Table 3).

The fat content (Table 3) increases continuously from first to fifth lactation.

This result is opposite to the findings of Heinrichs et al. (2017) in USA, who state that milk fat in dairy herds decreases ”as the animal becomes older”.

The differences between the values correspondent to first, fourth and fifth lactation are statistically significant if compared to the mean by all lactations (Table 4).

Protein, lactose, water and pH have fluctuant evolutions, lactose and pH characterized by an increasing evolution, while protein and water by a decreasing tendency (Table 3, Fig. 2). The differences between these means, by lactation, and means by all lactations are not statistically significant (Table 4).

Table 2. Descriptive statistics concerning the evolution of the climatic parameters within the experimental areal, February – April, 2016

| Parameter | N | Temperature (˚C) | Humidity (%) | Wind velocity (m/s) |
|---|---|---|---|---|
| Mean | 92 | 4.95 | 67.92 | 7.45 |
| Standard deviation | 92 | 1.82 | 11.17 | 1.92 |
| Standard error of mean | 92 | 0.19 | 1.16 | 0.20 |
| Minimum | 92 | -5.00 | 45.00 | 3.00 |
| Maximum | 92 | 7.00 | 96.00 | 11.00 |
| Coefficient of variation | 92 | 37.91 | 16.44 | 25.77 |

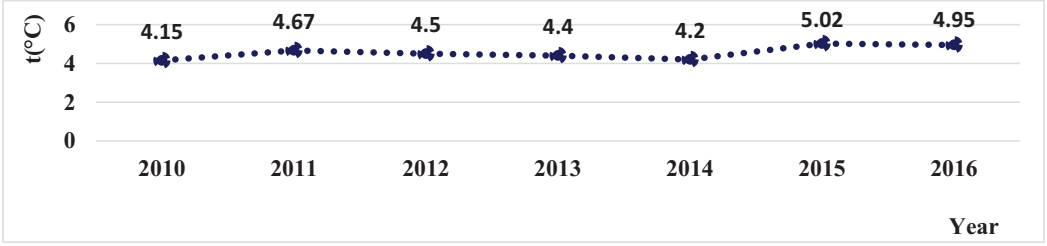



Figure 1. The evolution of the mean temperature by a three months period (February – April) in experimental area, 2010 - 2016

Table 3. Descriptive statistics concerning the evolution of donkey milk traits, function of lactation, February – April, 2016

| Lactation | I | II | III | IV | V |
|---|---|---|---|---|---|
| **Fat (%)** | | | | | |
| N | 10 | 10 | 10 | 10 | 10 |
| Mean | 0.86[a] | 1.68[b] | 1.97[b] | 2.30[a] | 2.46[a] |
| Standard deviation | 0.03 | 0.03 | 0.02 | 0.07 | 0.05 |
| Standard error of mean | 0.11 | 0.11 | 0.06 | 0.21 | 0.16 |
| Minimum | 0.67 | 1.55 | 1.87 | 2.01 | 2.20 |
| Maximum | 0.94 | 1.83 | 2.03 | 2.55 | 2.60 |
| Coefficient of variation (%) | 12.72 | 6.29 | 3.21 | 9.32 | 6.36 |
| **Protein (%)** | | | | | |
| N | 10 | 10 | 10 | 10 | 10 |
| $\bar{X}$ | 1.88 | 1.72 | 1.73 | 1.80 | 1.81 |
| $s_{\bar{X}}$ | 0.03 | 0.01 | 0.01 | 0.02 | 0.02 |
| s | 0.10 | 0.04 | 0.04 | 0.06 | 0.07 |
| Minimum | 1.77 | 1.68 | 1.68 | 1.71 | 1.73 |
| Maximum | 1.98 | 1.78 | 1.79 | 1.87 | 1.89 |
| CV(%) | 5.42 | 2.41 | 2.42 | 3.23 | 3.60 |
| **Lactose (%)** | | | | | |
| N | 10 | 10 | 10 | 10 | 10 |
| $\bar{X}$ | 6.80 | 6.72 | 6.78 | 6.77 | 6.80 |
| $s_{\bar{X}}$ | 0.03 | 0.02 | 0.01 | 0.01 | 0.01 |
| s | 0.11 | 0.08 | 0.05 | 0.04 | 0.05 |
| Minimum | 6.68 | 6.61 | 6.71 | 6.70 | 6.73 |
| Maximum | 6.92 | 6.82 | 6.84 | 6.80 | 6.85 |
| CV(%) | 1.58 | 1.17 | 0.78 | 0.62 | 0.67 |
| **Water (%)** | | | | | |
| N | 10 | 10 | 10 | 10 | 10 |
| $\bar{X}$ | 88.40 | 87.29 | 85.16 | 84.66 | 86.01 |
| $s_{\bar{X}}$ | 0.22 | 0.50 | 0.60 | 0.55 | 0.67 |
| s | 0.71 | 1.60 | 1.90 | 1.75 | 2.11 |
| Minimum | 87.50 | 84.78 | 82.70 | 82.50 | 82.75 |
| Maximum | 89.10 | 89.01 | 87.10 | 87.20 | 88.20 |
| CV(%) | 0.80 | 1.84 | 2.23 | 2.07 | 2.45 |
| **pH** | | | | | |
| N | 10 | 10 | 10 | 10 | 10 |
| $\bar{X}$ | 6.94 | 6.86 | 6.94 | 7.11 | 7.09 |
| $s_{\bar{X}}$ | 0.03 | 0.04 | 0.04 | 0.03 | 0.03 |
| s | 0.11 | 0.15 | 0.15 | 0.09 | 0.09 |
| Minimum | 6.80 | 6.71 | 6.70 | 7.01 | 7.00 |
| Maximum | 7.10 | 7.10 | 7.10 | 7.20 | 7.20 |
| CV(%) | 1.64 | 2.25 | 2.19 | 1.22 | 1.23 |

