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VETERINARY MEDICINE

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SUMMARY

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TOPOGRAPHY OF THE MAJOR SALIVARY GLANDS IN RABBITS

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Abstract
Salivary glands are important in research articles, because of their different functions (Asari et al., 2000). They develop in different locations, having a very various architecture, secreting different types of saliva (Jaskoll et al., 2002). The major salivary glands are parotid, mandibular and sublingual glands. These glands have an important role in aliments' digestion through their secretions (saliva), which is serous, mucous or sero-mucous, containing different enzymes, water, mucopolysaccharides and lubrefiant glycoproteins (Al-Saffar and Simawy, 2014; Boşca et al., 2014). In this study, we used five healthy male rabbits from a private breeder in Cluj, sacrificed by the owner for own consumption. Subsequently, the anatomical regions of major salivary glands were shaved and cleaned with alcohol. Macroscopical exam revealed that the rabbits' parotid gland is the most voluminous gland, having the aspect of a scythe blade. The mandibular gland has a lobate appearance. We noticed that there is an asymmetry between the two mandibular glands, the right one being more developed than the left one. Sublingual gland is the smallest gland, being covered by surrounding connective tissue. Inferior buccal glands gathered and formed a major salivary gland, the labial gland, situated at the lips commissure.

Key words*: anatomy, mandibular, parotid, rabbit, saliva, sublingual.*

INTRODUCTION

The salivary glands are considered parts of the upper gastrointestinal tract, having exocrine secretion (Al-Abbad, 2011). Some of the salivary glands are voluminous, compact, being well individualized - the major salivary glands. This category includes: parotid, mandibular and sublingual glands (Popovici et al., 2003; Barone, 2009; Papuc et al., 2009; Miclăuş, 2012). In addition, there are minor salivary glands, poor developed, spread in the labial mucosa, hard palate, soft palate and lingual mucosa.
Saliva prepares food for digestion, which takes place in the following segments of digestive tract: pharynx, esophagus, reaching into the stomach where the digestion begins, proceeding into the small and large intestine (Popovici et al., 2003; Reece, 2005; Stan, 2014, 2013).

MATERIALS AND METHODS

The biological material was represented by five healthy male rabbits, with an average weight of 1750 g, from a private breeder in Cluj, sacrificed by the owner for own consumption. Subsequently, the anatomical regions of major salivary glands were shaved and cleaned with alcohol. The materials used were the dissection instruments (forceps, scalpel, scissors, magnifying glass, gloves, gauze) and a Nikon D3000 camera.

RESULTS AND DISCUSSIONS

The **parotid gland** is the most massive of the major salivary glands, having a white colour and visible lobulation, being represented by a dorsal and ventral extremity. The dorsal extremity surrounds the base of the ear and the ventral one reaches the mandibular angle. We noticed that the ventral extremity is less wider than the width of the dorsal extremity, which surrounds both orally and aborally the base of the ear.
Cranial edge of the parotid gland follows closely the recurved branch of the mandible and implicit the aboral border of the masseter muscle. Because of the glands' aspect, we can say that it not only shows two extremities - dorsal and ventral - but also two edges - cranial and caudal - both concave cranially.

The ventral extremity is sharp in the oral part, bringing together the two edges - cranial and caudal - giving the gland the appearance of a scythe blade.
The dorsal extremity covers the base of the ear, temporo-mandibular region, the cephalic extremity of the brachiocephalic muscle and cleidomastoidian muscle.
The subjects in dorso-ventral recumbence point out that the ventral extremity of the parotid gland reaches the mandibular gland.
The excretory duct of the parotid gland has the trajectory on the surface of the masseter muscle, crossing directly over this muscle and opening up above the last upper molar (Fig. 1).

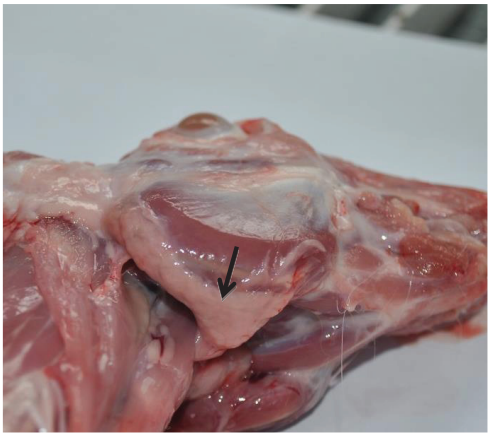

Figure 1. Parotid gland

Mandibular gland is lobated, being smaller than the parotid gland, located ventro-medial to the angle of the mandible.
These glands are located on both sides of the aboral extremity of the tongue. We noticed an asymmetry regarding the dimensions of the gland, 100% of the subjects examined having the right mandibular gland larger than the left one.
The previous trajectory mentioned before was encountered in all subjects in this study, the parotid duct being parallel to the facial nerve branches, more exactly to the buccal nerves.
Also, to all individuals studied by us, the ventral extremity of the parotid gland is in contact with the mandibular gland.
Mandibular gland presents anatomical relation with the masseter muscle, pterygoid medial and milohioidian muscles (Fig. 2).

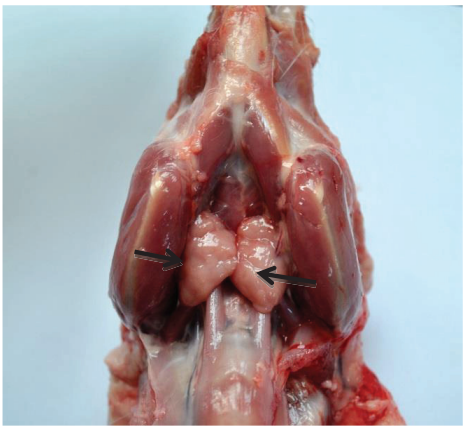

Figure 2. Mandibular glands

Sublingual gland is the smallest gland of the glands discussed above, being embedded in the surrounding connective tissue. After removing this tissue, we noticed that this gland has two extremities: one oral and another, aboral. This two extremities have an elongated shape which extend from the base of the tongue (aboral extremity) and 1.5 cm oral of the tongue (oral extremity). Sublingual gland ducts are detached from the superior edge of the gland, having dorsal trajectory (Fig. 3).

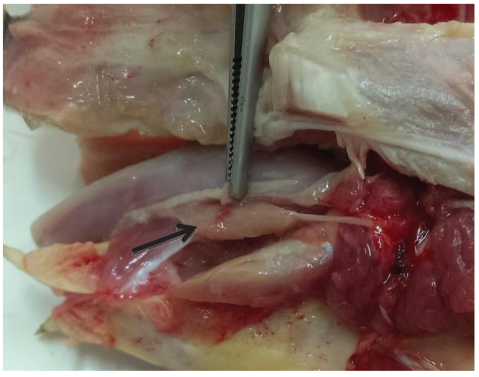

Figure 3. Sublingual gland

We mention that in all subjects examined, the inferior buccal glands gathered and formed a major salivary gland, found at the lips commissure, extending one cm aboral and following the dorsal edge of the horizontal branch of the mandible. This gland has multilobat appearance (Fig. 4).

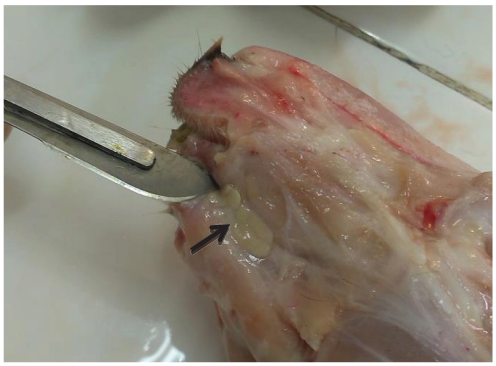

Figure 4. Buccal glands

As we also noticed, Zhang et al., 2005 and Zhou et al., 2010 show that in rabbit and mouse, ventral extremity of the parotid gland is in contact with the mandibular gland. We agree and confirm what Kimura et al., 1998 say, that mandibular gland is the second largest major salivary glands.

The main salivary glands are the parotid, mandibular and sublingual. The parotid gland in rabbit is the most developed, compared with the other major salivary glands - mandibular and sublingual. The gland extends from the base of the outer ear to the angle of the jaw.

The duct crosses rostral the lateral surface of the masseter muscle, very close to the facial nerve branches, entering in the oral cavity, next to the last molar. The colour of the gland is white, and the lobulation is visible (Al-Saffar and Simawy, 2014).

Mandibular gland in rabbits is smaller in comparison with the parotid, but bigger than the sublingual. It has pyramidal shape and a reddish-brown colour. The outer surface is smooth, with no signs of obvious lobulation. Both glands are located medial to the mandibular angle. They are located on the midline of the caudal part of the tongue (Kimura et al., 1998; Al-Saffar and Simawy, 2014).

Sublingual gland is smaller compared with the parotid and the mandibular glands. It is located rostral to the mandibular gland, ventro-caudal to the base of the tongue. It has an elongated shape and a reddish-brown colour. The outer surface is smooth, without lobulation (Al-Saffar and Simawy, 2014).

The ventral location at the base of the ear is comparable to the location of parotid gland in other species, such as rats (Kimura et al., 1998), rodents (Jonjic, 2001), koala (Mizuno et al., 2009), dogs (Weidner et al., 2012) and man (Amano et al., 2012).

Like in rabbit, also in human, sublingual gland is the smallest of the three pairs of major salivary glands, being also located deep under the mucous membrane of the oral cavity (Rana et al., 2012).

CONCLUSIONS

In rabbits, the ventral extremity of the parotid gland reaches the mandibular angle, ending sharp in the oral side, having the two edges concave cranially, the gland having the appearance of a scythe blade; parotid gland is in contact with mandibular gland.

In rabbits there is an asymmetry of the mandibular and the parotid glands regarding their size, those on the right side being slightly bigger compared to those on the left.

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THE *IN VITRO* EVALUATION OF ANTIBACTERIAL ACTIVITY OF A TOTAL CASEIN EXTRACT FROM GOAT MILK

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Abstract

The goat milk composition can be distinguished by significant proportions of biologically active proteins that can exert antimicrobial effects. In this context, the present work is focused on evaluating antimicrobial activity of total caseins in vitro, extracted from goat milk, concerning some reference bacterial strains such as Escherichia coli (ATCC 25922), Pseudomonas aeruginosa (ATCC 27853) and Staphylococcus aureus. The research was conducted on milk samples collected from two groups of clinically healthy goats, Carpathian (group I, n = 12) and Alpine (group II, n = 12) breeds. Milk samples were subjected to a procedure of total casein separation. In vitro test procedure consisted in evaluating the inhibitory effect of casein on the microbial strains listed, using the serial micro-dilution method in liquid broth, which led to the establishment of minimum inhibitory concentration (MIC), correlated with diffusion method. Thus, the concentrations tested were 10, 20, 30 and 40 mg casein / mL, and the interpretation was based on the measurement of the inhibition zones diameters. Our results indicate antimicrobial activity with variations dependent on the bacterial strain tested with no significant differences between the two goat breeds. Thereby, E. coli strain was proven to be very sensitive to all concentrations used, P. aeruginosa at the 20, 30 and 40 mg/mL concentrations, and the St. aureus to 30 and 40 mg casein/mL concentrations. Our results reveal the antimicrobial potential of milk proteins, analysing it in the current context of developing microbial resistance to antibiotics. In conclusion, caseins may be an alternative solution to diminish the consequences of the antibiotic resistance phenomenon, giving some dairy products the character of functional foods.

Key words*: goat, milk, casein, antibacterial effect*

INTRODUCTION

Goat milk is different from the milk of other ruminant species through richer content in proteins with intense functional activities, which also exercise a good antimicrobial activity (Korhonen and Pihlanto, 2001 şi 2009; Roncada et. al., 2012; Murata et al., 2013). The main protein of milk is casein, a phosphoprotein with phosphate groups attached to the side chains of the amino acids, primarily to the hydroxyl groups of serine and threonine. Actually, the casein is a mixt between at least three similar proteins, which differ mainly in molecular weight and in the amount of phosphorus it contains (Daniel, 2000). Casein exists in milk in the form of the calcium salt, calcium caseinate (Minard, 2002) and it is considered an important source of antimicrobial peptides (Lopez et al., 2006; Park et al., 2007; Eriksen et al., 2008). This salt has a complex structure, being composed of α, β and k caseins, which form a mycelium. Neither α-casein, nor the β-casein is soluble in milk. K casein has fewer phosphate groups and high carbohydrate content linked to it (Veloso et al., 2002; Miranda et al., 2004). It seems that all the k-casein contains serine and threonine (which have hydroxyl groups) and also carbohydrates, linked to only one side of the outer surfaces. This portion of the outer surface, it is easily solubilized in water. Calcium caseinate, has the isoelectric point at pH 4.6, therefore, it is insoluble in solutions with a pH below 4.6 (McMaster, 1997; Pavia et al., 2000; Minard, 2002; Yeditepe, 2006). Besides the major milk proteins (caseins and whey proteins), there are also small amounts of

minor proteins and peptides in milk. These bioactive peptides are inactive in the sequence of the protein they come from and can only be released by enzymatic proteolysis. As soon as they are released in the body, they can act as compounds with antimicrobial activity (Bellamy, 1992, 1993; Atanasova and Ivanova, 2010). Thereby, these peptides represent a health potential with applications in the food and pharmaceutical industry (Ariyoshi, 1993). Milk quality, both in terms of health (Ognean, 2001), as well as nutritional value and biologically active, is considered essential for consumer welfare and safety (Michel, 2001, Yadav, et al., 2014). Unlike endogenous bioactive peptides, peptides derived from milk are characterized by multifunctional properties with specific sequences, which have two or more biological activities. The bioactivity of immuno-peptides present in the milk has been proven and is characterized both in vitro and in vivo, immuno-peptides being derived from the amino acid residues present in casein (Migliore et al., 1989; Coste et al., 1992; Assarglrd et al., 1994; Kayser, 1996). Bio-peptides were defined as specific protein fragments that have a functionally beneficial impact on the body and, consequently, on health status (Haque and Chand, 2001). They are encrypted in milk proteins, obtained only by enzymatic hydrolysis, in vivo, during gastrointestinal digestion. This process is dependent on food processing by microbial enzymes specific for fermented products. Thanks to their physiological and physicochemical diversity, lactic proteins are essential components of dairy products, which may also have applications in the pharmaceutical industry (De-Felice, 1995; Hans, 1999).

Taking everything into account, the aim of our study is to test the casein extracted from the milk of Alpine and Carpathian goats, for the evaluation of the antimicrobial activity on some reference pathogenic bacteria strains.

MATERIALS AND METHODS

The group composition and milk sampling. The research was conducted during July-August 2016, on milk samples (n=20) from a goat farm situated in the heart of Transylvania. For this purpose, from a total of 60 animals, clinically healthy goats were selected and divided, by breed, into two groups: group I, including 10 Carpathian goats and group II, 10 Alpine goats. From the experimental groups of goats milk samples were collected, taking into consideration the hygienic conditions and the usual sanitation measures (Ognean, 2001). The collection procedure was finalized by reuniting the individual samples into an average sample per batch, which was the subject of the following investigation.

The casein separation from the milk. Immediately after collection, the samples were subjected to a procedure for the separation of casein, based on the initial acidification of milk. Thereby, a first separation of the milk was obtained, into its morphological components, represented by a solid fraction (curd) and a liquid fraction (whey), followed by the casein uptake from the solid component (Mohr and Wayne, 1994; Jahnavi et al, 2016; www.chemistry.mcmaster.ca).

Testing the antimicrobial effect of the casein extract. To evaluate the antimicrobial effect of the total casein extract, isolated from goat milk, reference microbial strains were used: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* isolated from clinical cases. Inoculum preparation was performed from 18-24 hours microbial strains. For this purpose 4-6 colonies were selected, which were diluted in saline solution (PBS, Sigma Aldrich) - representative inoculum. Microbial density was adjusted based on nephelometry, using the MacFarland scale 1, thus establishing a concentration of $5x10^8$ CFU/ml (Oxoid).

The obtained inoculum was seeded on Muller Hinton agar, by making the wells to about 15 mm from the periphery of the plate and, respectively, 30 mm apart from each other (Andrews, 2001). In order to dissolve the total casein extract and to obtain the dilutions, a saline solution was used PBS and the testing was made for the 10, 20, 30 and 40 mg/mL concentrations. The dilutions were performed according to the method proposed by (Costa et al., 2014; López-Expósito et al., 2006). The obtained solutions were distributed in the previously prepared wells and the plates were incubated at 37^0C. The results were evaluated after 24 hours of incubation. As a control

sample, micro tablets of 10 mg of Ampicillin (Amp, Oxoid) were used and, saline solution, as a negative control.
The interpretation of the results consisted in the measurement of the inhibition zones diameters, which are considered to be directly proportional to the sensitivity of the tested strain. Therefore, the more active the tested substance is the more extended the inhibition area of microbial growth will be.
The study was conducted using the serial micro-dilution method in liquid broth (Carson et al., 1995; Mann, 1998; Brut, 2004), method which led to the establishment of minimum inhibitory concentration (MIC) (Costa, 2014). The data were statistically interpreted using the GraphPad software and the "p" index value was calculated by ANOVA test.

RESULTS AND DISCUSSIONS

The inhibition of bacterial growth, evaluated by the micro dilution method in liquid medium and correlated with diffusion method used for this study, proved to be sufficiently relevant for evaluating the antibacterial action of goat milk casein. The results are comparable to those provided by conventional pathogen susceptibility (classical antibiogram - Kirby-Bauer method).
The measurement of the inhibition zones highlights the diameters size as directly proportional to the sensitivity of the bacterial strains tested. The diameter of the inhibition zones of *Echerichia coli,* strain showed multiple variations and the differences to standard antibiotic are statistically significant ($p \leq 0.005$). Zones of inhibition were seen in all casein concentrations tested. The larger diameters (15-16 mm) were recorded at a concentration of 40 mg/mL, followed by the 30 mg/mL (12-13 mm) and 20 mg / mL (Fig. 1). The data presented also shows very close degrees of antimicrobial activity concerning the concentrations of 30 and 40 mg casein/mL.
Based on the arithmetic averages obtained when testing the two casein samples we appreciated that they exhibit a proper antimicrobial activity towards the tested strains, with no significant differences between the two goat breeds. Thus, after the quantification of the values obtained evaluating the casein antimicrobial activity from the two milk samples, different levels of activities were found: The *E. coli* strain presented the highest degree of sensitivity towards the concentration of 40 mg casein/mL, followed in descending order by 30, 20 and 10 mg / mL concentrations (Fig.1); *P. aeruginosa* strain proved to be sensitive to 20, 30, 40 mg/mL concentrations, without any inhibition area at other concentrations towards the positive control (Fig. 2); For *St. aureus* strain, the diameter of the inhibition zones varied between 16 and 17 mm to 30 and 40 mg/mL concentrations, without having inhibition zones for the other dilutions (Fig. 3).
Statistical analysis of recorded values for the inhibition zones of *E. coli* strain has emphasized significant variations, with statistically significant differences ($p \leq 0.005$), between the values registered for 40 mg casein/mL concentration and those recorded in the case of positive control antibiotic (Fig. 1).

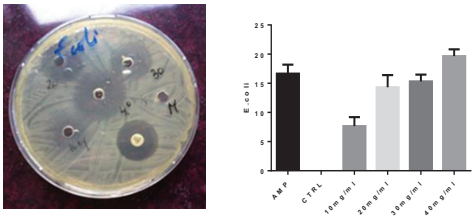
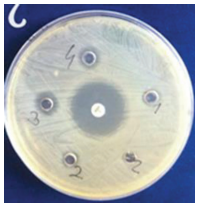


Figure 1. Emphasizing the inhibition zones of casein and witnesses dilutions tested against a standard strain of *E.coli* and the graphical representation of the obtained values.

The statistical analysis of recorded values in the inhibition areas of P. aeruginosa strain revealed a slight sensitivity to dilutions of 20, 30 and 40 mg. casein / mL, statistically significant ($P < 0.0001$) (Fig. 2).
The statistical analysis of recorded values in the inhibition areas of *St. aureus* strain revealed significant variations only at dilutions of 30 and 40 mg casein/mL, statistically significant ($P < 0.0001$). They present an inhibition zone which is superior to the positive control (Fig. 3).
The results obtained show that the higher the inhibition zone of microbial growth is the higher the antimicrobial activity of the tested substance is.



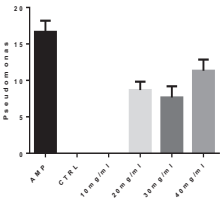


Figure 2. Emphasizing the inhibition zones of casein and witnesses dilutions tested against a standard strain of *P. aeruginosa* and the graphical representation of the obtained values.

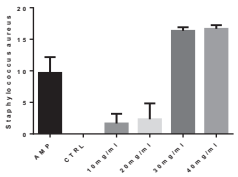


Figure 3. Emphasizing the inhibition zones of casein and witnesses dilutions tested against a standard strain of *St. aureus* and the graphical representation of the obtained values.

General analysis of the recorded values when testing the inhibitory effect of casein on bacterial growth, have revealed that it is influenced by the bacterial strain introduced in testing and ranged from the minimum concentration of 20µg /mL and a maximum of 40µg/mL.

The results of our study bring new arguments in favour of supporting the complex functions exercised by milk proteins, which are essential not only in terms of nutrition or biologically active, but also in terms of antibacterial protection of new born and, by default, the consumer. According to the consulted bibliographic data, antibacterial action exerted by the lactic proteins, suggest the possibility of their use for therapeutic purposes, contribute to obtain functional foods and nutritional formulas for infants (Malkoski, 2001; Vajiheh, 2012).

In the context of the above, we should also mention other research, which proved that the antimicrobial activity can be attributed mainly to milk caseins which release the active peptides against various pathogens (McCann, 2006; Haque, 2008; Faiza et al., 2013). Thereby, we should mention that there are some of bioactive peptides which are not released *in vivo*. They are encrypted in major milk proteins and activated only by enzymatic proteolysis (De-Felice, 1995). They could receive commercial/trading forms assigned to the functional foods category for the prevention and therapy of diseases with microbial component (Hans, 1999; Schmidl, 1993). In this regard, we should remind the results of the preliminary study on the inhibitory effects of a β-casein hydrolysate towards E. coli (JM103), without the identification of the peptides responsible for this action (Gomez, 2005; 2006). Later on, four antibacterial peptides from a αs2-casein hydrolysate have been identified (Lopez, 2006). It is also known that antimicrobial peptides derived from milk proteins are generally active against Gram positive bacteria (Bellamy, 1992; Recio, 1999; Pellegrini, 1999; 2001; 2003; Faiza et al., 2013).

CONCLUSIONS

The results of our study revealed that total extracts of caseins from goat milk exert inhibition activity against in vitro development of various bacterial strains, such as *E. coli, P. aeruginosa* and *St. aureus.* Such extracts can also be an alternative solution for reducing the consequences of antibiotic resistance phenomenon and can assign a character of functional foods to dairy products.

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THE MACROSCOPIC MORPHOLOGY OF THE OF THE THORACIC CAVITY LYMPH NODES AT COYPU (MYOCASTOR COYPUS)

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Abstract

The present study is performed on a number of 4 bodies of nutria. At this species, the azygos vein is placed on the right side, opening in cranial vena cava at the third intercostal space level, after intersecting from top to bottom the right side of the trachea and esophagus. At coypu, the vagal nerve is in direct contact with dorsolateral edge of the trachea. The parietal and visceral lymph nodes of the thoracic cavity were studied using the China 40% ink dye colouring method. The method used for highlight the lymph nodes was the stratigraphic and regional dissection, up to the limit of the visibility, and subsequently using the Nikon stereo microscope. The studied lymph centres are represented by the dorsal thoracal, ventral thoracal, mediastinal and bronchial lymph centers. No dorsal thoracic lymph centre was identifiable at the investigated animals. The left brhonchial lymph node has a smaller dimension compared to the right bronchial lymph node. The medial mediastinal lymph nodes are identifiable especially on the right side. There were no cranial mediastinal lypmh nodes found during the dissection. The caudal mediastinal lymph nodes were found macroscopical, but is but their presence is advisable to carry out histological examination certification.
The present lymph nodes from the thoracic cavity are reduced by number. In the discussion framework it were highlighted the medial mediasnital lymph nodes on the right side. On the right side of the stern, at the level of the second rib, it is well represented the cranial sternal lymph node. This lymph node belongs to the ventral thoracal lymph node and it appears constant as presence.

Key words: coypu, ink dye, lymph centers, thoracic cavity.

INTRODUCTION

The copyu belongs to the Rodentia Order, Mammalia Class, Myocastoridae Family, Myoscastor Genra, coypus species. This wild animal, semi-aquatic, lives in colonies or in pairs (Spătaru et al., 1997).
This rodent is grown for meat and fur. In the studied bibliography appear some relatively brief data on the visceral morphopathology lymphatic system. The study is realised to complete the existing data from the specific literature. (Dănacu et al.,2013; Gheție et al., 1967; Predoi et al., 1997; Predoi et al., 2003).
In the present study, before presenting the morphology and the topography of the thoracic lymph nodes at coypu, there are described in summary some intracavitary structures particularities following the anatomical and morphological base. (Barone, 1996; Coțofan et al., 1994).

MATERIALS AND METHODS

It were used 4 coypu bodies. The material was heterogenous regarding to the physiological age. For the injection of the Chinese inky dye solution 40% the coypu were previously anesthetized. For the thoracal cavity lymph centres dissection, the animals were euthanized after 2 hours from the injection of the dye. The administration was made intracavitary.

RESULTS AND DISCUSSIONS

In the thoracic cavity, the ceiling is formed from 13 thoracic vertebraes that presents a wiped ventral ridge. As exceptions are the last three vertebraes, where the ventral ridge is more prominent. At this species, the ventral face of all verebraes bodies is covered by

muscle, whereas the psoas muscle origin is ”advancing more”, getting to meet along the neck muscle.
The species is presenting nine pairs of sternal ribs, one asternal and three floating ribs. The first two pairs are relatively short, which makes the cranial thoracic aperture to be narrow, and the whole thoracic cavity structure is being like a funnel. The sternum is formed from seven sternal vertebraes at the young animals. At adults, the VI-th and the VII-th sternal vertebraes are welded together. At the level of the precordial mediastinal right phrenic nerve that accompanies on the dorsal path the cranial cava vein is passing lateral the sinus of the cava vein, continuing in the caudal cava vein meso to the diaphragm, at the dorso-lateral side of this vein. At this species the azygos vein is disposed on the right side and it is opening in the cranial cava vein, at the level of the third intercostal space, after crossing from top to bottom the right face the trachea and esophagus. Until the VIII-th thoracal vertebrae, the azygos vein is masked by the psoas muscles origin. At the level of the first two intercostal spaces, the esophagus is located on the right side of the trachea. Taking into account this particular aspect, the right vagus nerve is coming directly related with the dorso-lateral edge of the trachea. The two vagus nerves, arrived in the posterior mediastinum, will flank the esophagus forming a single cordon, dorsal from this one, at the entry in the abdominal cavity. The aorta emit the dorsal intercostal arteries, starting with the fifth intercostal space.
The thoracic cavity lymph nodes are reduced by number, being grouped in lymph centers: **dorsal thoracal, ventral thoracal, brochial and mediastinal.**
The ventral thoracal lymph centre is represented only by the cranial sternal lymph nodes. These are highly represented, placed at the second rib level, under the cranial cava vein. It has a diameter of about 5-7 mm, they are easily to be isolated and identified (Figure 1-*1*, Figure 4-*6*).

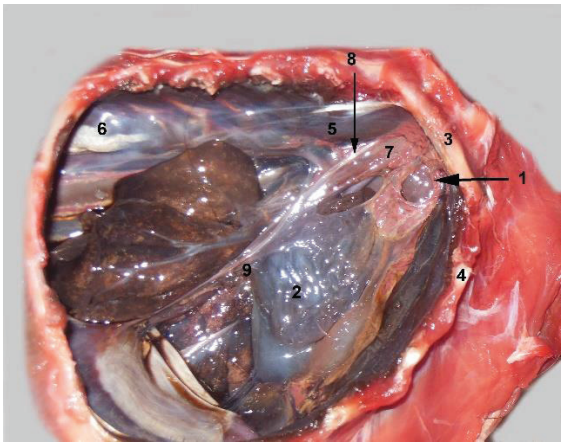

Figure 1. The topography of the cranial sternal lymph node – View on the right side, after opening the thoracic cavity (original)
1- the cranial sternal lymph nodes, 2 – the cordon coated in pericardium, 3 – the first rib, 4 – sternum, 5 – the long muscle of the neck, 6 – the origin of the psoas muscles, 7 – cranial cava vein, 8 – right phrenic nerve, 9 – caudal cava vein with the right phrenic nerve

It was not possible to be identified by macroscopic view the lymph nodes belonging to the dorsal thoracal lymph centre.
The bronchial lymph centre is represented on the right side by a right bronchial lymph node with a discoid form, with a diameter about 3 mm. It is situated lateral the trachea, behind the affluence of the azygos vein to the caudal cava vein. (Figure 2-*1*, Figure 4-*5*). On the left side, the left bronchial lymph node is more reduced compared with his congener from the right side (Figure 3-*1*). It appears situated at the origin of the costo-cervical

trunk, lateral by the trachea, at the ventral edge of the esophagus.
The mediastinal lymph centre appear constantly represented by the medial medistinal lymph nodes, especially visible on the right side, cranial by the affluence of the azygos vein in the cranial cava vein, lateral by the trachea, in relation with the vagus nerve (Figure 2-*2*).

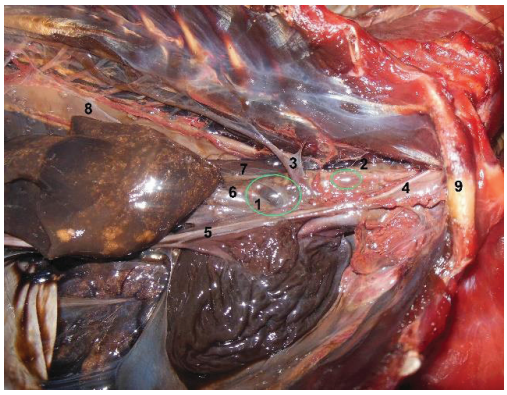

Figure 2. The topography of the right bronchial and medial mediastinal lymph nodes – Right side view of the thoracic cavity (original)
1 – right bronchial lymph node, 2 – medial mediastinal lymph node, 3 – right azygos vein, 4 – cranial cava vein with the phrenic nerve, 5 – caudal cava vein with the phrenic nerve, 6 – tracheal bifurcation, 7 – esophagus, 8 – aorta, 9 – first rib

It was not identified any cranial mediastinal lymph nodes. In the postcordial mediastin it could be observed a elongated group of reduced lymph nodes, which could correspond to the caudal mediastinal lymph nodes, by structure, situated nearby the ventral descendent edge of the aorta, which have not been described in the speciality literature (Figure 4-*1*).
However, harvesting and making histological preparations could clarify this.

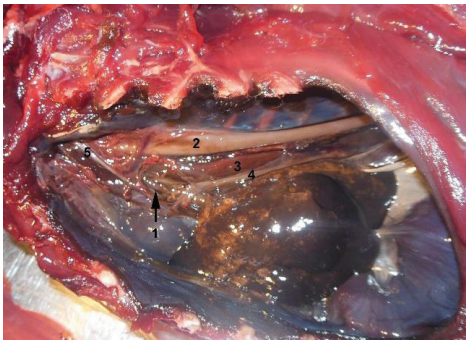

Figure 3. Topography of the left bronchial lymph nodes – Left side view of the thoracic cavity (original)
1 - left bronchial lymph node, 2 – aorta, 3 – esophagus, 4 – left vagus nerve, 5 – left phrenic nerve

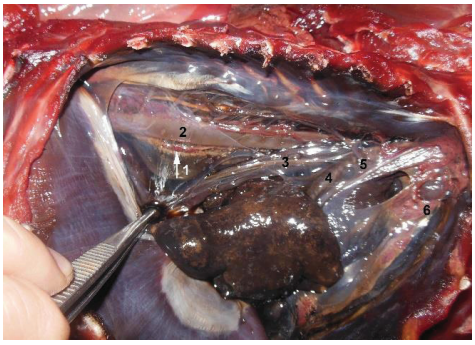

Figure 4. The topography of the caudal mediastinal lymph nodes – right side view of the thoracic cavity (original)
1 – caudal mediastinal lymph nodes grouping, 2 – aorta, 3 – esophagus with the right vagus nerve, 4 – trachea, 5 – right bronchial lymph nodes 6 – cranial sternal lymph node

CONCLUSIONS

The thoracic cavity lymph nodes are reduced by number, being grouped in the lymph centres: dorsal thoracal, ventral thoracal, bronchial and mediastinal.

It couldn't be identified by macroscopic view the intercostal and toraco-aortic lymph nodes.

The bronchial lymph centre is represented by the left and right bronchial lymph nodes, the right one being better represented by the left one.

The mediastinal lymph centre is represented constantly by the medial mediastinal lymphnodes, being easily to identify after opening the right side of the thoracic cavity.

The most better represented is the cranial sternal lymph node, constantly, situated on the dorsal face of the sternum, on the distal extremity of the second rib.

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MACROSCOPIC ANATOMY OF THE GALLBLADDER AND EXTRAHEPATIC BILIARY TRACT IN THE GUINEA PIG (*CAVIA PORCELLUS*)

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Abstract

In mammals the variations of the anatomy of the extrahepatic biliary tree have long been recognized. The aim of this study was the macromorphological description of the gallbladder and extrahepatic biliary tract in guinea pigs (Cavia porcellus).Using dissection techniques the gallbladder topography, anatomic particularities regarding the shape and the connecting elements were assessed. Also, the macroscopic appearance of extrahepatic biliary tract and its path was described. The round and well developed gallbladder, exceeds the ventral border of the liver being visible on both, the visceral and the diaphragmatic surfaces of the liver. The gallbladder was connected with the right medial lobe and with the quadrate lobe of the liver by two tiny ligaments. Proximal, the unique cystic duct shows an obvious constriction and a conspicuous swelling. On its path on the hepatoduodenal ligament, the cystic duct joined the left hepatic duct to form the common bile duct. The right hepatic duct drained the right territory of the liver and joined the common bile duct itself. The left territory of the liver and in some cases the quadrate lobe was drained by the left hepatic duct. Distally, the common bile duct shows a unique ampullary dilatation from which a small duct drains into the first segment of duodenum. The major duodenal papilla was located at 1.5 cm distal to the pylorus.

Key words*: gallbladder, extrahepatic biliary tract, macroscopic anatomy, guinea pigs*

INTRODUCTION

The anatomy of the biliary tree involves complex relationship between the liver, intrahepatic biliary canaliculi, bile ductules, gallbladder and extrahepatic biliary tract (Barone 2009; Ellis, 2011). In animals, the obstruction of the extrahepatic tract is frequently present more than it is reported (Bacon et al., 2003; Amsellem et al., 2006; Miwa and Sladky 2016). Considering the many anatomical variations of biliary tract in mammals (Oldham-Ott and Gilloteaux 1997) a proper knowledge of the normal anatomy of the extrahepatic biliary tract is essential in order to prevent its injury during the surgery. Most liver anatomical descriptions contain descriptions of biliary tract too. Due to the extensive use of rats as experimental model in liver transplantation, the most studied is the rat liver (Higgins 1931; Madrahimov et al., 2006). With the exception regarding the absence of the gallbladder in rats, the macroscopic anatomy and connecting elements of the liver resemble the human anatomy of the liver. From the caviomorphs order, the chinchilla's liver was described having four lobes and a well developed gallbladder (Spotorno et al., 2004). The gallbladder is located between the right and medial lobes, having more than one cystic duct and a complex hepatic ducts system (Nowak et al., 2014). In guinea pigs the liver was described having six lobes (Cooper and Schiller, 1975; Breazile and Brown 1976; Stan 2014) and a well developed gallbladder attached to a fossa which delineates the quadrate lobe, drained by a cystic duct which receives several hepatic ducts to form the common bile duct (Higgins 1927; Quesenberry et al., 2004). In guinea pigs, the angioarhitecture, the inervation and the musculature of gallbladder and and bile ducts was assessed by scanning electron microscopy of vascular corrosion casts, histochemical methods and light electron microscopy (Aharinejad and Lametschwandtner 1992; Cai and Gabela 1983). In rabbit, the absence of the common hepatic duct was reported (Brewer

2006). Also, in humans, the biliary tract and its vascular anatomy show numerous anatomical variations (Lamah et al., 2001; Horiguchi and Kamisawa 2010). The aim of this study is to perform a detailed morphological description of gallbladder and extrahepatic biliary tract 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 performed by administration of an overdose of isoflurane. The abdominal cavity was opened and the wall of it was carefully removed. The gallbladder topography, its connecting elements and the extrahepatic biliary tract was recorded after the displacement on right and left side of the liver lobes, without altering hilum topography and its components.

RESULTS AND DISCUSSIONS

Gallbladder topography

The rounded shape of gall bladder (*Vesica fellea*) exceeded the ventral border of the liver, being visible both on diaphragmatic and visceral surface of the liver, in all examined specimens (Figure 1).

Its diameter was 9 mm ± 0.3 mm and the total length was 11 mm ± 0.1mm. The gall bladder was attached to a fossa (*Fossa vesicae felleae*) situated at the delineation of the right medial lobe and the quadrate lobe (Figure 1), showing an obvious transition zone at the neck and a small swelling on the beginning of the cystic duct (Figure 2).

Ventral edge of right medial lobe embraces the gallbladder fundus (*Fundus vesicae felleae*) being attached to the later by a small but conspicuous ligament. Also, on the left side, the gallbladder fundus was attached to the quadrate lobe by a second small ligament (Figure 3).

Figure 1 The rounded shape of the gallbladder in guinea pigs. The gallbladder exceeds the ventral border of the liver being visible on both visceral and diaphragmatic surfaces of the liver.

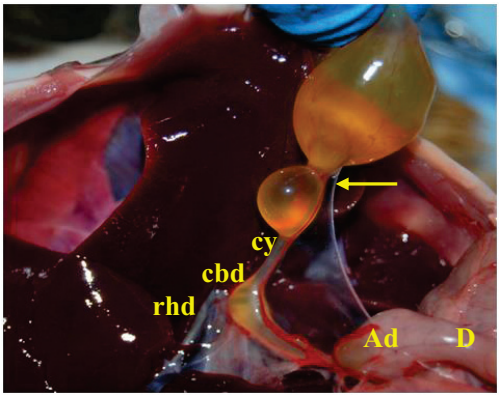

Figure 2 An obvious constriction and a small swelling found at the beginning of the cystic duct - arrow. Rhd –right hepatic duct join the common bile duct –cbd. Ad – ampularry dilatation.

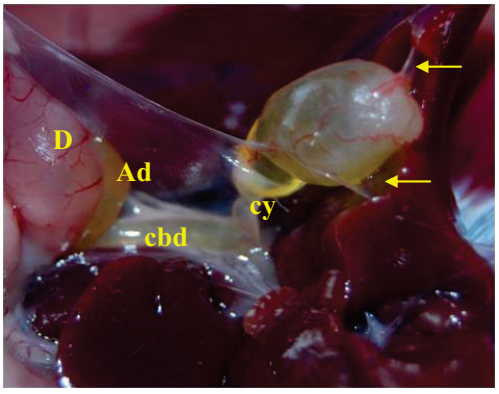

Figure 3 The gallbladder ligaments – arrows. The cystic duct – cy, join the common bile duct – cbd. Ampullary dilatation – Ad, of distal segment of common bile duct. D - duodenum

The extrahepatic biliary tract

The cystic duct (*Ductus cysticus*) diameter was 2 mm ± 0.2 mm making an acute angle with the common bile duct. (Figure 4).

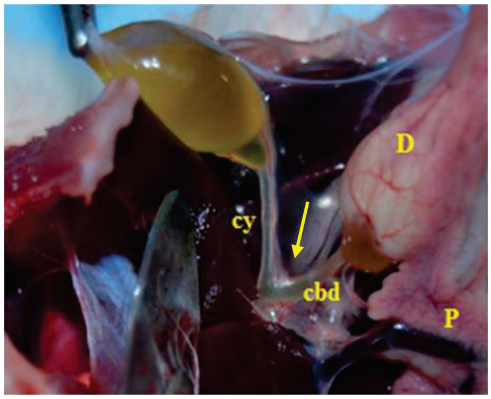

Figure 4 The cystic duct made an acute angle with the common bile duct - arrow

First, the cystic duct joins the left hepatic duct to form the common bile duct, the right hepatic duct draining at short distance after the mentioned union, on the right side of the common bile duct (Figure 5).