a - $p < 0.01$; b - $p > 0.05$.

Mean fat and protein contents from donkey milk resulted from our trial (Table 4), are bigger compared to the values (0.53% fat, and 1.63% protein) obtained by Martini et al. (2013) and (0.4% fat, 1.57% protein) obtained by Ragona et al. (2016) in Amiata donkey, but lactose content of donkey milk (Table 4) is lower compared to the same trials, where a mean of 7.12% is reported by Martini et al. 92013), and 7.23% by Ragona et al. (2016). Compared to the values obtained by Conti (2013) in Sicily, for donkey milk content in lactose (6.23-6.24%), and fat (1.61%) we report bigger values (Table 4).

If the protein mean content of the donkey milk analyzed in our trial (Table 4) frames with the range of variation of 1.5-1.8% mentioned by Guo et al.

(2007), similar with human milk protein content (0.9-1.7%), but smaller compared to cow milk, of 3.1-3.8% (Polidori et al., 2016), it is bigger compared to limits mentioned by Barłowska et al. (2011), of 1.59-1.74%.
The fat content (Table 4) is slightly over the range (0.3-1.8%) mentioned by Guo et al. (2007) and (0.28-1.82%) reported by Barłowska et al. (2011), but smaller compared to the limits mentioned by Polidori et al. (2016), for human milk (3.5-4%), and cow milk (3.5-3.9%). On the contrary, pH (Table 4) is slightly under the ranges mentioned by Guo et al. (2007) for donkey milk (7-7.2%), and human milk (7-7.5%). Lactose identified in our trial in donkey milk (Table 4) frames within the ranges mentioned by both Guo et al. (2007), 5.8-7.4%, respectively, and also by Barłowska et al. (2011), of 5.87-6.88%.

Table 4. Descriptive statistics for the evolution of donkey milk traits by all lactations, February – April, 2016

| Parameter | N | Fat (%) | Protein (%) | Lactose (%) | Water (%) | pH |
|---|---|---|---|---|---|---|
| Mean | 50 | 1.85[a,b] | 1.79 | 6.77 | 86.31 | 6.99 |
| Standard deviation | 50 | 0.12 | 0.02 | 0.01 | 0.42 | 0.03 |
| Standard error of mean | 50 | 0.02 | 0.01 | 0.01 | 0.06 | 0.01 |
| Minimum | 50 | 0.67 | 1.68 | 6.61 | 82.50 | 6.70 |
| Maximum | 50 | 2.60 | 1.98 | 6.92 | 89.10 | 7.20 |
| Coefficient of variation | 50 | 31.82 | 4.74 | 1.04 | 2.42 | 2.14 |

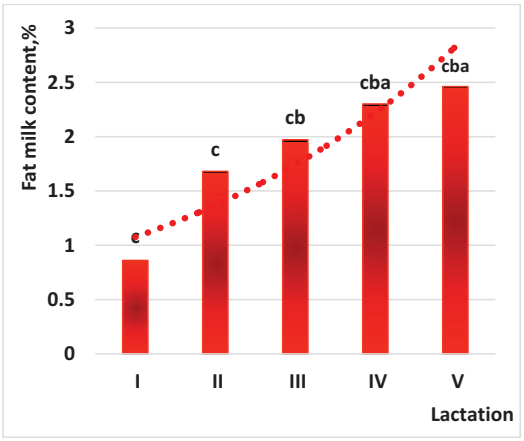
a - $p < 0.01$; b - $p > 0.05$.