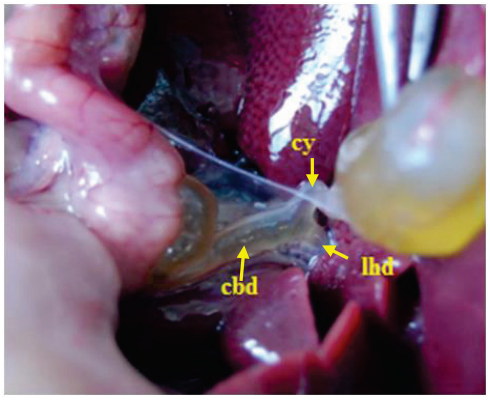

Figure 5 The union of cystic duct – cy, with the left hepatic duct – lhd, to form the common bile duct – cbd.

The right hepatic duct was formed by the union of the hepatic duct that drains the caudate process of the caudate lobe with the channel that drains the right lateral and right medial lobes. The left medial and lateral lobes and the quadrate lobe were drained by the biliary channels which merged to form the left hepatic duct. In three cases the quadrate lobe was drained by a separate channel that joined the cystic duct itself. Distally, above and attached of duodenal wall, the common bile duct shows a unique ampullary dilatation from which a small duct drains into the first segment of duodenum. Proximal of this dilatation the common bile duct showed a small contraction. The opening of the common bile duct observed through the duodenal lumen appeared like a slight elevation of the duodenal mucosa. The major duodenal papilla was located about 1.5 cm distal to the pylorus inside of the first segment of the duodenum (Figure 6).

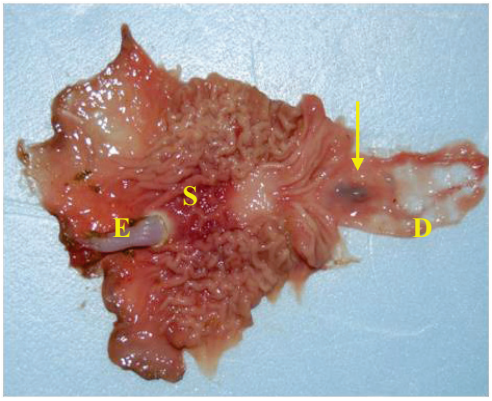

Figure 6 The major duodenal papilla (arrow) located about 1.5cm distal to the pylorus inside of the first segment of the duodenum – D. E – esophagus; S – stomach.

Like most of the mammals, excepting horse, deer, rat, European hamster which have no gall bladder, (Higgins 1931; Shiojiri 1997; Martin and Neuhaus 2007; Barone 2009; Hunyh and Pignon 2013) the guinea pigs show a well developed gall bladder. Different from humans, rabbit, chinchilla and hamster, the guinea pig gall bladder have a rounded shape. However, in contrast with many other species in which the gall bladder is firmly attached and covered by the liver lobes, the guinea pigs gall bladder, exceed the ventral edge of the liver. It was stated that the guinea pigs gall bladder is suspended by a single membrane from the liver with the reminder of the gall bladder hanging freely. Our result pointed out the presence of two small ligaments which connect the medial margin of the right middle lobe on the right

side of the gall bladder and a second one between the gallbladder and the ventral edge of the quadrate lobe. Also, the obvious neck zone constriction is typical for the guinea pigs. This feature and the acute angle formed by the cystic duct with the gall bladder is the morphological explanation of bile stasis. Based on this feature and on the fact that guinea pigs form cholesterol gallstone when given 1% cholestyramine and a weight loosing pellet diet, using guinea pigs as experimental models in gallstone disease in human, is common. The bile stasis, abnormal bile composition, or infections are involved in gallstone formation both in human and guinea pigs (Wagner and Manning 1976). Both in normal or pathological conditions in humans has been found the presence, just below the gall bladder neck, of a dilated pouch at the cystic beginning, named Hartmans pouch (van Eijck et al. 2007; Ellis 2011). In six cases we found an equivalent dilatation, issue that has not been mentioned so far in guinea pigs. As in humans, this is a morphological aspect rather than an anatomic and constant feature.

The gallbladder and the extrahepatic ducts are subject to numerous variations, both in humans and animals, which are best understood by considering their embryological development (Shiojiri, 1997; Uemura et al., 2015). In some animals, including the rat, the deer, the horse and the pigeon, the gallbladder do not develop in embryonic state (Shiojiri 1997; Hisami, 2010; Uemura et al., 2015; Hill, 2017). On the other hand, it was stated that the variability of gallbladder anatomy in mammals is mainly dependent upon diet. Frequent eating of pigeons, rat and deer, which eat almost continuously, imply a continuous secretion and a constant flow of bile from the liver to the intestine, the presence of a gallbladder is not required. In other mammals, like human, cattle, dog or hamster which eats at times the bile is storage and concentrated in the gallbladder, being concentrated one to two times in cattle, four to ten times in dogs and eight to ten times in human and hamster . The anatomical variants of extrahepatic biliary tract include differences in terms of number of ducts, their length, and the manner of union and how the drainage in duodenum is made (Mahandevan 2014). In humans it is clearly stated formation of the common bile duct by joining the common hepatic duct (resulting from the union of the right and left hepatic ducts corresponding to the left and right territories of the liver) with cystic duct (Ellis 2011; Mahadevan, 2014). In rabbit Barone (2009) describe the presence of two hepatic ducts, the left one which drain the left lobes and the quadrate lobe too, joins the cystic duct to form the common bile duct, which receive the right hepatic duct, made by the union of the ducts which drain the right lobe and the caudate lobes. This description is in compliance with the description of Aharinejad and Lametschwandtner (1992) and Jackowiak and Lametschwandtner, (2005), regarding the absence of a common hepatic duct, but the latter authors, studying the angioarchitecture of the rabbit extrahepatic bile ducts and gallbladder, by scanning electron microscopy of vascular corrosion casts, have shown that, there is four or five hepatic ducts which individual join the cystic duct to form the common hepatic ducts. Also, in chinchillas, Novak et al, 2014 state the presence of a complex system of extrabiliary tract by description of multiple cystic ducts which drain the gallbladder together with a multiple anastomosing hepatic ducts running in the hepatoduodenal ligament.

The same pattern was found by Martin and Neuhaus,(2007) who noted that the extrahepatic biliary ducts of the rats are more superficial and also has intercommunicating branches, which implies the existence of a biliary network. Due to the absence of the gallbladder in rats, the common bile duct is made by the junction of the main hepatic ducts. Compare to all mentioned, in guinea pigs the extrahepatic biliary tract is simpler, the absence of a common hepatic duct is obvious and the variability consist of the presence of a different pattern of union of hepatic ducts with the cystic ducts to form the common bile duct, which make the guinea pigs a suitable model to gall stones pathogenesis.

Our results concerning the presence of the distal ampullary dilatation of common bile duct in guinea pigs, are in accordance with those of Higgins (1927) and Cai and Gabela (1983), who also described the common bile duct ampulla and its attachment to the duodenal wall. This is a unique feature of guinea pigs

common bile duct and has not been described in other species.

CONCLUSIONS

The rounded gallbladder of guinea pigs exceeds the ventral margin of the liver. It presents a small constriction and an obvious swelling at the beginning of the cystic duct. The common bile duct is formed by the union of the cystic duct with the left hepatic duct. The common hepatic duct is missing in guinea pig. Distally and attached of duodenal wall, the common bile duct shows a unique ampullary dilatation from which a small duct drains into the first segment of duodenum. The major duodenal papilla opens at 1.5 cm distal to the pylorus.

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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.

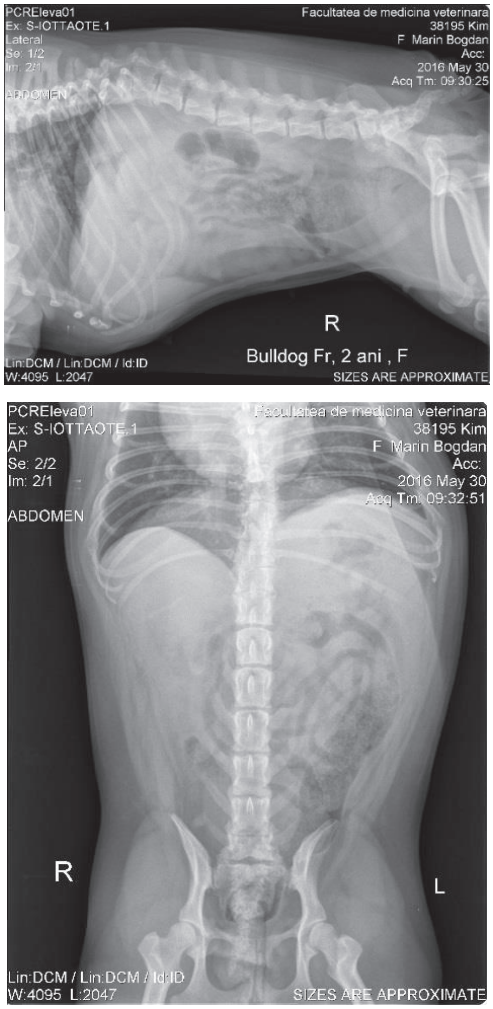
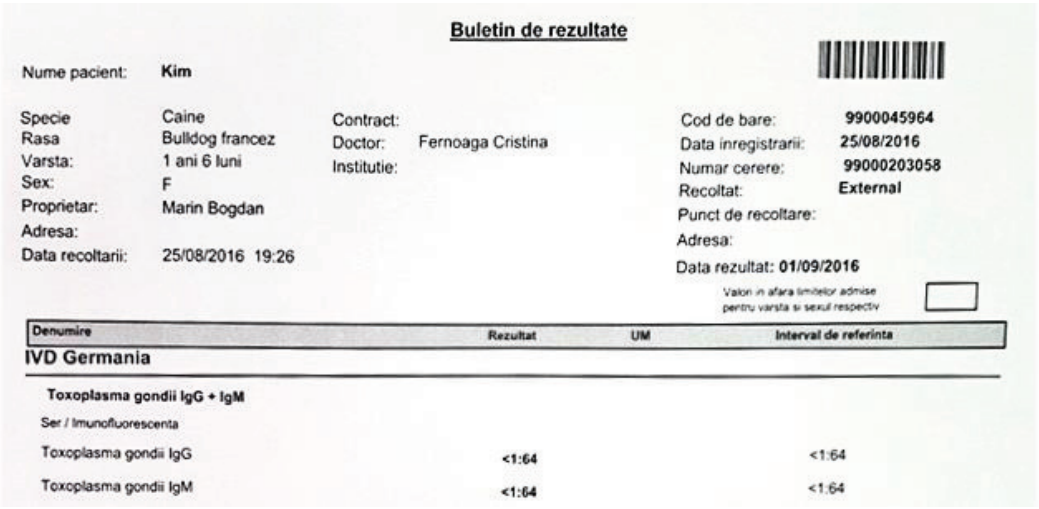


Figure 1.T8-T10-T13 Hemivertebras. T7-T9 united spinous processes. Spinous processes sclerosis at T11-T10. Deposition of new bone on the surface of the ventral vertebral bodies at T12-L2 level. Transitional vertebra - S1 (S1 vertebra lombarization), (dr. Nicolae Tudor permission)



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).

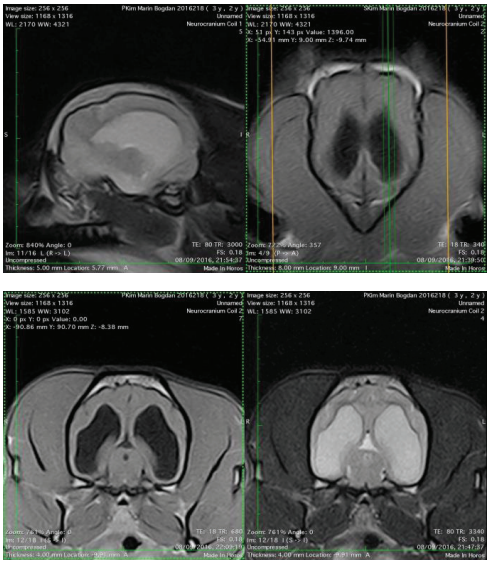


Figure 3. Bilateral ventriculomegaly hydrocephalus (dr. Florin Grosu permission)

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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TIME EVOLUTION OF IMMUNOGLOBULIN Y (IgY) TITER IN THE EGG YOLK HARVESTED FROM HENS AFTER THREE INOCULATIONS WITH MULTIPLE ANTIGENS

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Abstract

The present research focuses on the determination of the immunoglobulin Y (IgY) levels derived from egg yolks obtained from hens previously inoculated with a combination of bacterial and fungal antigens (multiple antigens).
The purpose of the work consists of establishing the frequency of the inoculations, in order to obtain a high level of antibodies throughout the duration of the experiment. The antigens were obtained from inactivated bacterial and fungal humans pathogenic strains. Egg-laying hen lots were formed out of individuals at the beginning of the egg-laying period. The hens were inoculated with multiple antigens, three times, at the first, 14th and the 28th day of the experiment. The control for the immune response was performed by sampling eggs on the 14th day from the third inoculation process. The IgY was extracted from the egg yolk in order to obtain the aqueous phase. The characterization of the IgY titers was performed every 30 days, for a period of 9 months, by using qualitative and quantitative ELISA assays.
Following the end of the 9 months period since the 3rd inoculation process, the specific IgY titers maintained at high levels, another (4th) inoculation shouldn't be necessary.

Key words: *ELISA, IgY, inoculation, multiple antigens.*

INTRODUCTION

Antibodies represent the main molecular effectors of the immune system, being glycol-proteins synthesized under the influence of antigens, with which they interact specifically both in vivo, as well as in vitro.
They are multiple chain proteins resulted from the combination of two multiple peptide chains, with different molecular weight and sequences of amino acids.
The multiple peptide chains are bound by disulfide bridges (-S-S-), and generally have the Y letter shape (Răpuntean G., 2010).
In 1983, Klemperer published the work that stated that there are virus neutralizing proteins in eggs. Between 2004 and 2006, worldwide experts have started, for the first time ever, to acknowledge eggs like a pharmaceutical product. The egg yolk was reviewed as an important source for immunoglobulins that can be used for prophylaxis, diagnosis and therapy purposes in humans and animals (Klemperer, 1893).
Leslie and Clem proved, in 1969, that IgY is different from IgG. IgY does not interfere with the activity of drugs in the digestive tract, nor with their blood circulation in the human body. IgY does not induce antimicrobial resistance and it does have remanence in the organism (Leslie et al., 1969).
The antibodies presently used for research, diagnosis and therapy purposes originate from mammals. These are monoclonal or polyclonal antibodies. In order to produce polyclonal antibodies, animals such as horses, sheep, pigs, rabbits, rats are used. For monoclonal anti-bodies, it is the case of rabbits or mice. Both technologies have advantages and disadvantages (Mojca N., 2003).
It has been noticed that the obtaining of mammalian antibodies requires complex technologies, with a modest yield. The technology used in mammals induces stress for

them both in the immunization stage, as well as when control blood is sampled in order to produce the antibodies (Larsson et al., 2003).
The past 25 years have shown an increase in the use of hens as a replacement of mammals in the production of antibodies. The foremost advantage is that the antibodies are obtained from the eggs, and not from the serum. At the same time, the quantity of produced antibodies by an egg-laying hen is considerably higher than that of a mammal of the same size (Carlander et al., 2002).
The purification of immunoglobulin from mammals requires considerable time and is an expensive process. Presently, hens are recognized as a cheap and convenient source for the production of antibodies. The quantity of immunoglobulin produced from a single egg is comparable to that prepared from 300 mL of blood sampled from rabbits (Wang et al., 2012).
Opposite mammalian antibodies, avian origin immunoglobulins can be produced in high quantities from the egg yolk and are an ideal source for medical and scientific applications (Chiurciu et al., 2017; Leslie et al., 1969).
The egg yolk antibodies have been intensely studied and IgY is considered to be ancestral when compared to mammalian IgG and IgE. IgY technologies possesses a series of advantages to those used in extracting antibodies from mammals, such as: bird husbandry is considerably cheaper, egg collection is non-invasive, and IgY is easily obtainable (Kim et al., 1998).
Furthermore, the phylogenetic distance between the species allows for avian antibodies to be more efficient in inducing immunity responses than those of mammalian origin (Larsson et al., 2003).
IgY technologies have been evaluated for the case of therapeutic application, by oral administration in the prophylaxis or treatment of various bacterial pathogens in humans. IgY differs both structurally, as well as functionally from the mammalian IgG and does not cross-react with mammalian IgG.
Due to the fact that hens can lay eggs almost on a daily basis, the IgY rich immunized egg yolks have become the main source of antibodies used for scientific research, both fundamental and applicative (Pauly et al., 2011; Scheett et al., 2007).
The present study focused on the evolution of specific IgY antibodies obtained after three inoculations, during a 9 months experimental period of time.

MATERIALS AND METHODS

Animals. The study was developed within the Research & Development Laboratory of Romvac Company S.A., over a 9 months experimental period. All procedures were compliant with Directive EU 2010/63 relating to the manipulation of animals used in scientific purposes. The study was approved by the Ethics Committee of Romvac Company S.A.
Clinically healthy, 2.5 kg and 19 weeks old egg-laying hens (*Gallus domesticus*) were inoculated. The environmental conditions consisted of individual halls, battery-based, controlled temperature, humidity, noised and light. The birds were fed a standard *ad libitum* diet.

Antigens. The microbial strains used in the study were obtained in the laboratories of the Victor Babes Hospital for Infectious and Tropical Diseases in Bucharest, as well as from isolates from the patients that were consulted at the Alternative Therapies Medical Practice of ROMVAC Company S.A. The Victor Babes Hospital provided the following strains: *Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii* and *Escherichia coli.* The strains isolated and characterized in the ROMVAC microbiology laboratory were: *Pseudomonas aeruginosa, Clostridium difficile, Salmonella ssp., Enterococcus faecalis, Salmonella enteritidis, Salmonella typhimurium, Streptococcus mutans, Streptococcus group B, Proteus mirabilis, Helicobacter pylori, Candida albicans, Candida glabrata,* and *Candida krusei.*

Inoculum preparation. The bacterial strains were cultivated on nutrient broth, and the Candida strains on liquid Sabouraud medium. The cultures were incubated at 37°C for 24-48 hours, were washed twice every day using sterile PBS, pH=7,2 and were inactivated using formaldehyde 0.5% for 18 hours. The suspensions were adjusted for 0.05 at OD 600

by using a micro plate reader (Spectra Max 190), corresponding to a cellular density of approximately $1x10^5$ CFU/ml.

Inoculation of the hens. Egg-laying hens lots were formed, at the beginning of their egg-laying period, for which a mixture of antigens was prepared, previously described.
The hens were inoculated three times with the multiple antigens, in four different inoculation sites belonging to the chest muscles. The 2nd and 3rd inoculations were performed at 14 and 28 days difference from the initial one. The hyperimmune eggs were collected on a daily basis, starting with the 14th day from the last inoculation. The eggs were stored at 2^0 - 8^0C. For comparative testing, 30 weeks old hens SPF eggs were used. These hens were housed in individual halls.

IgY extraction. Presently, the literature highlights numerous methods for extracting and purifying immunoglobulins from the egg yolk (Kim et al., 1998; Pauly et al., 2011). The present study used the aqueous phase supernatant for the extraction of IgY. The yolk was separated from the white, 1 mL of yolk was sampled in 15 mL tubes. Each tube was added 7 mL of Milli Q water in order to achieve the 1:8 dilution. The mixture was homogenized, and the pH was adjusted at 4.5-5. The tubes were incubated for 20 hours at -20^0C, followed by defrosting, centrifugation at 10500 rpm for 20 minutes and Millex 0.45 µm filtering. The samples were mantained at la 2^0-8^0 C until the moment of testing.

Immunoenzymatic testing (ELISA). Quantitative and qualitative ELISA assays were performed in order to identify and quantify the IgY immunoglobulin from the hyperimmune eggs. The testing was performed every 30 days for the 9 months experimental period.

Qualitative determination. It was performed to identify the IgY immunoglobulins through the Elisa assay, by using the MyBioSource kit, the Elisa plate reader Spectra Max 190, an automatic ELISA plate washer (MultiWash III TRICONTINENT). As a negative control for the reaction, IgY from SPF hens was used. The reaction plates were padded with 100 µl of the antigens used for inoculation. The maximum positive dilution was considered that where OD is equal of higher than 0,200. The padded plates were kept overnight at 2^0-8^0C, and then washed three times with a PBS –Tween 20% washing solution. 300 µl/fixing pads were added, and the plate was maintained for 30 minutes at room temperature. The blocking liquid was removed and 100 µl of IgY suspension was added in dilution from 1:100 to 1:64000.
As a positive control, ROMVAC specific reference IgY was used. After the incubation of the plates for two hours at 37°C, 100 µl of 1:10000 anti-avian IgY IgG enzyme conjugate was added, followed by introducing 100 µl TMB for 5-15 minute, and then 100 µl of blocking solution.
The absorbance rate was read by using the plate reader at 450 nm wavelength. The reaction was validated when the blank control is lower than 0,060 OD, the negative control between 0,060 and 0,090, and the OD positive control is 1,400 to 1,800.

Quantitative determination. For the quantitative determination of IgY from hyperimmune eggs, the direct in-house ELISA assay was performed, by using International Reference IgY (Lampire Laboratories). We used ELISA plated (Falcon), reference IgY, enzyme conjugate anti-avian IgG (MyBioSource), diluted in stability buffer 1:10000, plate reader (Spectra Max 190), automated ELISA plates washer (MultiWash III-TRICONTINENT).
The samples represented the IgY obtained from hyperimmune eggs collected every 30 days for the 9 months experimental period. From these samples decimal dilutions were performed, from 1:100to 1:64000 and added on the plates.

RESULTS AND DISCUSSIONS

Based on the absorbance values measured for the Reference IgY, the calibration curve was obtained, having a straight equation of OD (450 nm) = 0,00667 C (ng/ml) – 0,0154 and a correlation coefficient of R^2 = 0,9785. By using this equation, we calculated the concentration of the IgY to be analyzed (Fig. 1).

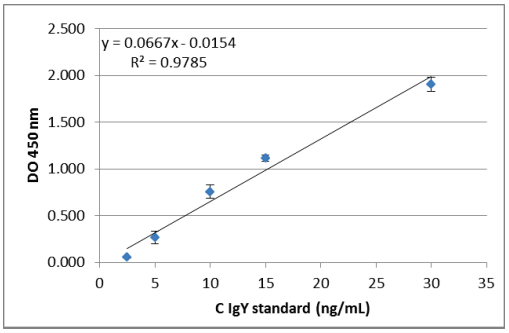

Fig. 1: Calibration curve for the Reference IgY used for the calculus of IgY concentration

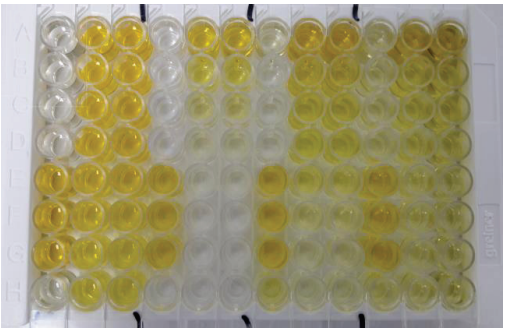

Figure 1: Qualitative ELISA test to establish the SPF IgY specificity to a component antigen from the multiple inoculum.

The results obtained via the qualitative ELISA testing have allowed the confirmation of the presence of IgY in the collected samples from immunized hens with multiple antigens over the 9 months experimental period. Figure 1 highlights that IgY manifests specificity for the antigens used in the immunization process. The tire of the antibodies is elevated for each individual antigen, indicating that the immune system of the hens reacts equally for the inoculated antigen stimuli. For the confirmation of the results, negative control SPF eggs were used (Figure 2).

Table 1 presents the optical densities (OD), read at 450 nm wavelength, for IgY antibodies against-*Streptococcus group B*, *Salmonella enteritidis*, *Proteus mirabilis*, *Salmonella sp*., *Candida krusei* and *Klebsiella pneumoniae* throughout the experimental period.

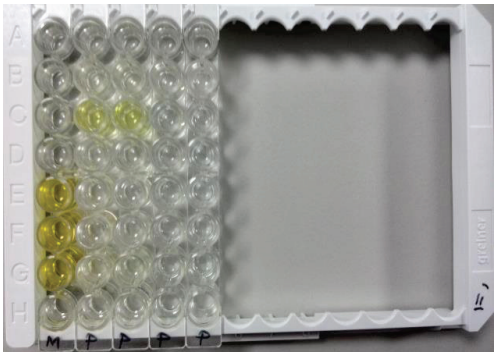

Figure 2: Qualitative ELISA test used for establishing the specificity of antibodies for the 4 component antigens of the multiple inoculum.

Table 1. ELISA test: IgY antibodies titer against -*Streptococcus group B, Salmonella enteritidis, Proteus mirabilis, Salmonella spp., Candida krusei* and *Klebsiella pneumoniae*

Antigen/ Dilution	**OD values of specific antibodies**								
	Months								
	Dec. 1	**Jan. 2**	**Feb. 3**	**Mar. 4**	**Apr. 5**	**May 6**	**Jun. 7**	**Jul. 8**	**Aug. 9**
Streptococcus group B	2,859	2,862	1,964	3,471	3,407	3,275	2,803	2,476	3,201
Salmonella enteritidis	3,143	3,160	2,116	3,598	3,468	3,696	1,624	1,333	2,221
Proteus mirabilis	1,496	2,654	1,731	3,130	3,137	3,323	2,488	1,639	3,077
*Salmonella sp*p.	3,456	2,834	3,196	3,757	3,551	3,701	3,009	1,574	3,005
Candida krusei	2,848	1,590	1,24	3,406	3,744	3,268	3,447	1,670	3,431
Klebsiella pneumoniae	Not tested	3,358	2,355	3,325	3,643	3,643	3,568	3,167	3,228

For the testing of IgY antibodies, 1:100 to 1:64000 dilutions were performed and then assigned to ELISA plates padded with specific antigens *(Streptococcus group B, Salmonella enteritidis, Proteus mirabilis, Salmonella spp., Candida krusei* and *Klebsiella pneumoniae).*
The titer of IgY anti *Streptococcus group B* is elevated in the first two months from the third inoculation (OD = 2,859 - 2,862), while noticing an increase of the OD values at 4,5 and 6 months (OD=3,471; OD=3,407; OD=3,275), and also maintaining at a constant level until the end of the experimental period (9th month OD=3,201); the results are highlighted in Table 1 and Fig. 3.

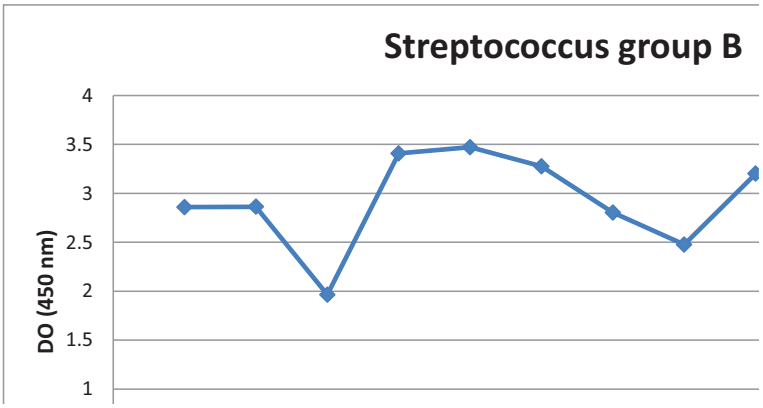

Fig. 3: The titer of IgY anti *Streptococcus group B*

Following the titration of IgY anti *Salmonella enteritidis* , we notice that the OD values are elevated for months 4,5 and 6 since the last inoculum (OD = 3,598; OD =3 ,468; OD = 3,696), and slowly decreasing at months 7 and 8 (OD= 1,624; OD = 1,333), followed by an increase at month 9 (OD = 2,221). The results are highlighted in Table 1 and Fig. 4.

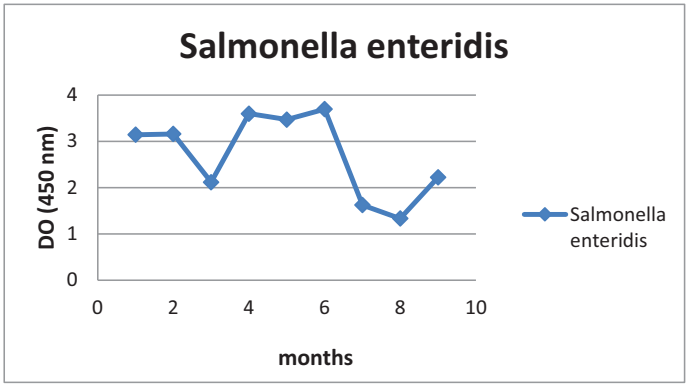

Fig. 4: The titer of IgY anti *Salmonella enteritidis*

The level of IgY anti *Proteus mirabilis* antibodies is relatively elevated for the first three months, offers higher OD values at months 4,5 and 6 (OD=3,130; OD=3,137; OD=3,323), and at 9 months from the start of the experiment (OD=3,077). The registered values are shown in Table 1 and Fig. 5.

By analyzing the results for the testing of the IgY anti *Salmonella spp*. antibodies, we notice that the 1:1000 titer is elevated throughout the experimental period (OD=3,456 for month 1 and OD=3,005 at month 9). The data are shown in Table 1 and Fig. 6.

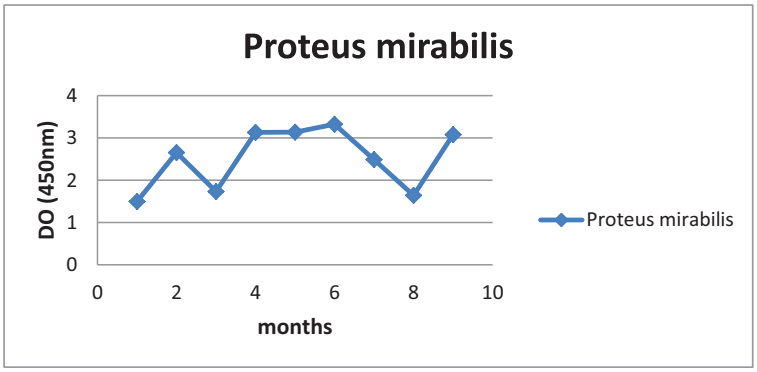

Fig. 5: The titer of IgY anti *Proteus mirabilis*

By analyzing the results for the testing of the IgY anti *Salmonella spp*. antibodies, we notice that the 1:1000 titer is elevated throughout the experimental period (OD=3,456 for month 1 and OD=3,005 at month 9). The data are shown in Table 1 and Fig. 6.

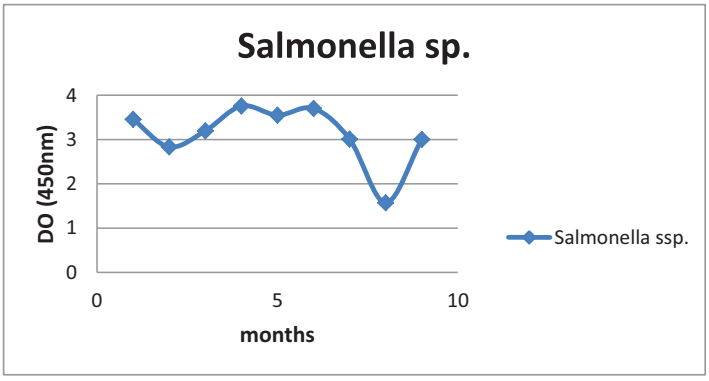

Fig. 6: The titer of IgY anti *Salmonella sp.* antibodies

The evaluation of IgY anti *Candida krusei* antibodies has shown elevated titers for months 4-7 (OD= 3,406; OD= 3,744). We notice that even at 9 months since the last inoculum the titer is high (OD= 3,431). The results are shown in Table 1 and Fig. 7.

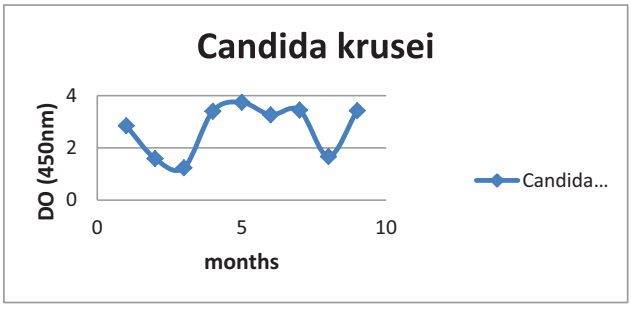

Fig. 7: The titer of IgY anti *Candida krusei*

Following the analysis of the results for the levels of IgY anti *Klebsiella pneumoniae* antibodies, we notice elevated levels throughout the experiment (OD between 2,355 – 3,643). The results are shown in Table 1 and Fig. 8.

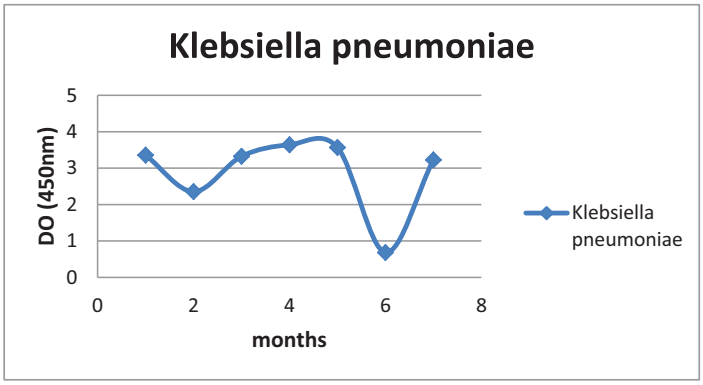

Fig. 8: The titer of IgY anti *Klebsiella pneumoniae*

Gathered data suggests that IgY antibodies extracted from hyperimmune eggs show specificity for the utilized antigens in the hens' immunization process. For the entire array of studied antigens, a decrease of the antibody levels was noticed during the 8th month, followed by an increase in month number 9.

CONCLUSIONS

Igy antibodies have attracted a significant degree of attention from the researchers, due to the structural differences from mammalian IgG and reactivity in the human body. The characteristics that differentiate IgY from IgG make it a hope for medicine, due to its potential use in several specialties.

The experiments performed have proven that Immunoglobulin Y extracted hyperimmune eggs show specificity to the epitopes of bacterial and fungal antigens used for the immunization of hens.

The determination of the titer for IgY antibodies, by using ELISA assays, has shown elevated levels over a 9 months period from the last inoculation process.

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RETROSPECTIVE GIS ANALYSIS OF BLUETONGUE OUTBREAKS IN ROMANIAN CATTLE, BETWEEN 2014 AND 2015

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Abstract

Bluetongue is an insect-borne viral disease of ruminants produced by an Orbivirus. The first case of bluetongue in Romania was recorded on August the 23rd, 2014 in the South-Eastern county of Buzau, and in the coming years hundreds of new cases were documented. The purpose of the paper was the application of GIS methodologies to create a retrospective analysis of bluetongue outbreaks in Romanian cattle between 2014 and 2015. The GIS analysis used a tool for registering epidemiological data that was developed in Microsoft Office Excel 2013 with add-in Power Map. Addresses of individual animal cases were registered, and GPS coordinates were determined automatically by Power Map application for each village of interest. The same application generated GIS maps of bluetongue outbreaks. As a conclusion, results obtained using interactive GIS maps showed a relative improvement in the perception and understanding of the overall situation of Bluetongue throughout the country.

Key words*: Geographic Information System, computational analysis, cattle pathology, bluetongue epidemiology*

INTRODUCTION

Bluetongue is an insect-borne viral disease transmitted to ruminants by bite of several *Culicoides* midges. The most susceptible animal species to BTV are sheep, goats, cattle, buffaloes and deer (Verwoerd and Erasmus, 2004). The Bluetongue virus belongs to *Orbivirus* genus of the *Reoviridae* family (Attoui et al., 2012). The infection can evolve from unapparent to fatal disease. Cattle and goats usually develop subclinical infections while sheep develop acute form of disease (Verwoerd and Erasmus, 2004). However, severe acute disease of cattle has been documented in bluetongue with serotype 8 (Zanella et al., 2013).

Bluetongue has a recent history in Romania, although the presence of BTV vectors was confirmed several years before the presence of the clinical disease (Ivana et al., 2009). The first case was reported on the 23rd of August, 2014 in the South-Eastern part of Buzau county (WAHIS, 2014a), and in the next months hundreds of new cases were documented (WAHIS, 2014b, 2015).

Geographic Information System (GIS) is a computer system designed to analyse spatial/geographical databases. In medicine, GIS is a valuable tool used in spatial analyses of diseases incidence/spread and risk. Also, GIS is a decision support tool in application of contingency plans mainly in major events or emerging diseases (Gattrell and Loytonen, 2003).

The purpose of this paper was the application of GIS methodologies to achieve a retrospective analysis of bluetongue outbreaks in Romania, between 2014 and 2015.

MATERIALS AND METHODS

Materials used for GIS analysis were the archives of Romania's reports to the World Organisation for Animal Health (OIE) between 2014 and 2015, available in the database of World Animal Health Information System (WAHIS, 2014b, 2015).

The GIS analysis used a tool for registering epidemiological data that was developed in Microsoft Office Excel 2013 with add-in Power Map. Addresses of the individual animal cases were registered, and GPS coordinates were

determined automatically, by Power Map application for each village of interest. The same application generated GIS maps of the bluetongue outbreaks.

RESULTS AND DISCUSSIONS

During 2014 Romania reported 1113 outbreaks of bluetongue while in 2015 the number of reported outbreaks was 29 (WAHIS, 2014b, 2015).

Statistical analysis of Romania's reports to World Organisation for Animal Health (OIE) revealed the following data concerning bluetongue, between 2014 and 2015: 102,923 susceptible animals (7,717 cattle), 4191 clinical cases (1079 cattle) and 881 fatalities (25 cattle) (tables 1 and 2) (WAHIS, 2014b, 2015).

Table 1. Animals affected in bluetongue outbreaks, 2014 (WAHIS, 2014b)

Species	Susceptible	Cases	Fatalities
Cattle	***7575***	***1037***	***25***
Buffaloes	3	0	0
European bison	12	2	2
Goats	4478	21	3
Sheep	82535	3001	851
Sheep/ goats	8074	88	0
Total	**102677**	**4149**	**881**

Table 2. Animals affected in bluetongue outbreaks, 2015 (WAHIS, 2015)

Species	Susceptible	Cases	Fatalities
Cattle	***142***	***42***	***0***
Goats	104	0	0
Total	**246**	**42**	**0**

GIS analysis revealed that in August 2014 (fig. 1): the first cases occurred in Sub-Carpathian hills of Muntenia and Oltenia, crossing the area between Gorj and Buzau counties, through the Valcea, Arges, Dambovita, and Prahova, where casualties were reported. The peak of prevalence was in Buzau County. In the next months, the bluetongue has spread to Vrancea County and other Moldavian Counties. Thereby, in September 2014, the number of outbreaks increased in Oltenia area, mainly in Gorj and Olt Counties. The disease has disseminated outside of the Muntenia's hills in September 2014, reaching Dobrogea, Banat, and Transylvania area (fig. 2). In October 2014 bluetongue outbreaks were reported in several Transylvanian counties - Sibiu, Covasna, Mures, Brasov, in Timis county and in Moldavia and Bucovina area (fig. 3). In November 2014, Transylvania and Muntenia areas continuously reported bluetongue outbreaks, while Arad County reported the first cases. The outbreaks reported in Arad County could be the effect of vectors propagation from Timis County (fig. 4). In December 2014 Oltenia area reported new cases, in Dolj, Gorj, Valcea and Olt Counties. Muntenia and Moldavia areas were affected too (fig. 5).

In 2015, outbreaks of bluetongue were reported between September and November (fig. 6-9). The first outbreaks of the year were present in Bucovina area (Botosani and Suceava Counties) (fig. 6), followed in October by outbreaks in Moldavia area (Iasi and Vaslui Counties) (fig. 7). In November 2015, new outbreaks were reported in Botosani and Vaslui Counties (fig. 8).