Except lactose (Fig. 2c), all investigated donkey milk traits exhibit differences between different stages of lactation (Fig. 2). Fat increases very significantly from the I$^{st}$ up to the V$^{th}$ lactation (Fig. 2a), while protein records a fluctuation between lactations, with significand differences at significance threshold of 5% (Fig. 2b). The milk pH has a positive trend from the first to the V$^{th}$ lactation, the differences being statistically significant at significance threshold of 5% (Fig. 2d).
Donkey milk water content records a negative trend from first to V$^{th}$ lactation, the differences between III$^{rd}$ and V$^{th}$ lactations being significant and distinctly significant (Fig. 2e).
Between the donkey milk traits positive and negative simple correlations, from very weak to moderate intensity may be emphasized (Table 5). Moderate significant ($p<0.01$) correlations, positive between the protein and lactose, fat and pH, and negative between fat and water are recorded. No significant correlations are found between the other studied traits (Table 5).
Negative weak correlation was observed between fat and protein, and positive moderate between water and protein. Positive weak and very weak correlations may be identified between lactose and fat, water, pH, and also between water and pH (Table 5).
Our data are not consistent with those obtained by Conty (2013), as result of an experiment performed in warm climate of Sicily, on milk from common donkey breed, where weak to moderate correlation (R=0.372) resulted between fat and lactose.

Table 5. The correlation matrix of the donkey milk traits Decriptive statistics for the the evolution of donkey milk traits by all lactations, February – April, 2016

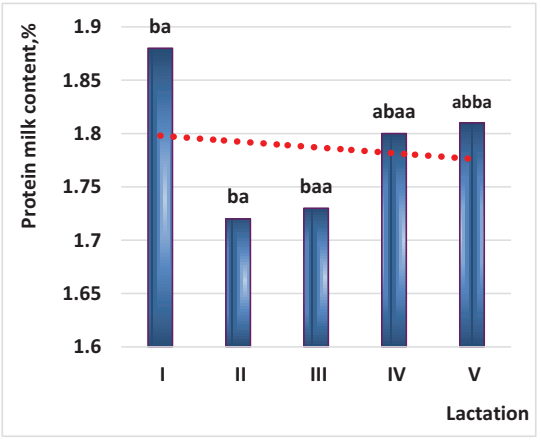
| Issue | Fat (%) | Protein (%) | Lactose (%) | Water (%) | pH |
|---|---|---|---|---|---|
| Fat (%) | - | -0.239 | +0.060 | -0.449[a] | +0.477[a] |
| Protein (%) | - | - | +0.451[a] | +0.370 | +0.264 |
| Lactose (%) | - | - | - | +0.242 | +0.120 |
| Water (%) | - | - | - | - | +0.089 |

a - $p < 0.01$.



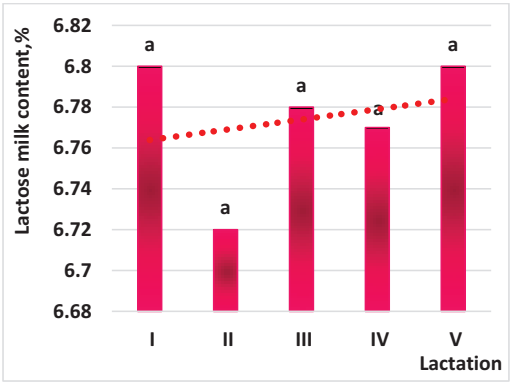

a - p>0.05; b - p<0.01; c - p <0.001;

a. Fat (%)



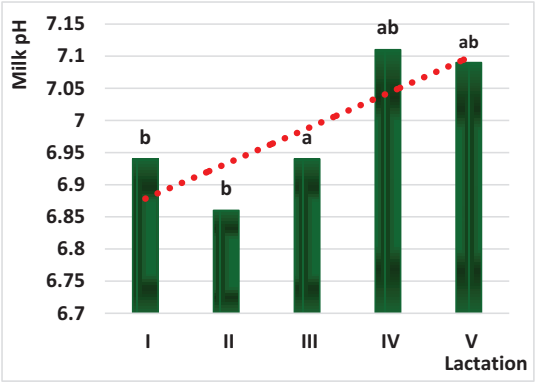

a - p >0.05; b – p<0.05;

b. Protein (%)