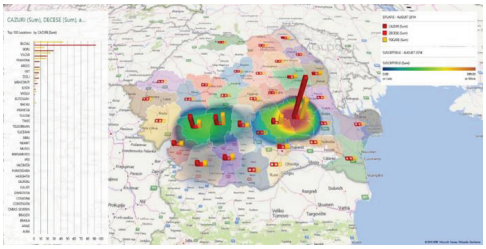

Figure 1. Spatial distribution of bluetongue cases during August, 2014

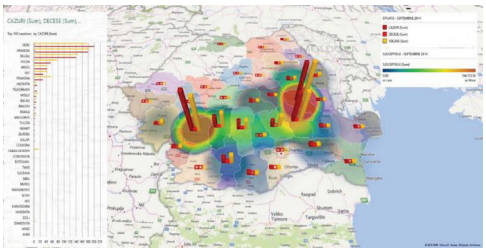

Figure 2. Spatial distribution of bluetongue cases during September, 2014

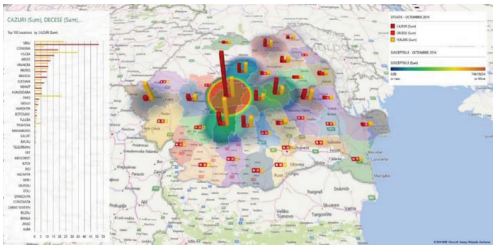

Figure 3. Spatial distribution of bluetongue cases during October, 2014

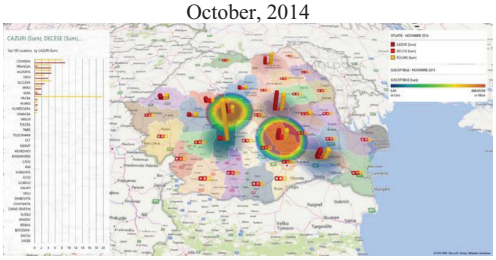

Figure 4. Spatial distribution of bluetongue cases during November, 2014

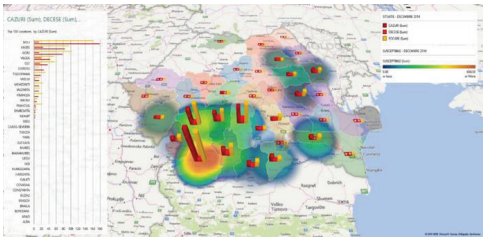

Figure 5. Spatial distribution of bluetongue cases during December, 2014

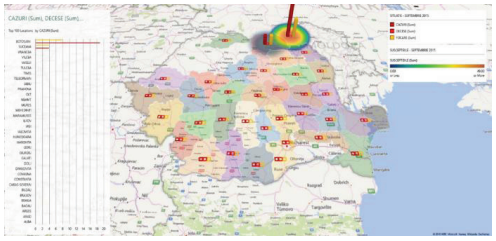

Figure 6. Spatial distribution of bluetongue cases during September, 2015

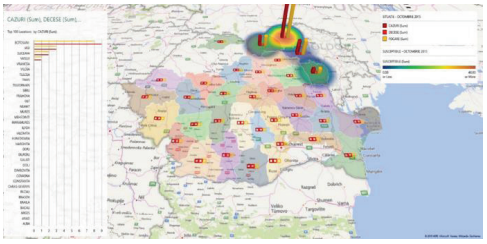

Figure 7. Spatial distribution of bluetongue cases during October, 2015

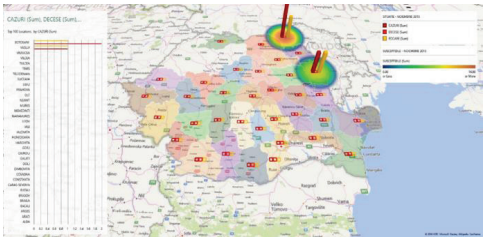

Figure 8. Spatial distribution of bluetongue cases during November, 2015

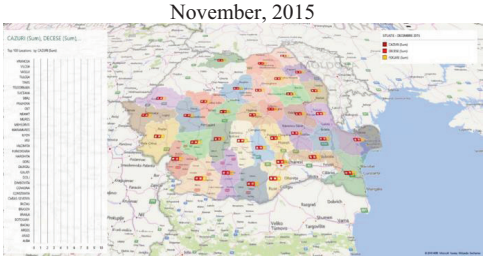

Figure 9. Spatial distribution of bluetongue cases during December, 2015

By using Microsoft Office Excel 2013 with add-in Power Map we drew a simple and useful epidemiological analysis of bluetongue dynamic in Romania. This geographic information system tool supplied:

• Forecasts on place, time and pattern of disease;

• Scenario development regarding strategies to be adopted at the onset of disease;

• Data to correct the control strategy, following the outbreak, in order to reduce the damage and return to normal.

CONCLUSIONS

In 2014, Romania reported 1,037 cases of bluetongue and 25 casualties in cattle, while in 2015 only 42 cases, without mortality. The results obtained using interactive GIS maps improved the perception and understanding of the overall situation on Bluetongue disease throughout the country. Achievement by GIS interpretation of the disease evolution over a certain period provides solutions for preventive actions to be implemented in areas exposed to bluetongue occurrence.

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COMPARATIVE THERAPIES IN KERATOCONJUNCTIVITIS SICCA IN DOGS

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Abstract

The study aimed to present the importance of the proper treatment in Keratoconjunctivitis Sicca in dogs depending on the evolution stage, approaching all the phases. It highlights the necessity of an urgent and accurate treatment in order to avoid blindness. The study is based on the clinical activity of The Department of Surgery from The Faculty Of Veterinary Medicine of Bucharest, during 18 months. During the analyzed period were evaluated 472 dogs with keratoconjunctivitis Sicca, divided in three groups according to the evolutive stage. The treatment used the following products: for the first phase, artificial tear products based on hialuronic acid and carbomers such as HyCare®, Vidisic® and Xanternet®; for the second phase were used artificial tear products and cyclosporine 2% and for the third phase the treatment consisted in eye drops with prednisolone and ointment with tacrolimus. The healing process was complete in 4 to 24 weeks depending on the perseverance of the owner in administrating the treatment and the severity of the disease.

Key words*: Keratoconjunctivitis Sicca, , tacrolimus, cyclosporine 2%, artificial tears, dog.*

INTRODUCTION

Keratoconjunctivitis Sicca is also known as "the dry eye syndrome". It consists in a qualitative or quantitative alteration of the tear film and it can affect dogs of all ages and breeds, most frequently brachycephalic breeds which tend to have a large orbit which allows the eye to be more exposed, therefore the evaporation of the tear film being faster. (Gelatt, 2007; Ionașcu, 2015; Sheppard, 2003)

The etiology of Keratoconjunctivitis Sicca varies widely from congenital aplasia or hypoplasia to hormonal influence or infectious disease, to trauma or drug-induced and due to the physiological process of aging. (Barnett, 2006; Christof, 2001; Ionascu, 2012; Ionașcu, 2015; Martin, 2010).

The qualitatives or quantitative alteration of the physiological characteristics of the tear film, leads to the drying of the cornea and the conjunctiva. In time, depending on the severity of the alteration, the vascularization on this two structures becomes visible, the cornea becomes opaque and in the final phase, the cornea and in some cases even the conjunctiva are pigmented, leading to blindness. (Gelatt, 2007; Slatter, 2008).

Although the simptomatology of Keratoconjunctivitis Sicca depends on the evolutionary phase, there are some clinical signs which are common to all three stages and include enoftalmia and blefarospasm, dried cornea and muco-purulent secretions which are in fact scraped cells from the cornea. The first phase has minimal clinical signs, but the Schirmer Tear Test can reveal severe alterations, the result being sometimes 0 mm/min. The second phase is characterized by the vascularization and the opacification of the cornea determined by the corneal oedema. The third and final stage is characterised by the pigmentation of the cornea which affects the sight therefore the dog becomes blind. (Ionașcu, 2015; Sheppard 2003; Slatter, 2008)

Diagnosis of Keratoconjunctivitis Sicca's is established after a clinical examination, Schirmer Tear Test, Bengal Pink Test and Fluorescein Test. Each of these steps is important in order to eliminate other lesions such

as corneal ulcers, superficial lesions of the cornea and conjunctiva, or infectious keratoconjunctivitis. The reference test is the Schirmer Tear Test. It reveals both types of qualitative and quantitative alterations, being also useful to evaluate the therapeutic response. (Gelatt, 2007; Ionașcu, 2013)

The therapy has to be specific for each phase in order to stop the evolution. The main goals are to eliminate the secretions for the wellbeing of patient but also for assuring a proper field for drug administration; to stimulate the natural tear film secretion, using products that contain cyclsporin or pilocarpin, and tacrolimus, as well. The cyclosporin and the tacrolimus also help remove pigmentation; to replace the precorneal tear film with artificial tear film products, liquid or ointment, depending on the need; to reduce the local inflammation using eye drops with corticosteroids such as prednisolone; to stop the secondary infections when these appear, using local antibiotic. It is extremely important to evaluate the therapeutic response, therefore a periodic re-evaluation is imperative, specifically when using products containing anti-inflammatory or antibiotics. There are also other treatments which include androgens eye drops when considering a hormonal etiology or surgery: partially or completely close the tear ducts, the submandibular gland duct transposition in severe cases. (Berdoulay, 2005; Geerling, 1998; Gelatt, 2007; Gumus, 2009; Ionașcu, 2015; Lemp, 2000; Moore, 2001; Pflugfelder, 2004; Slatter, 2008; Strong, 2005;)

MATERIALS AND METHODS

In a period of 18 months, at the Surgery Department of The Faculty of Veterinary Medicine Bucharest were diagnosed with Keratoconjunctivitis Sicca a number of 472 dogs. The patients were brought for medical care in different stages of the disease, therefore 159 dogs were diagnosed in the first stage, 30 patients were in the second phase and the majority (283 dogs) were in the last stage.

Diagnosis of Keratoconjunctivita Sicca's was established following a certain protocol that includes a clinical examination in order to evaluate the periorbital, palpebral, conjunctival and corneal lesions; the Schirmer Tear Test as a refference method for qualitative and quantitavine alterations, used for diagnosis and therapeutic response evaluation; Fluorescein Test used for differential diagnosis (the lessions on the cornea remain stained with green if present). The next step to follow is the differential treatment according to each stage of the disease. (Table 1.)

Table 1. Therapeutic protocol in Keratoconjunctivitis Sicca according to each phase

Stage	Number of dogs	Therapeutic protocol				
		Artificial tears	Stimulation of lacrimal secretion	Stimulation of lacrimal secretion and depigmentation	Anti-inflammatory	Antibiotics
Phase I	159	+	-	-	-	+/-
Phase II	30	+/-	+	-	+/-	+/-
Phase III	283	-	+	+	+/-	+/-

RESULTS AND DISCUSSIONS

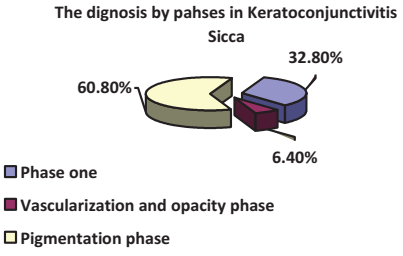

Figure 1. The diagnosis by phases in KCS

The patients in this study were from 1-year-old to 12 years of age, Keratoconjunctivitis Sicca having diverse etiology (Figure 2.).

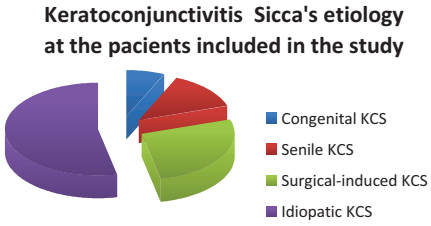

Figure 2. Etiology of Keratoconjunctivitis Sicca

The dogs with idiopatic Keratoconjunctivitis Sicca are brachycephalic and tend to have a wide pathology which demonstrate the existence of an immune-mediated mechanism that subjects the organism to constant metabolic disorders (Peterson- Jones, 2002; Slatter, 2008).
The role of testosterone in determining Keratoconjunctivitis Sicca is not yet established, but regarding the patients from this study, there has been a higher incidence in males than in females (Figure 3) (Peterson-Jones, 2002)

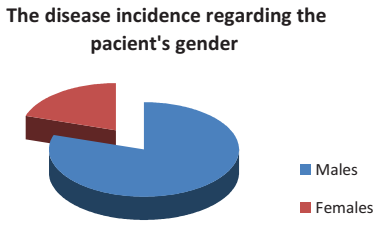

Figure 3. The incidence
of the disease regarding the gender

Unlike other studies, this study approaches all the stages of Keratoconjuctivitis Sicca in therms of differential diagnosis and treatment according to the local pathogenetic processes.
First stage of Keratoconjunctivitis Sicca was diagnosed at a lower number of patients compared with the other two phases, because of the minimal clinical signs. The best results in this phase were obtained using artificial tears products, mainly HyCare. If untreated, the degenerative processes continue towards more severe stage, therefore the diagnosis is frequently between-phases.
The patients diagnosed with phase two of keratoconjunctivitis Sicca had a satisfying recovery following the treatment with Ciclosporin 2% and HyCare. When the diagnosis was between-phases, the dogs recived an additional treatment, the one for the third phase that included Tacrolimus and Prednisolone.
The recovery in first stage patients was from 4 to 8 weeks, whereas for the third phase patients the recovery lasted from 20 to 24 weeks.
It is necessary for the treatment to be administrated for a long period of time, sometimes for the entire life of the dog because the lesions of lacrimal apparatus could be irreversible.
Recurrences are frequent in patients who stop the treatment or in case of an improper administration.

CONCLUSIONS

The treatment in Keratoconjunctivitis Sicca must be administrated according to each stage in order to stop the degenerative processes that can lead to blindness.
The simptomatology can be reduced in 4 to 24 weeks, the therapeutic response being conditioned by the time elapsed until diagnosis and the owner's accuracy in administrating the treatment.
The clinical signs improvement rate is up to 66.6% in the cases from this study, but the other 33.3% of the patients in witch the clinical signs were aggravated had other associated ophthalmological pathology or the treatment was not properly administrated, the treatment response also depending on the owner consistency in following the treatment.
The treatment initiated in the first stage had satisfying results, but the minimal clinical signs do not alarm the owner.
It is essential for the treatment to be administrated for the entire life of the dog because of the irreversible lesions on the lacrimal apparatus that induce the relapse.
A very important step in therapeutic protocol of Keratoconjunctivitis Sicca l, is the removal of the cellular debries which by the hydrophilic properties absorbes the local administrated products, lowering the disponibility of these products for the eye structures.
The associated pathologies to the ocular adnexa that involve the cornea determine the exacerbation of the simptomatology.

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DEVELOPMENT OF A VARIANT OF DIRECT IMMUNOFLUORESCENCE TECHNIQUE IN THE DIAGNOSIS OF PRRS

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Abstract

Porcine reproductive and respiratory syndrome (PRRS) is an infectious disease spread in intensive growth with endemic evolution and with economic significance. In Romania the disease was for the first time diagnosed in 1998 and nowadays the disease is prevalent in many farms of pigs. For detection of viral antigens were sampled lymph nodes with pathological lesions macroscopic characteristic of the PRRS, from cadavers of piglets from farms where the disease evolves, and from pigs with clinical evolution of the disease was taken oro-nasal fluid. Viral nucleocapsid antigen was detected using kit - Anti PRRSV monoclonal antibody labelled with fluorescein isothiocyanate -BIO 268. The cytoplasm of cells, infected with PRRS virus, had a brilliant greenish yellow appearance due to the presence of the viral nucleocapsid antigens coupled with monoclonal antibodies labeled with fluorescein. Epithelial cells were rare, smaller, and fluorescent appearance of the cytoplasm was very evident. In case of the oro-nasal fluid smears, in the microscopic field were highlighted cells agglomerations with highly fluorescent cytoplasm. 19/30 (63.33%) of the examined samples (lymph nodes and oro-nasal fluid) were positive, respectively, 12/30 (40%) of the lymph nodes and 7/30 (23.33%) of oro-nasal fluid. Samples of lymph nodes and oro-nasal fluid were examined also through RT-PCR in order to create a correlation between the results provided by direct immunofluorescence (DIF) and RT-PCR, regarded as the reference method. The results confirm that the DIF can be adapted but more research is required to establish the sensitivity and specificity of this method.

Key words*: DIF, lymph nodes, oro-nasal fluid, PRRS.*

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is an infectious disease spread in intensive growth with endemic evolution and economic significance.

In Romania, the disease was for the first time diagnosed in 1998, and nowadays the disease is prevalent in many pigs' farms worldwide (Stanuica, 1999, 2005; Zimmerman et al., 2012).

Since 1998, several PRRS studies have been conducted in Romanian pig farms, some of them focused in serological evaluation of virus circulation by using ELISA (Stanuica, 1999; Baraitareanu et al., 2009; Campeanu et al., 2010), and other in detection of PRRS virus RNA by different RT-PCR techniques (Zaulet et al., 2011; Fluerașu et al., 2016).

The PRRS syndrome has rapidly expanded in intensive rearing pigs farm, where is producing significant economic losses.

This syndrome is produced by a ribovirus, placed in the *Arteriviridae* family, genus *Arterivirus*, with two genotypes respectively, Type 1 (European) and Type 2 (American) with a considerable variability of gene sequences, with similarity of only 50-60% (Stanuica, 2005; Zimmerman et al., 2012).

Confirmation of PRRS syndrome can be made (based) through several laboratory exams that aim detection of the virus, characterization of the isolated strains and antibody detection. For the rapid diagnosis techniques are preferred the immunohistochemical and immunofluorescence exams, who detects viral nucleocapsid antigen existing in the cytoplasm of infected cells in the lymph nodes, lungs and other organs (Mengelling et al., 1995; Botner, 1997; Zimmerman et al., 2012).

The immunofluorescence reaction (DIF) use monoclonal antibodies marked with fluorochromes for the detection of viral antigen from cryosections made it in lungs and lymph nodes (Botner, 1997; Jing et al., 2009; Zimmerman et al., 2012).
The aim of study was evaluation of a DIF as a rapid diagnostic method by using fingerprint smears of lymph nodes and oro-nasal fluid from pigs with clinical or pathological suspicion of PRRS.

MATERIALS AND METHODS

For detection of PRRS virus (PRRSV), 20 lymph nodes were sampled from piglet cadavers with characteristic lesions of PRRS. From pigs with respiratory syndrome was taken 10 oro-nasal fluid samples. All cadavers and oro-nasal fluid samples were supplied by a pig farm with a recent history of PRRSV infection.
Because the use of cryosections requires an endowment with cryotome, in the framework of research, DIF technique has been adapted in order to use the fingerprint smears made from lymph nodes and oro-nasal fluid.
Viral nucleocapsid antigen was detected using the commercial diagnostic test BIO 268 (Bio-X Diagnostics, 2016), a reagent for detection of Porcine Respiratory Reproductive Syndrome Virus: anti PRRSV monoclonal antibody labelled with fluorescein isothiocyanate.
DIF technique consists of the following steps: degreasing glass coverslips with ethyl alcohol exhibiting of samples of lymph fingerprint, desiccation of the wells and fixing in acetone for 15 minutes, desiccation of the wells for two hours, washing the slides with PBS-Blue Evans, and the desiccation of the wells. Oro-nasal fluid samples were centrifuged at 3000 rpm for 10 minutes at room temperature. The supernatant was siphoned off and the sediment transferred to a microscope slide.
Fluorescein-conjugate BIO 268 (0.1 µl) was added on dried and fixed slides, after which the slides were incubated for 1 hour at 21℃, and finally examined under the microscope with fluorescent light OLYMPUS.
PRRS virus RNA detection has been done by using RT-PCR technique developed at SN Institute Pasteur SA Bucharest (Romania), and previously described (Fluerașu et al., 2016). For this purpose were used four extraction kits (Qiagen and Roche, Germany) and two primers specific to ORF 7 area (Fluerașu et al., 2016).

RESULTS AND DISCUSSIONS

Inguinal lymph nodes were taken from fresh corpses of piglets, from primary outbreaks. Lymph nodes were swollen, oedematous, with firm consistence, and red on the section. Inguinal lymph nodes were preferred because they present macroscopic lesions well cast.
The results of DIF and RT-PCR exams are shown in Table 1.

Table 1. DIF and RT-PCR results

No.	Type	DIF		RT-PCR	
		+	–	+	–
1	lymph node	+		+	
2	lymph node	+		+	
3	lymph node		-	+	
4	lymph node		-	+	
5	lymph node	+		+	
6	lymph node		-	+	
7	lymph node	+		+	
8	lymph node		-	+	
9	lymph node		-		-
10	lymph node	+		+	
11	lymph node	+		+	
12	lymph node		-	+	
13	lymph node	+		+	
14	lymph node	+		+	
15	lymph node	+		+	
16	lymph node		-	+	
17	lymph node	+		+	
18	lymph node		-		-
19	lymph node	+		+	
20	lymph node	+		+	
21	oro-nasal fluid		-		-
22	oro-nasal fluid	+		+	
23	oro-nasal fluid	+		+	
24	oro-nasal fluid	+		+	
25	oro-nasal fluid	+		+	
26	oro-nasal fluid	+		+	
27	oro-nasal fluid	+		+	
28	oro-nasal fluid		-		-
29	oro-nasal fluid	+		+	
30	oro-nasal fluid		-		-

On the smears made from lymph nodes, the image from microscopic field was different being viewed isolated cells, cells grouped, or large clusters of cells. In case of positive samples, microscopic field predominated in medium and small lymphocytes, and large lymphocytes, plasma cells, and epithelial cells were rare. On medium and small lymphocytes, cell outline was obviously the nuclei were well individualized, the ratio between nucleus and cytoplasm was about equal. Large lymphocytes have a similar aspect, the plasma had an

elongated oval shape and cytoplasm was dominant.
The cytoplasm of cells, infected with PRRSV had a brilliant greenish yellow appearance due to the presence of the viral nucleocapsid antigens coupled with monoclonal antibodies labelled with fluorescein. Epithelial cells were rare, smaller, and fluorescent appearance of the cytoplasm was very evident.
In case of the oro-nasal fluid smears, in the microscopic field were highlighted cells agglomerations with highly fluorescent cytoplasm.
Smears made from lymph nodes and the oro-nasal fluid, when was identified in microscopic field the described aspects, were considered positive. Thus, 19/30 (63.33%) of the examined samples (lymph nodes and oro-nasal fluid) were positive, respectively, 12/30 (40%) of the lymph nodes and 7/30 (23.33%) of oro-nasal fluid.
Samples of lymph nodes and oro-nasal fluid were examined also through RT-PCR in order to create a correlation between the results provided by direct immunofluorescence and results from RT-PCR, regarded as the reference method.
The results obtained by RT-PCR were communicated through a previously published scientific paper (Fluerașu et al., 2016).
Samples of lymph nodes, and oro-nasal fluid confirmed as positive by direct immunofluorescence technique were confirmed as positive also by RT-PCR technique. Six DIF-negative samples of lymph nodes and oro-nasal fluid were confirmed as positive by RT-PCR technique, and five samples were negative in both techniques.
Until now, DIF was used only for nucleocapsid antigen detection of PRRSV by using cryosections from lymph nodes, lungs and other lymphoid organs. In this research was aimed adaptation of DIF method in order to be taken like a quick method of PRRS diagnosis using fingerprint smears made from the lymph fluid or oro-nasal sediment (Botner, 1997; Jing et al., 2009).
The results confirm that the DIF technique can be adapted but more research is required to establish with certainty the sensitivity and specificity of this method.

CONCLUSIONS

The operating-method, allowed us to obtain smears from lymph nodes and oro-nasal fluid, which with the aid of monoclonal antibodies fluorescein-labeled could be detected different types of cells infected with PRRS virus.
The IFD reaction made it by the methodology described, detected the presence of viral antigens in 63.33% of samples.
For using IFD as a rapid diagnostic method, is necessary to continue the researches on a larger number of samples and compared with more diagnostic methods.

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THE IMMUNE RESPONSE INDUCED BY E. COLI IN CHICKENS AND AVAILABLE VACCINES

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Abstract

The paper aimed to present the available vaccines for E.coli in chicken and discuss the immune response induced by E.coli. It is based on statistical data provided by already published articles and data interpreted from specialised journals. Avian pathogenic E. coli (APEC) represents the most economically significant disease which has a negative impact on the industry of broilers. In many countries, a common practice during the last decades has been to administer antibiotics in order to suppress infection with APEC, but lately market pressures, by trade and statutory restraints, have limited the use of antibiotics and this has led to the development of specially designed E.coli vaccines, to stimulate an immune response against pathogenic E. coli, besides vaccination against primary respiratory and immuno-suppressive pathogens. Chickens display different mechanisms to protect against and to combat pathogenic infection. Typically APEC is inhaled or ingested, by crossing the mucosal barrier; it cannot penetrate the skin, which generally acts as a protective element. In broilers, the source of infection are, usually, the contaminated drinking water or the inhaled dust, laden with APEC. Antimicrobial drugs remain an important tool in reducing of incidence as of mortality, associated with this disease, but a vaccine-based approach for the disease control remains highly desirable, which is why the focus of this paper will be to analyze and criticize each available type of E.coli vaccine, including the advantages and disadvantages of each, in terms of preparation, efficiency, costs and other important factors. The latest developments in molecular biology have created new vaccine strains effective against APEC, like the modified gene-deleted vaccines which stimulate both, tissue (cellular) associated immunity and humoral (circulating antibody) immunity. The first commercial E. coli vaccine for chickens was licensed by the US Department of Agriculture in 2006, so this paper will also try to focus on the newest research, directed after this year.

Key words*: E.coli vaccines, immune response*

INTRODUCTION

E. coli can enter the body by various routes, all of which can lead to colibacillosis. Avian pathogenic E. coli (APEC) represents the most economically significant disease for poultry, which has a negative impact on the industry of broilers. Two major problems currently make it difficult to control poultry colibacillosis, namely, the lack of a dependable method to identify the pathogenic strains of E. coli and the not totally effective available vaccines, also vaccines against E. coli are not widely used, and this may be due to the large variety of serogroups involved in field outbreaks. Septicemic disease may be rapidly fatal or chronic, manifested by debilitation, diarrhea and respiratory distress. Other pathological features are pericarditis, synovitis, salpingitis and panophthalmitis (Hirsh and Zee., 1999).

Avian colibacillosis is a complex syndrome characterized by multiple organ lesions with airsacculitis and associated pericarditis, perihepatitis and peritonitis. Environmental factors as well as the constitution of poultry or initial viral infections, influence the outcome of APEC-infections. Experimental studies have shown that the respiratory tract, mainly the gas exchange region of the lung and the interstitium of the air sacs are the most important sites of entry for avian pathogenic E.coli (Ewers et al., 2005).

E. coli vaccines induced mucosal and humoral immunity, but were not effective in the

prevention of E.coli infections, due to highly serotype diversity.
Researchers have proposed different types of vaccines in attempts to induce protective immunity against colibacillosis in chickens. The earliest studies involved evaluation of killed organisms, subunit vaccines and live vaccines. (Maas, 2013).
This paper will discuss, in the results and discussion section, the benefits of live, attenuated, inactivated, subunit, recombinant, conjugate and toxoid vaccines.

MATERIALS AND METHODS

Specific articles were selected from hundreds of articles analysed in PubMed, Google Academic databases, NCBI, Research Gate, vet research, Biomed central, and Science Direct. The research method followed three main steps.
1-scientific databases research of the relevant articles concerning E. coli available vaccines.
2- Analysis and selection of the relevant data
3- Extraction and summarization of the results. When necessary and available, the data have also been statistically processed and interpreted.

RESULTS AND DISCUSSIONS

Respiratory tract infection often presents itself with one or more birds sneezing, having a runny nose and foamy running eyes. Septicemic disease course may be rapidly fatal or chronic, manifested by debilitation, diarrhea and respiratory distress. Other infections are pericarditis, synovitis, salpingitis and panophthalmitis (Hirsh and Zee., 1999). In severe cases these birds can have swollen sinuses, stop eating and, in extreme cases, die. Chicken respiratory infections are usually spread by direct contact between infected and uninfected chickens, but also by contact with objects that infected chickens have sneezed or coughed on, such as transport coops or clothing can carry the infectious organisms from place to place, too. Escherichia coli infections are widely distributed among poultry of all ages and categories. They are primarily related to poor hygienic conditions, to neglected technological requirements or to previous respiratory and immunosuppressive infections. A common sequel of navel infections is the local or diffuse peritonitis. Bacteriophages are viruses that can infect and kill bacteria. Pathogens that cause respiratory or enteric disease typically enter via mucosal surfaces and stimulate a local immune response, involving production of the mucosal secretory immunoglobulin A (sIgA), the most important immunoglobulin involved in the immune response of mucous in many species (McGhee et al., 1992).
Despite the results of many studies, carried to determine the efficacy of the bacteriophage to prevent an Escherichia coli respiratory infection in broiler chickens, the data proved that this can protect birds from a respiratory challenge with E. coli, the adding the bacteriophage to the drinking water did not protect the birds against E.coli challenge. (Huff et al, 2002).
Avian colibacillosis is an infectious disease of birds caused by Escherichia coli. Avian colibacillosis has been noticed to be a major infectious disease in birds of all ages. This disease has an important economic impact on poultry production worldwide. The majority of economic losses results from mortality and decrease in productivity of the affected birds.
Two major problems currently make it difficult to control poultry colibacillosis, namely, the lack of a reliable method to identify the pathogenic strains of E. coli and the limited efficacy of the available vaccines, limiting the use of vaccines against E. coli, and this is due also to the huge variety of serogroups identified in outbreaks. (Hirsh and Zee., 1999).
Avian colibacillosis is a complex syndrome characterized by multiple organ lesions with airsacculitis and associated pericarditis, perihepatitis and peritonitis. Environmental factors as well as the biological status of poultry and/or previous chronical/unapparent viral infections, influence the outcome of APEC-infections. Experimental studies have shown that the respiratory tract, principally the gas exchange region of the lung and the interstitium of the air sacs are the most important sites of entry for avian pathogenic E.coli (Ewers et al., 2005).
Compared to bacterins and subunit vaccines, live vaccines are attractive because they are much cheaper than purified proteins, and can be used for mass immunization via aerosols,

feed, or drinking water (Peighambari, et al, 2012). Immunogenicity (SC injection) of an oil-emulsified E. coli (O1:K1) bacterin with an aqueous-phase-to-oil-phase ratio of 1:4 in chickens was evaluated by Panigrahy M. in 1984 (Maas, 2013).

The great diversity among APEC strains limits the possibilities of vaccination, and vaccines are not used on a large scale. Vaccines based on killed or attenuated strains have been tested experimentally. Generally, they induce a satisfactory protection against infection with homologous strains, but lesser or none protection against heterologous. Passive immunization of young birds by the yolk antibodies is protecting for the next two weeks after hatching, if the birds are challenged with homologous strains. Vaccines based on virulence factors like fimbriae, also give a good homologous protection, i.e., against APEC possessing the same fimbriae.

The Mucosal Immune System (MIS) is the main inductor sites for the immune response induction in poultry, when the primary lymphoid organs are subjected to atrophy (20 weeks old). (Janeway et al, 2001).

Although have been reported many studies on bacteria isolation and methods of detection, host immune responses to APEC infection remain unclear (Li et al, 2016). For hosts, the innate immune response represents the first line of defense against enteric pathogens (Mogensen, 2009). When pathogens invade, the innate immune response manages the invasion by inducing programmed cell death and secreting pro-inflammatory compounds that direct immune cells to infection sites (Takeuchi and Akira, 2010). In that context, toll-like receptors (TLRs) serve as important pattern recognition receptors (PRRs) that can recognize various pathogenic organisms (Keestra et al., 2013).

Infection is associated with the induction of both, humoral and cell-mediated immune response. It is unclear whether such events are also induced by existing commercial vaccines. Passively immunity can be acquired either vertically via egg-yolk antibody or administering antibodies intravenously. Environmental factors as well as the biological status of the poultry or the presence of the previous viral infections influence the outcome of APEC-infections.

In poultry, immunity can be innate or acquired. Innate immunity refers to the natural or inherited ability to resist disease. Acquired immunity can be active or passive. Active immunity refers to an active immune response in a bird as a result of recovery from the disease or as a response to exposure to vaccine antigen. The bird produces its own immune cells and/or antibodies to provide protection. Active acquired immunity is divided into non-cellular (humoral) and cellular components (Janeway et al, 2001).

In the last few years, emphasis has been made about the epithelium intestinal biology, the defence mucosa mechanisms and the regulation of induction and expression of immunity, which is reflected in the productive performance of the broiler farm. It is because these poultries grow in intensive condition and receive vaccines to induce immunity (Peralta, 2016).

Mucosal immunity is short-lived and mucosal immunogens do not induce a memory response (Keestra et al., 2013). However, others contend that the induction of mucosal memory response to certain proteins is possible (Vajdy and Lycke, 1995). Generally, it is assumed that eliciting of T and B cells in mucosal tissues such as GALT by microbial antigen presented to that site, leads to priming of B cells.

Antimicrobial drugs remain an important tool in reducing both, incidence and mortality associated with this disease, but a vaccine-based approach for the disease control remains highly desirable, which is why the focus of this paper will be to analyse and discuss each available type of E.coli vaccine, including the advantages and disadvantages of each, in terms of preparation, efficiency, costs and other important factors.

The latest developments in molecular biology have created new vaccine strains effective against APEC, like for example modified gene-deleted vaccines which stimulate both tissue (cellular) immunity and humoral (circulating antibody) immunity. The first commercial E. coli vaccine for chickens was licensed by the US Department of Agriculture in 2006 (Ferguson et al., 1995).

The great diversity among APEC strains limits the possibilities of vaccination, and vaccines are not used on a large scale. Several vaccines based on killed or attenuated strains have been tested experimentally. In general, they induce a satisfactory protection against infection with homologous strains, but protection against heterologous strains is less efficient. In vaccines against enteric infections, gut antibodies can provide antibacterial protection by two mechanisms: direct action against the bacteria or combination with the bacterial products. The first mechanism can result in immobilization, agglutination or prevention of mucosa adherence. The combination of bacterial products like toxins or enzymes can cause inactivation and can help in the destruction by proteolytic enzymes (Ferguson et al., 1995; Smith and Beal, 2008). Previous exposure to the same antigen (Ag) or cross-reaction can guide the mucosal immune response (MIR).

Attenuated vaccines mainly induce better MIR than the inactivated one (Meeusen, 2011). The new generation of orals avian vaccines include genes that guide the vaccines to dendritic cells, as these cells have an important function in the antigenic presentation mechanism, leading to cellular or humoral immunity (De Geus and Velverde, 2013; Parra et al., 2013). Live vaccines are widely used throughout the world because they are commonly effective when mass applied and relatively economical.

Inactivated vaccines or killed vaccines used in poultry are generally whole bacteria or virus preparation, delivered with an adjuvant designed for subcutaneous or intramuscular injection. A study conducted by D.R. Anuruddhika Dissanayake and T.G. Wijewardena (2016) determined the LPS core specific antibody titters of chickens immunized with a single dose of heat killed rough mutant E. coli strains comprising of LPS core types R1, R2,

R3 and R4. Thus, the heat killed mixture of rough mutant E. coli strains can be used as a vaccine to enhance LPS core specific antibodies in chickens. They are frequently used in laying hens, to stimulate long-lasting immunity toward specific antigens. Most inactivated vaccines, however, induce a weaker immune response than do live vaccines. Compared to bacterins and subunit vaccines, live vaccines are attractive because they are much cheaper than purified proteins, and can be used for mass immunization via aerosol, feed, or drinking water.

Live virus vaccines usually induce high levels of immunity, long-lasting in the host. Instead of using the whole bacterial body, subunit vaccines include only those selected antigens, that better stimulate the immune system. In some cases, these vaccines use epitopes—the very specific parts of the antigen that antibodies or T cells recognize and bind to. Because subunit vaccines contain only the essential antigens and not all the other molecules that make up the microbe, the risk of side reactions to the vaccine are lower.

Recombinant vaccines are made using live viruses or bacteria, as a vector to host and transport the gene coding for the protective antigen of a second infection agent, for which immunity is desired. Recombinant technologies provide a very safe and economical way for large-scale production of subunit vaccines (Ribot et al., 2006). The technology facilitates identification, extraction, amplification and modification of DNA/RNA fragments encoding the antigenic part of a pathogen for recombinant expression. The first step is the identification of a suitable antigen with the ability to elicit a protective, long lasting immune response. Recombinant vaccines can be divided into two major groups: DNA and antigen-based vaccines.

DNA based-vaccines are a new type of vaccine which started to evolve in the late 1990s. They can achieve both humoral and cell-mediated immunity and are very similar to live-vaccines, and have the safety associated with inactivated or vector vaccines. DNA vaccines can be used successfully in poultry for avian diseases, but they still have technological and economical challenges to overcome, so are not very cost-effective (Ribot et al., 2006). The main disadvantages of DNA vaccines are the need for large amounts of DNA to achieve protective immune response in large animals (Brun et al., 2011)

The use of DNA as vaccine is a new and promising approach in vaccination since it is safe (no infectious antigen is involved), specific, easy and economical. The term refers

to induction of immune response against a protein(s) expressed in vivo (vaccine recipient) subsequent to the injection of plasmid DNA, therefore mimicking the live pathogen in immune system induction. The plasmid DNA is carrying an antigen-coding sequence under a host-specific promoter. This method is attractive due to its simplicity. It only requires plasmid construction, proliferation and extraction (Dertzbaugh, 1998).

Antigen-based vaccine (subunit recombinant vaccine) are also called protein based subunit vaccines and they present an antigen to the immune system without viral particles, using a specific, isolated protein of the pathogen. A weakness of this technique is that isolated proteins, if denatured, may bind to different antibodies than the protein of the pathogen. Recently, virus-like particles (VLPs) and subviral particles (SVPs) have received great attention due to their potential application in vaccine development as well as in drug targeting and gene therapy (Zhao et al., 2011). VLPs are composed of one or more recombinantly expressed viral proteins, which spontaneously assemble into supermolecular structure. VLPs have the same size and morphology as the parent virus, whereas SVPs are smaller in size or possessing a lesser degree of organization than the whole intact viral particle, because the parent virus comprises several different coat protein subunits while SVPs are chromomeric (Manzenrieder et al., 2011).

Conjugate subunit vaccines create a response against the molecules in the pathogen's capsule. These types of vaccines can prevent common bacterial infections. Producing conjugate vaccines in recombinant E coli is potentially easier and more cost-effective than alternatives, but the method suffers from low yields. A conjugate vaccine is a substance that is composed of a polysaccharide antigen fused (conjugated) to a carrier molecule. This enhances the stability and the effectiveness of the vaccine (Ree, 1985).

Toxoid vaccines are made from the bacterial toxins. The body learns how to fight off the bacterial's natural toxin once exposed to, through producing antibodies against. It has been possible to serologically demonstrate that the vast majority of the birds having an E. coli septicaemia had been infected with E. coli strains that contain fimbriae of the F11 type. Such fimbriae contain subunit proteins. In the past have already been cloned F11 fimbriae from a wild-type uropathogenic E. coli strain (Ree, 1985). Toxoid E.coli vaccines may contain, together with or instead of F 11 fimbriae, immunogenic sections of F11 fimbriae. Other E. coli antigens can be included, such as E. coli flagella toxins. (Ree, 1985)

CONCLUSIONS

Active immunization involves administration of vaccines containing antigenic molecules (or the genes controlling these molecules) derived from APEC. As a result, vaccinated animals should generate specific immune response and to develop prolonged, strong immunity against APEC.

When properly used, vaccines against APEC are highly effective in controlling infectious diseases. Several criteria determine whether a vaccine can or should be used. First, each APEC producing a disease outbreak must be identified and characterized. Although this appears self-evident, it has not always been followed in practice.

An ideal APEC vaccine for active immunization should confer prolonged, strong immunity in vaccinated animals, as well as rapid onset of immunity. It should not cause side effects, should be stable genetically and thermostable and, for poultry, should be economically affordable and proper to mass administration. It should allow discriminate between the post-infection immune response and the vaccinal one, so the vaccination and the eradication may proceed simultaneously. Modified live vaccines are more difficult to create for bacteria: bacteria have thousands of genes and thus are much harder to control. Scientists working on a live vaccine for a bacterium, however, might be able to use recombinant DNA technology to remove several key genes.

Avian pathogenic Escherichia coli (APEC) cause a wide range of economically significant infections in chickens. Control of these infections by antimicrobial drug is no longer possible due to high prevalence of multidrug resistance strains.

Involvement of large number of serotypes in these infections left none serotype specific vaccine as the only option. A study conducted by D.R. Anuruddhika Dissanayake and T.G. Wijewardena (2016) determined the LPS core specific antibody titters of chickens immunized with a single dose of heat killed rough mutant E. coli strains comprising of LPS core types R1, R2, R3 and R4. Thus, the heat killed mixture of rough mutant *E. coli* strains can be used as a vaccine to enhance LPS core specific antibodies in chickens.

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TREATMENT OPTIONS FOR CRANIAL CRUCIATE LIGAMENT RUPTURE IN DOG – A LITERATURE REVIEW

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Abstract

Cranial cruciate ligament (CrCL) breaks in dogs can be treated by surgical and non-surgical methods. Choice of the treatment method of cranial cruciate ligament rupture in dog continues to constitute a real problem for veterinarian clinicians. This topic has been the subject of many studies. The investigation of the speciality literature data concerning the surgical treatment options in the management of cranial cruciate ligament breaks in dog, remains in the conditions of an informational avalanche a present concern. The purpose of this study was to analyze additional evidence which have appeared in the literature in the period of 2006 - January 2017 and which advocate with concrete evidences in the favour or disfavour of a particular method of dog's cranial cruciate ligament breaks treatment. Analysis of online searches using PubMed engine in 403 articles suggest that the data analyzed do not allow accurate comparisons between different treatment procedures of cranial cruciate ligament deficiency in dogs and did not show significant differences and major changes compared to previous reports (from 1963 to 2005). New long-term clinical studies must designed and further biomechanical and kinematic analyses are required to determine the optimal technique, and whether these procedures are superior to other stabilization methods.