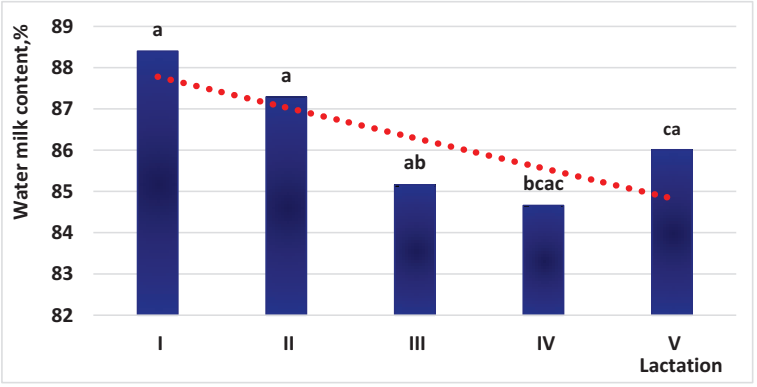

a - p>0.05;

c. Lactose (%)




a - p >0.05; b – p<0.05;

d. pH




a - p >0.05; b - p>0.01; c - p>0.05.

e. Water

Figure 2. The evolution of the donkey milk traits function of lactation

Between temperature, humidity and wind velocity and donkey milk fat, protein, and water, moderate to strong multiple correlations are reported, while between above mentioned climatic parameters, pH and lactose from donkey milk, moderate correlations may be mentioned (Table 6).
According to regression lines, temperature and humidity contribute to decrease of the donkey milk traits analyzed in the present study, while wind have a small positive contribution (Table 6). The correlation between fat and climatic factors explains 63.8% of the variability of this donkey milk trait, for protein they explain 52.70% of variability, while for water 58.50%.
The variability of donkey milk pH and lactose content is explained in a less extent by the interaction with considered climatic factors (Table 6).

Table 6. The multiple correlations (R) and determination coefficients ($R^2$) reported between the climatic factors characterizing the environment (temperature, humidity, wind velocity) and donkey milk traits

| Traits | R ($R^2$) | Regression line |
|---|---|---|
| Fat (%) | R = 0.799 ($R^2$ = 0.638) | Fat (%) = 4.038-3.251t(˚C)-2.597H(%)+0.133v(m/s) |
| Protein (%) | R = 0.726 ($R^2$ = 0.527) | Protein (%) = 2.466-1.429t(˚C)-0.527H(%)+0.073v(m/s) |
| Lactose (%) | R = 0.590 ($R^2$ = 0.348) | Lactose (%) = 7.286-1.178t(˚C)-0.354H(%)+0.034v(m/s) |
| Water (%) | R = 0.765 ($R^2$ = 0.585) | Water (%) = 87.084-0.283t(˚C)-0.685H(%)+0.047v(m/s) |
| pH | R = 0.530 ($R^2$ = 0.281) | pH (%) = 9.356-1.366t(˚C)-1.988H(%)+0.156v(m/s) |

Concerning environmental influence on fat and protein from donkey milk, our results are consistent to those emphasized, in cow milk, by Milani et al. (2015), and also Linn (1988).
Linn states that in summer, when heat and humidity record high values, a decrease of fat and protein from cow milk is noticed. Barash et al. (2001) also noticed the negative influence of temperature upon protein content in cow milk.

## 4. CONCLUSIONS

Because of hypoallergenic and nutritional traits, together with composition similar to human milk, donkey milk is a very good candidate for replacing human milk. Our study emphasizes, besides the heat, humidity, and wind velocity influence on qualitative donkey milk traits (fat, protein, lactose, water, and pH), the variation of these traits function of lactation and also, the interactions between them. Majority donkey milk traits, fat, lactose and pH, respectively, exhibit increasing tendency from first to fifth lactation, while protein and water content, a decreasing one. The evolutions of fat and lactose contents in donkey milk, and water and pH, respectively, may be considered not correlated. Noticeable significant positive moderate correlations are identified between fat and donkey milk pH, and protein and lactose, while between donkey milk water content and fat, the correlation, even significant and moderate, is negative, suggesting the divergent evolution of these parameters.Between protein, fat and water from donkey milk and climatic factors (temperature, humidity and wind velocity) strong interrelationships are reported. Donkey milk pH and lactose content is less influenced by the above mentioned climatic factors. Temperature and humidity recorded within the experimental areal have bigger influence on the donkey milk traits discussed in this study, contributing to decrease of all studied traits, while wind have a weak positive influence upon the increase of these traits. Within the climatic landscape of the experimental areal characterized by a noticeable warming, meaning about 0.8°C during a 6 year period, and taking into consideration the importance of heat stress upon donkey milk qualitative traits, this study suggest farmers interested in valuating donkey milk for consumption, the urgency of adopting appropriate managerial conditions in order to diminish the potential effects of increased heat stress upon fat, and protein milk content.

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