Key words*: cranial cruciate deficiency, dog, treatment procedures, trend.*

INTRODUCTION

Choice of the treatment method of cranial cruciate ligament (CrCL) rupture in dog continues to constitute a real problem for veterinarian clinicians.

This topic has been, since 1963 (Foster et al., 1963), the subject of many concerns and studies. CrCL breaks in dogs can be treated by surgical and non-surgical methods.

The latest study investigating the speciality literature on surgical treatment options in the management of CrCL ruptures in dogs was published in 2005 (Aragon and Budsberg, 2005) after an online bibliographic search through Medline, PubMed, Veterinary Information Network, and Commonwealth Agricultural Bureau Abstracts were found and analyzed 240 sources and it ends with the conclusion „*At this time, the application of evidence-based medicine in analyzing the current available evidence suggests that there is not a single surgical procedure that has enough data to recommend that it can consistently return dogs to normal function after CCL injury*".

Referring only to the surgical procedures used to treat dog's CrCL ruptures, a review from 2011 (Tonks et al., 2011) was focused only on extracapsular procedures and shows that there is no data to allow recommendation of a specific technique being necessary „*future studies should be directed toward outlining the virtues and inadequacies of the current techniques"* and another study investigating the literature (444 paper works) with constant referring only on surgical procedures occurred in 2014 (Berg et al., 2014) conclude that tibial plateau levelling osteotomy (TPLO) is superior to the extracapsular lateral side suturing procedures, but there are insufficient data to properly assess other surgical methods.

There are also several studies comparing the effectiveness of surgical and non-surgical therapeutic methods, the latest being dated in 2013 (Baker and Baker, 2013; Comerford et al., 2013; Wucherer et al., 2013).

The purpose of this study was to analyze additional proof which have appeared lately in the speciality literature in the favour / disfavor of a particular method of CrCL breaks treatment in dog.

MATERIALS AND METHODS

In January 2017 was conducted an online search, using three searching engines: Google scholar (https://scholar.google.ro), PubMed – US National Library of Medicine National Institutes of Health (https://www.ncbi.nlm.nih.gov/pubmed) and Taylor & Francis Online (http://www.tandfonline.com).

There have been used as basic search term "cranial cruciate ligament rupture in dog" preceded into three main additional insights by the terms „treatment", „nonsurgical treatment", „surgery treatment", last with the following secondary insights „lateral extracapsular stabilization treatment", "tibial plateau levelling osteotomy - TPLO", " tibial tuberosity advancement - TTA", " triple tibial osteotomy - TTO" and "Maquet".

Filtering of the results was done using „most recent" and „best match" for PubMed engine, „article" for Google scholar and „subject" and „publication date" for Taylor & Francis Online.

The results obtained by investigating PubMed were analyzed and classified according to the method proposed by Aragon and Budsberg, 2005, after the evaluation method used: 1 - force plate analysis, 2 - subjective and objective evaluation by the clinician and 3 - subjective evaluation by the pet owner, being considered relevant in this order (1 – maximum and 3 - minimum).

RESULTS AND DISCUSSIONS

Results of the bibliographic online introspection are shown in Table 1.

The analysis of search results with PubMed engine reveals 403 articles (for the period 1963 - January 2017), respectively 391 after manual excluding of those in whose abstract was not evidenced a connection with CrCL. In the period 2006 - January 2017 were found 216 articles about CrCL in dog, data that reveals a near doubling of number of articles reviewed by Aragon and Budsberg until August 2004 (Aragon and Budsberg, 2005).

In the period of 2006 - January 2017 have appeared instead only 115 articles for therapeutic results evaluation of dog's CrCL ruptures from which only 23 were relevant articles of level 1, according to the criterion (evaluation by force plate analysis), 86 articles of level 2 (subjective and objective evaluation by the clinician) and 6 articles of level 3 (subjective evaluation by the pet owner).

Table 1. Number of scientific papers identified online

Descriptors	Search engines		
	Google scholar (https://scholar.google.ro)	PubMed – US National Library of Medicine National Institutes of Health https://www.ncbi.nlm.nih.gov/pubmed)	Taylor & Francis Online (http://www.tandfonline.com)
cranial cruciate ligament rupture in dog	10.600	403	62
treatment cranial cruciate ligament rupture in dog	9550	267	51
surgery treatment cranial cruciate ligament rupture in dog	8530	251	41
lateral extracapsular stabilization treatment in cranial cruciate ligament rupture in dog	735	5	2
tplo treatment in cranial cruciate ligament rupture in dog	983	33	4
tta treatment in cranial cruciate ligament rupture in dog	465	21	1
tto treatment in cranial cruciate ligament rupture in dog	384	3	1
tightrope in cranial cruciate ligament rupture in dog	140	4	0
maquet procedure in cranial cruciate ligament rupture in dog	162	3	0
bone anchor in cranial cruciate ligament rupture in dog	1340	3	20
nonsurgical treatment in cranial cruciate ligament rupture in dog	1120	3	1

Of the 23 articles of level 1, six articles promotes kinematics and force plate analysis methods for the diagnosis of dog's CrCL ruptures (Fanchon and Grandjean, 2007; Sanchez et al., 2010; Pillard et al., 2012; Nelson et al., 2012; Souza et al., 2014; Krotscheck et al., 2014; Wüstefeld et al., 2016), 14 articles describe or compares the results obtained with different techniques of surgical treatment (Thieman et al., 2006; Robinson et

al., 2006; Havig et al., 2007; Schaijk, 2008; Voss et al., 2008; Gordon-Evans et al., 2010; Pozzi et al., 2010; Morgan et al., 2010; Gordon-Evans et al., 2011; de Medeiros et al., 2011; Böddeker et al., 2012; Nelson et al., 2012; Mols et al., 2014; Rey et al., 2014; Berger et al., 2015) and two articles describe and compares the results obtained with different nonsurgical treatment techniques versus surgical (Siva et al., 2013; Wuchereria et al., 2013).

If in 2005 there is the opinion that a correct assessment of effectiveness of dog's CrCL rupture treatment method only the investigations of level 1 can be considered reliable (Aragon and Budsberg, 2005; Houlton, 2013), reaffirmed subsequently (Fanchon and Grandjean, 2007; Sousa et al., 2014; Wüstefeld et al., 2016) there are some studies appeared quite recently (Mols et al., 2014) which concludes that „*ground reaction forces may be inadequate as a sole method for assessing functional outcome after cranial cruciate ligament repair*".

Articles of level 2, for the most part, makes the inventory of postoperative complications of different treatment's types (Butterworth and Kydd, 2017; Dymond et al., 2010; Fitzpatrick and Solanio, 2010; Frey et al., 2010; Haaland and Sjöström, 2007; Hishenson et al., 2012; Imholt et al., 2011; Rotherford et al., 2012; Stauffer et al., 2006; Proot and Cooke, 2009; Taylor et al., 2011; Thompson et al., 2011; Wolf et al., 2012; Moles et al., 2009) or shows mixed results of level 2 with 3 (Christopher et al., 2013; Stein et al., 2008; Steinberg et al., 2011).

The estimated costs of surgical treatment for cranial cruciate ligament ruptures in dogs in USA in 2003 were 1.32 billion dollars (Wilke et al., 2005).

Surgical techniques for the repair of cranial cruciate ligament deficiency can be classified into three categories: intra-articular grafts, extracapsular suture stabilization and proximal tibial osteotomy (Hulton, 2013).

Intraarticular stabilization techniques utilize autografts, allografts, xenografts, and synthetic materials to replace the affected CrCL (Paatsama, 1952; Leighton et al., 1976; Arnoczky et al., 1979; Hulse et al., 1980; Meyers et al., 1979; Andnsh and Woods, 1984; Curtis et al., 1985; Yoshiya et al., 1986; Yoshiya et al., 1987; Vasseur, 1984; Stead et al., 1991; Aiken et al., 1994; Tonks et al., 2011).

Vasseur, 2003, makes the inventory of the main reasons of intra-articular procedures low use: - autografts present inferior stiffness and strength compared with normal ligament; - allografts present the inconvenience of collection and storage; - synthetic materials caused intra-articular fibrosis, bone abrasion and chronic inflammatory response; which has limited their use in veterinary medicine.

Extra-articular stabilization techniques include: lateral fabellar suture (LFS), percutaneous placement of the lateral fabellar suture (pLFS), Tightrope (TR) (Cook et al., 2010), transcondylar toggle system (Kunkel et al., 2009), modified lateral extra-capsular technique with bone anchor.

The treatment using lateral fabellar suture (LFS) remains at this moment the most practiced method, applied particularly in small dogs (Hulton, 2013).

The major shortcoming of the method (overloading of suture anchor points) (Fischer et al., 2008) has benefited from the contribution of several studies (Roe et al., 2008; Hulse et al., 2010; De Sousa et al., 2014; Cinti, 2015) which introduced the concept of anchoring in isometric position (relatively isometric) but also the anchor through bone anchors (Guenego et al., 2007; Hulse et al., 2011; Choate et al., 2012; Rask et al., 2013; Citi, 2015).

Efforts to identify the ideal material for suture when lateral fabellar suture is applied were materialized by the dethronement of nylon wires as the main option (Caporn and Roe, 1996; Lewis et al., 1997; Sicard et al., 2002; Sicard et al., 2012; Ledecky et al., 2012; Igna et al., 2014) and by promoting polyethylene wires which are stronger, stiffer and elongate less than nylon leader (Burgess et al., 2010; Tonks et al., 2010; Choate et al., 2012), promising options offers the polyblend wires (Rose et al., 2012) and braided polyester (Guenego et al., 2007).

Securing of the suture reveal the existence of three systems: a square knot (SQ), a slip knot (SL) and a crimp clamp (CR).

Existing data show no new information being maintained the recommendation (Nwadike et

al.,1998) "*that 27-kgt nylon leader line be secured with a SL, and 27-kgt nylon fishing line be secured with a SQ*" as the "*clamping the first throw of a square knot in monofilament nylon leader material who increases failure load by two percent and stiffness by 16%, and decreases elongation by 12%*" (Caporn and Roe, 1996) although there are studies showing that "*crimping suture alters the biomechanical properties of the loop*" (Burgess et al., 2010). Securing the suture through CR remains a superior method of knotting techniques (Anderson et al., 1998; Vianna et al., 2006) and the *wave pattern crimp system* is more efficient then the *single crimp system* (Maritato et al., 2012). Using tensioning sutures systems before the applying of a crimp clamp does not bring significant advantages over manual tightening (Moores et al., 2006).

Difficulties in various procedures execution are reported to be the bone tunnels creation in TR and anchoring around fabella in LSF and pLSF (Biskup and Griffon, 2014).

Evaluation of extracapsular therapeutic methods efficiency although it is the subject of several studies (Moore et al., 1995; Jevens et al., 1996; Caporn and Roe, 1996; Anderson et al., 1998; Budsberg et al., 1998; Conzemius et al., 2005; Guenego et al., 2007; Kunkel et al., 2009; Cook et al., 2010; Hulse et al., 2011; Havig et al., 2007; Schaijk, 2008) with mostly positive reports, many of these studies are subjective (level 2 and 3). Studies based on analysis of data obtained through force plate measurements show that peak vertical force was 93% and vertical impulse was 96% of normal values in the limbs of dogs that had extra-articular stabilization at six months following surgery (Conzemius et al., 2005), recorded differences being insignificant compared to normal preoperative values in all studies which appeared before 2006 (Jevens et al., 1996; Budsberg et al., 1998) and after (Havig et al., 2007; Schaijk et al., 2008).

Postoperative complications reported after the application of extra-articular stabilization techniques are between 4.2 and 17.4% (Casale and McCarthy, 2009; Frey et al., 2010) and a 7.2% of them required reintervention (Casale and McCarthy, 2009).

Proximal tibial osteotomy techniques include: tibial plateau levelling osteotomy - TPLO, combined tibial plateau levelling osteotomy and tibial tuberosity transposition (TPLO-TTT), tibial tuberosity advancement – TTA with the variants TTA-1, TTA-2 and TTA-rapid, triple tibial osteotomy - TTO and modified Maquet procedure – MMP.

All procedures impart primarily change the biomechanics of the stifle and required specialized and custom equipments. The choice of source of this equipment depend of surgeon preferences or/and their affiliated a product companies (Igna, 2013).

Recent assessments of the effectiveness of therapeutic methods of tibial osteotomy reveals unanimously that locomotor function of the limb with CrCL insufficiency can be improved using the techniques of tibial osteotomy (Bruce et al., 2007; Kim et al., 2008; Dymond et al., 2010; Schaijk, 2008; Christopher et al., 2013; Nelson et al., 2013) described so far (Slocum and Devine, 1984, 1993; Slocum, 1996; Leonard et al., 2016; Montavon et al., 2002; Maquet, 1976; Samoy et al., 2014; ***, 2012; Damur et al., 2003).

More prospective and retrospective studies (Pacchiana et al., 2003; McCarthy, 2002; Priddy et al., 2003 ; Carrey et al., 2005; Staufer et al., 2006; Bruce et al., 2007; Haaland and Sjöström, 2007; Lafavere et al., 2007 ; Stein et al., 2008; Voss et al., 2008; Duerr et al., 2008; Proot and Cooke, 2009; Moles et al., 2009; Dymond et al., 2010; Fitzpatrick and Solano, 2010; Frey et al., 2010; Conkling et al., 2010 ; Imholt et al., 2011; Taylor et al., 2011; Thompson et al., 2011; Gatineau et al., 2011; Coletti et al., 2011; Steinberg et al., 2011; Etchepareborde, 2011; Hishenson et al., 2012; Rotherford et al., 2012; Wolf et al., 2012; Christopher et al., 2013; Etchepareborde, 2014; Butterworth and Kydd, 2017) reports one or more complications (osteomyelitis, incisional infections, fractures of the tibia or fibula, broken drill bits, hemorrhage, intra-articular implant placement, intra-osteotomy screw placement, retained surgical sponges, broken holding pins or screws, septic arthritis, loose implants, draining tracts, ring sequestrum, incisional inflammation, dehiscence and swelling, osteomyelitis, oedema and seroma formation, bruising, premature staple removal, patellar tendon swelling, and late meniscal

injury) after proximal tibial osteotomy procedures.
In TPLO postoperative complication rate, until 2006, ranged between 45.7 and 28% (Carrey et al., 2005; Pacchiana et al., 2003; McCarthy, 2002; Priddy et al. 2003) compared to 22.2-8.4% after 2006 (Staufer et al., 2006; Stein et al., 2008 ; Duerr et al., 2008 ; Imholt et al., 2011 ; Fitzpatrik and Solano, 2010; Frey et al., 2010; Conkling et al., 2010; Gatineau et al. 2011, Coletti et al., 2011) and 4.8% of the cases requiring implant removal (Thompson et al., 2011).
In TTA, the method introduced in practice in 2002 (Montavon et al., 2002), postoperative complication rate was between 35.5% and 11% (Lafavere et al., 2007 ; Voss et al., 2008; Hurt et al., 2009 ; Dymond et al., 2010, Steinberg et al., 2011, Hirshenson et al., 2012; Wolf et al., 2012) with 5.2% reinterventions (Wolf et al., 2012).
In TTO postoperative complication rate was between 18% and 23% (Renwick et al., 2009; Moles et al., 2009).
For MMP two complications were documented (subsequent meniscal injury) from a series of 12 cases (Etchepareborde et al., 2011; Etchepareborde, 2014) and 10.8% postoperative complications with 3.1% reintervention in a series of 65 cases (Bruce et al., 2007).
Comparative analysis of the obtained data (2006-2007) which assess the therapeutic efficiency by force plate measurements or kinematic data between extra-articular stabilization methods and tibial osteotomy methods (Schaijk, 2008; Nelson et al., 2012; Boddeker et al., 2012; Mols et al., 2014; Berger et al., 2015) as well as between different methods of tibial osteotomy (Lee et al., 2007; de Medeiros et al., 2011; Rey et al., 2014) does not show significant differences between methods and no major changes compared to previous reports.
Non-surgical treatment methods include administration of non-steroidal anti-inflammatory drugs, weight control, restriction of spontaneous locomotion, physiotherapy including hydrotherapy (Baker and Bake, 2013; Comerford et al., 2013; Wuchereria et al., 2013). These methods are usually applicable to small dogs with a body weight below 15 kg (Comerford et al., 2013).
In the treatment of obese dogs with ruptured CrCL surgical methods had a success rate (was defined as an affected limb net ground reaction force > 85% of the value for healthy dogs and a ≥ 10% improvement of the initial values) at 52 weeks after surgery by 75% compared to 63.6% in those treated by non-surgical methods (Wuchereria et al., 2013). The data presented are similar to those of previous studies, based only on clinical examination and with a success rate of 85.7% reported for small dogs with body weight below 15 kg (Vasseur, 1984).
Latest data concerning non-surgical methods of treatment (Baker and Bake, 2013; Comerford et al., 2013; Wuchereria et al., 2013) and veterinarians options for these therapeutic modalities (Comerford et al., 2013) did not show a change in trend compared to previous reports (Korvick et al., 1994), the majority of doctors preferring surgical approaches.

CONCLUSIONS

Currently available data does not allow accurate comparisons between different treatment procedures of cruciate cranial deficiency in dogs.
New long-term clinical studies must designed and further biomechanical and kinematic analyses are required to determine the optimal technique, and whether these procedures are superior to other stabilization methods.

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CLINICAL STUDY AND PATHOLOGICAL FINDINGS ON BABESIOSIS IN DOGS, ON SEASIDE OF ROMANIA

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Abstract

Canine babesiosis is a protozoan tick-borne disease affecting dogs worldwide. Knowledge on the prevalence and clinico-pathological aspects of Babesia species infecting dogs is of epidemiological and veterinary medical interest. Here we describe twenty cases of clinical babesiosis diagnosed in dogs, between March-June 2016, in a Veterinary Clinic located in the seaside (Dobrogea region) of Romania. Dogs with clinical signs compatible for babesiosis and positive when tested for the presence of intraerytrocytic protozoan parasites using the blood smear method were included in the study. Of the total animals, the most affected were adults (over 8 years of age); dogs of Bichon Maltese breed and male animals appear in higher numbers. The clinical presentation, pathological findings and therapeutic approaches are discussed in three clinical groups: mild, moderate, and severe babesiosis. Group one comprises dogs (n=9) with mild babesiosis characterized by lethargy, anorexia, fever without major changes in the hematological parameters; no other organ pathology. Dogs (n=5) with moderate babesiosis showed at least one change in the hematological (anaemia, thrombocytopenia, lymphopenia, neutropenia/neutrophilia, leukocytosis) and/or biochemical (elevated levels of liver enzymes, blood urea nitrogen, and creatinine, low albuminemia) parameters, reflecting an organ disorder. The third group included dogs (n=6) which developed complications associated with babesiosis, presenting at least two of the following complications: acute renal failure (n=3), hepatopathy (n=4), pancreatitis (n=2), acute respiratory distress syndrome (n=1). All animals were treated with imidocarb. Additionally, for dogs with moderate and severe babesiosis, a symptomatic treatment (intravenous fluid therapy, antiinflamatory, antypiretic, antiemetic, antispastic, procoagulant drugs) was administrated, while four dogs with severe anemia (PCV: 14 -27.57%) needed blood transfusion, too. The recovery rate (100%, 100%, and 50%, respectively) for the three clinical groups and mortality rate (0, 0, and 50%, respectively) revealed that a successful treatment is depending on the severity of diseases and the individual response of the host.

Key words: *clinical signs, pathology, therapy, babesiosis, dogs.*

INTRODUCTION

Babesiosis is an important tick-borne zoonotic diseases caused by intraerytrocytic protozoan species of the genus *Babesia* (Berger and Marr 2006; Uilenberg, 1995). These parasites usually affect vertebrate animals and are transmitted by various species of ticks. In dogs, infection with these hemoparasites leads to a wide range of clinical signs, of subclinical disease up to serious illnesses characterized by fever, jaundice, splenomegaly, weakness and collapse associated with intra- and extravascular hemolysis, hypoxia, systemic inflammatory response, thrombocytopenia and haemoglobinuria (Jacobson, 2006).

Traditionally, babesiosis in dogs is diagnosed based on the morphology of the intraerythrocytic piroplasm merozoites observed by microscopic examination in peripheral blood smears. By this method, the piroplasms can be classified as large (e.g. *Babesia canis*,) or small forms (*Babesia gibsoni*). However, currently, the large piroplasm forms previously considered to be *B. canis*, include *B. canis*, *Babesia rossi* and *Babesia vogeli* as distinct species (Carret et al. 1999).

Despite of their identical morphology, although they have significant differences in their clinical presentation, geographical distribution and vector specificity (Uilenberg, 1996).

Clinically, canine babesiosis can evolve in various forms from subclinical to super-acute. The severity of clinical signs and therefore the lesion framework depends on several factors such as species of the causative agent, immune status of the host and the existence of inter-

current illness (Irwin, 2009; Solano-Gallego and Baneth, 2011).
For example *B. rossi*, the dominant species in South Africa, is very virulent, with highly acute clinical evolution.
It is believed that the clinical signs are due to tissue hypoxia caused by anemia and systemic inflammatory response (Lobetti, 2006).
Anemia pathogenesis is not fully understood, intra- and extravascular hemolysis could be a cause, on the one hand and weak bone marrow response on the other hand. Mortality in the case of infection with *B. rossi* reaches up to 12% and 1% in the case of infection with *B. vögeli* (Lobetti, 2006).
Cases of babesiosis in dogs have been reported in different parts of the world. Global incidence of clinical babesiosis in dog is of 0.7% with variations depending on the countries and regions (Lenaig et al., 2014).
The status of the endemic country for *B. canis* was assigned to countries such as France, Spain, Hungary, Scotland, Germany, Belgium, Netherlands (Jacobson, 2006); for endemic *B. vögeli* are considered: the USA, Japan, Australia, South Africa, Brazil and for *B. gibsoni*: the USA, North East of Africa, Asia, Australia, Hungary, Italy (Jacobson, 2006).
In Romania, canine babesiosis is registering an increased prevalence (Imre et al., 2010; Ionita et al., 2012), particularly in the areas where in the last several years an increase in the tick population has been reported (Ioniţă and Mitrea 2003; Ionita et al. 2010).
Knowledge on the prevalence and clinico-pathological aspects of *Babesia* species infecting dogs is of epidemiological and veterinary medical interest.
Therefore, the aim of this study is to describe the clinical presentation and laboratory abnormalities of canine babesiosis, assessing its implications in the pathology of other organs and in the effectiveness of the babesiicid treatment.

MATERIALS AND METHODS

Within the study, there are included twenty dogs diagnosed with babesiosis, during March-June 2016, in a veterinary clinic for pets, located in the Dobrogea area (Constanta city) (table 1).
Suspicion of babesiosis was established based on clinical examination, respectively animals that displayed the following symptoms: febrile syndrome, anaemic mucous, jaundice, lethargy, anorexia, vomiting, haemoglobinuria, petechiae on gingival mucosa.
To confirm the diagnosis, blood samples have been collected for haematological (blood counts, smear) and specific biochemical (glucose-Glu, amylase-AMY, Glutamate oxaloacetic transaminase-GOT, Glutamate pyruvate transaminase-GPT, creatinine-CRE, blood urea nitrogen -BUN, total-billirubin- T-BIL, Ca, lactatdehidrogenase-LDH, alkaline phosphatise-ALP, albumine-Alb) parameters.
Blood analysis was performed using the Abacus Vet Jr. haemo-analyzer.
Biochemical determinations were carried out with the SPOTCHEM EZ SP-4430 device.
Thin blood smears were prepared, stained using a Dia-quick Panoptic kit and subsequently examined by light microscopy at 1,000x for detection of intraerytrocytic piroplasms) (Ionita and Mitrea, 2013).
In addition to haematological and biochemical analysis, rapid tests were used to detect the presence of other haemoparasites (4Dx IDEXX SNAP Test) or check the exocrine pancreatic function (CPL IDEXX) or exclude the presence of viral diseases (CDV Test).
The final diagnosis of babesiosis was established by the correlation of clinical signs, haematological analysis and identification of intraerytrocytic forms of *Babesia* spp. (Ionita and Mitrea, 2013).
Consequently to laboratory tests and clinical evolution, animals diagnosed with babesiosis were divided into 3 groups: mild, medium, and severe form of babesiosis.
Animals have been treated with specific babesiicid drug, but also supporting medications, according to their clinical status.
Anamnesis, clinical observations and the subsequent evolution of the animals were recorded in the database of the hospital.

RESULTS AND DISCUSSIONS

The clinical study, carried out on a total number of 20 dogs of different breeds, aged between 1 and 10 years old. All the animals had an owner and they originated from the

urban area of Dobrogea region, respectively: Constanta (n=16), Tulcea (n=3) and Cogealac (n=1) (table 1).

Table 1. General data on the dogs diagnosed with clinical babesiosis, included for the clinical follow-up

Nr. crt.	Place of origin (city)	Breed	Age (years)	Gender	Clinical form of babesiosis
1.	Constanta	Maltese Bichon	8	F	mild
2.	Constanta	Maltese Bichon	8	M	moderate
3.	Constanta	Maltese Bichon	1	M	moderate
4.	Constanta	Maltese Bichon	3	F	moderate
5.	Constanta	Maltese Bichon	6	M	severe
6.	Constanta	Maltese Bichon	1	M	mild
7.	Constanta	Maltese Bichon	2	F	mild
8.	Constanta	Maltese Bichon	4	F	moderate
9.	Constanta	Golden Retriver	1	F	mild
10.	Constanta	Golden Retriver	2	M	mild
11.	Tulcea	Metis	8	F	severe
12.	Constanta	Labrador Retriever	9	F	mild
13.	Constanta	Beasle	7	M	mild
14.	Cogealac	Shepherd of Central Asia	2	F	severe
15.	Tulcea	Carpathian Shepherd	3	M	severe
16.	Constanta	Metis	2	M	moderate
17.	Constanta	Labrador Retriever	8	M	mild
18.	Constanta	Rottweiller	6	M	mild
19.	Tulcea	Metis	10	M	severe
20.	Constanta	Alaskan Malamute	1	M	severe

Consecutively to clinical examination and laboratory tests, animals diagnosed with babesiosis have been divided into 3 groups: group 1- mild, group 2 - moderate, and group 3- severe babesiosis.

In the group 1, consisting of 9 animals who displayed mild forms of babesiosis, were included those animals showing on clinical examination at least one of the following clinical signs: lethargy, anorexia, fever, but without major changes on the haematological and biochemical parameters.

Dogs in the second group, with moderate forms of babesiosis (n = 5), in addition to clinical signs described in the group 1, had pronounced abnormalities in the blood and biochemical parameters, respectively: thrombocytopenia, lymphopenia, neutropenia/neutrophils, leukocytosis; increasing glucose, pancreatic amylase, creatinine, glutamate pyruvate transaminase and aspartate aminotransferase, and decreased albumin (Table 2, 3).

The third group includes animals who have presented severe form of the disease characterized by complications associated with babesiosis: renal failure (n=3), liver disease (n=4), pancreatitis (n=2) and respiratory failure (n=1).

Table 2. Haematological abnormalities in dogs (number; %) with different clinical forms of babesiosis

Haematological abnormalitiies	Dogs (n=20)		
	(n)	(%)	ND
Neutropenia	4	20.0	0
Neutrophilia	4	20.0	0
Lymphocytopenia	13	68.4	1
Granulocytosis	4	20.0	0
Mild thrombocytopenia (PLT > 70)	2	10.0	0
Moderate thrombocytopenia (PLT < 70)	4	20.0	0
Severe thrombocytopenia (PLT = 0)	14	70.0	0
Non-regenerative anaemia	5	25.0	0
Regenerative anaemia	2	10.0	0

Most dogs have been diagnosed with babesiosis in the beginning of the warm season (March-April) which shows the correlation between the appearance and the increased activity of vectors that transmit the parasite and the number of cases recorded (Ionita et al., 2010).

The majority of cases was registered in March (n = 8) with a small decrease in April (n= 5) and May (n= 5), then a significant reduction in June (n = 2).

In terms of gender, we have find that males have held the majority of new cases diagnosed with a prevalence of 60% (n=12) whereas females accounted for 40% (n= 8) of the total cases diagnosed with babesiosis.

In terms of clinical forms of babesiosis manifested, the mild form was registered in 5 males (55%) and 4 females (45%), the moderate form in 3 males (60%) and 2 females (40%) and the severe form in 3 males (50%) and 3 females (50%).

As the age at which animals have been diagnosed with babesiosis, the occurrence average is of 4.5 years old; the highest incidence was registered in dogs of ≥8 years old- 30% (n = 6), the rest were animals with age varying from 1 to 7 years old.

In terms of breed, high rate of infection (40%) occurred in the Maltese Bichon breed (n = 8).

As clinical manifestations, the febrile syndrome was recorded in all 20 dogs on their first consultation. Clinical manifestation such as vomiting was seen in 5 animals (25%), haemoglobinuria was detected in 3 (05%), pale mucous membrane was recorded on 19 (95%) and jaundice in a single dog (5%).

From the haematological point of view, dogs diagnosed with babesiosis presented neutrophilya / neutropenia, granulocytopenia, lymphocytopenia, thrombocytopenia of different degrees, as well as in the moderate and severe cases we have ascertained the presence of non-regenerative anemia (Table 2). In some dogs (n=2), severe anemia (PCV: 14 - 27.57) was registered.

According to biochemical analysis, within the classified groups there could be medium and severe cases, we have recorded overall increases in hepatic transaminases levels (GOT / GPT), pancreatic amylase, creatinine, BUN, total bilirubin, alkaline phosphatase, lactate dehydrogenase and glucose (Table 3, 4).

Table 3. Biochemical changes recorded in dogs who presented a moderate form of babesiosis

Biochemical parameter	No. of dogs in which it was determined	High values (n)	Low values (n)
Glu (glucose)	3	1	0
ALP (alkaline phosphatase)	1	1	0
GPT (glutamate pyruvate transaminase)	4	3	0
CRE (creatinine)	4	2	0
Amy (amylase)	2	1	0
T-Bil (total bilirubin)	3	1	0
GOT (glutamate oxaloacetic transaminase	3	2	0
Alb (albumin)	2	0	2
LDH (lactat dehydrogenase)	1	1	0

Table 4. Biochemical changes recorded in dogs with a severe form of babesiosis

Biochemical parameter	No. of dogs in which it was determined	High values (n)
BUN (blood urea nitrogen)	5	3
ALP (alkaline phosphatase)	1	1
GPT (glutamate pyruvate transaminase)	4	4
CRE (creatinine)	6	3
AMY (amylase)	5	3
T-Bill (total bilirubin)	4	2
GOT(glutamate oxaloacetic transaminase)	6	2
Albumine	1	1

Treatment was based on two main objectives: stabilizing the patient and babesiicid medication administration at the right time in terms of clinical status of the patients. Treatments were performed generally in outpatient and only 4 dogs have been admitted, of which 2 have died.

All animals were treated with imidocarb (dose of 4.25 mg/kg). In addition, to dogs with mild and moderate forms of babesiosis, a symptomatic treatment was administered (intravenous fluids, anti-inflammatory and antipyretic medications, antispasmodics and procoagulant medications) (Table 5).

Table 5. Therapeutical approaches (medications) used for the symptomatic and etiologic treatment of babesiosis

Drug / Medication	Effect	Dosage and administration
NaCl 0,09%	Rehydration, correction of electrolyte balance	l0-20ml/kg iv
Glucose 5%	Energetic support	5ml/kg iv
Lactated Ringer	Acidosis, uremia	5ml/kg iv
Duphalyte	Support of amino acids, energetic substrate	5ml/kg iv
Sodium Metamizol	Anti-pyretic	50 mg/kg iv, im
Drotaverine hydrochloride	Anti-spasmodic	1-2 ml sc. Im at 12-24 hours
Metoclopramide	Anti-vomiting	0.2-0.5 mg/kg sc at 6-8 hours
Maropitant	Central anti- vomiting	1 mg/kg sc. once a day
Ranitidine	Antacid	2 mg/kg iv 8-12 hours
Vitamin B Complex	Support	1-2 ml sc./ im
Vitamin C	Antioxidant, vascular tonic	0,5-1 g iv
Epinephrine	Cardiac arrest resuscitation	0.01 -0.02 mg/kg initially and if an appropriate response is not obtained, was increased up to 0.1-0.2 mg/kg iv
Pancreatic enzymes	Digestion maintenance after an episode of pancreatitis	according to the prospectus
Sylimarina	Hepatoprotector	according to the prospectus
Imidocarb Dipropionate (imizol solution 12%)	Babesiicid	6.05 mg/kg boosted 14 days

Furthermore, dogs who have shown severe anemia (PCV: 14 - 27.57) blood transfusion was required. The process of blood transfusion was carried out in 4 dogs (20%) of the patients which showed a severe form of babesiosis. Besides these clinical findings such as pale mucous membranes / jaundice, tachycardia,

tachypnea, they have also showed decreasing values of RBC, HGB, HCT at the haematological examination (RBC<3.85 $x10^{12}$/1; HGB < 9.2 mg/dI; HCT < 27.57%).
The recovery rate was of 100% in dogs with a mild form and moderate babesiosis and 50% in dogs with severe babesiosis.
The mortality rate for dogs who have manifested mild and moderate babesiosis and 50% for patients with a serious form suggests that successful treatment depends on the severity of the disease and on the individual response of the host.

DISCUSSIONS

In Romania, dog babesiosis has become a quite common parasitic disease in recent years, with a variety of clinical signs ranging from mild to moderate signs, from nonspecific clinical signs to collapse and death (Ionita et al., 2011). The severity of clinical babesiosis is characterized by marked hemolytic anemia severe acid-base imbalances (Leisewitz et al., 2001) resulting in kidney failure, liver disease with jaundice, hypoglycemia (Keller et al., 2004), acute respiratory failure, cerebral pathology and immune-mediated hemolysis (Jacobson, 2006). The severity of clinical signs and therefore the lesion framework depends on several factors such as breed causative agent, host immune status and inter-existing diseases (Lobetti, 2006).
According to this study, in Dobrogea, babesiosis in dogs presents an increased incidence in the period of March-June months, with a different aspect than the incidence described in Hungary (Mathe et al., 2006) where the authors observed an increased incidence in the summer-autumn months.
The haematological framework as well as other clinical changes were similar to those described in the literature (Duh et al., 2004; Furanello et al., 2005; Gopequi et al., 2007).
Among complications, liver failure, renal failure and pancreatitis were the most common deviations that we have found in dogs infected with *Babesia*. Liver failure in most cases was mild thus it did not even affect the course of therapy and recovery. Jaundice occurred in a single patient was not caused by the suffering of hepatocytes but it was determined by hemolysis.
Renal failure occurred in 5 dogs; in two of them an increased pancreatic amylase was also observed. As far as pathology is concerned, kidney failure is a common complication in dogs with babesiosis. Its gravity is directly responsible for the clinical course and survival rate (Mathe et al., 2006). In the study carried out on the pathogenesis of canine babesiosis in Hungary, the authors have established a threshold limit of creatinine in terms of survival rate (CRE = 275 μmol/l). In our study, a dog had an increased creatinine of 12.4 mg / dl (1096.41 μmol/l) while at another it was of 10.3 mg/dl (910.73 μmol/l). In these two patients, the major importance for survival was given by age and by inter-current pathologies, thus the individual with higher levels of creatinine survived while the other not, which demonstrates the relationship between clinical babesiosis and inter-current pathologies.
As a therapeutic babesiicid option, we have used imidocarb dipropionate with very good results, as suggested by other studies (Mathe et al., 2006; Uilenberg et al., 1981, Solano Gallego, 2008).

CONCLUSIONS

According to this study, babesiosis can have different types of clinical evolution, depending on different factors.
Clinical manifestations were represented by fever, anorexia, anemia / jaundice, haemo-globinuria, prostrate condition, muscle weakness. As babesiosis involvement in the pathogenesis of other organs, we have noticed alterations in the liver, renal and pancreatic function.
Treatment success was largely depended on the pathological form of the disease, on the evolutionary type and presentation time of animals for investigations. Age and inter-current pathologies have played an important role in the evolutionary process of the disease.
We have achieved good therapeutic results using dipropionate imidocarb as babesiicid, associated with supportive symptomatic medication, depending on each case, with a high recovery success.

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MFK PROVIDES ANALGESIA AND CARDIOVASCULAR STABILITY DURING LAPAROSCOPIC CHOLECYSTECTOMY IN SWINES

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Abstract

Laparoscopic surgery gained a lot of field in human and veterinary medicine, replacing successfully many invasive surgical techniques. Laparoscopic cholecystectomy is a reality and units must convert from open to endoscopic technique. To this end we have used the pig, whose biliary anatomy resembles the human, as a laboratory in vivo training model. This aspect has led to imagining and establishing cholecystectomy techniques in pigs that were subsequently applied to humans. We conducted the current study to address the need for a systematic investigation of anaesthetic and analgesic protocols in Landrace pigs. The goal of this study was to evaluate a partial intravenous anaesthetic (PIVA) protocol for Landrace pigs that yielded sufficient sedation for peripheral vascular catheterization, analgesia and miorelaxation for laparoscopic techniques and would have minimal cardiovascular effects while being safe for the patient. The study was carried out during several training session for surgeons on laparoscopic techniques. Sixteen pigs (weight between 15 and 30 kg, with a mean weight of 21 kg) were anaesthetized for the procedures. Pigs were randomly allocated to one of the following groups: MFK group (receiving midazolam 0.2 mg/kg/h, fentanyl 10µg/kg/h and ketamine 10µg/kg/min) or KL group (receiving ketamine 10 µg/kg/min and lidocaine 30µg/kg/min). Heart rate, respiratory rate, blood pressure and saturation of oxygen were monitored throughout the anaesthesia and recorded every 5 minutes using a vital signs monitor. Muscle relaxation was appreciated using a subjective scale. Midazolam-fentanyl-ketamine provided better analgesia and muscle relaxation, with minimal cardio-vascular effects compared to the KL protocol. Both protocols can be used in swine and ensure stable cardiovascular parameters during general anaesthesia in this species.

Key words*: laparoscopy, cholecystectomy, midazolam-fentanyl-ketamine, anesthesia*

INTRODUCTION

Laparoscopic surgery gained a lot of field in human and veterinary medicine, replacing successfully many invasive surgical techniques.

Laparoscopic cholecystectomy decreases postoperative pain, hence reducing the need for postoperative analgesia, shortens the hospital stay from 1 week to less than 24 hours, and returns the patient to full activity within 1 week (compared with 1 month after open cholecystectomy) (Tanase et al. 2015). If such techniques become standard, as seems likely, it will be necessary to develop safe and realistic learning and training models (Draghici et al. 2014).

The swine biliary tract closely resembles the human one, making this species a suitable anatomical model (J Sˇ tembı´rek et al. 2012; Kirwan et al. 2001).

Swine (*Sus scrofa*) are also common models for cardiovascular injury and intervention that largely have replaced traditional canine cardiology models (swine have similar coronary artery distribution and effective collateralized blood flow to the myocardium after coronary artery blockage) (Jan R Linkenhoker et al. 2010).

Anaesthesia and analgesia is frequently required for swine in research due to their use as preclinical models (translational research). Selection of an appropriate protocol which considers the physiologic effects of the pharmacologic agents for anesthesia/analgesia is an important aspect of designing an experiment (Smith AC et al. 2008). However, pigs are difficult to restrain and anaesthetize due to their size, temperament and resistance to

sedative drug combinations, including those with morphine (Kaiser 2006). Xylazine is an α2 agonist used for sedation of pigs. It is short acting, so it is suitable for continuous rate infusion (CRI) administration. It also has analgesic properties, provides miorelaxation, but the main side effects are related to cardiovascular depression (bradycardia and transitory hypertension), hyperglycemia and increased diuresis (Grimm et al. 2015).

Ketamine is a dissociative anaesthetic agent with a wide range of safety in swine (11-33 mg/kg IV, IM or SC). It is generally an effective restraint agent, safe cardio-respiratory (sympathomimetic effect) and analgesic (antagonist action on the N-methyl-D-aspartate (NMDA) receptors), but does not provide miorelaxation (Ajadi et al. 2008). It is best used in combination with other agents (Swindle MM 2015). Ketamine (20 mg/kg) with xylazine (2 mg/kg) has been recommended as a general anaesthetic protocol in swine for short procedures (Riebold TW et al. 1995). The addition of another analgesic agent and/or general anaesthetic is necessary to perform invasive surgery (Snjezana Golubovi 2009).

Propofol is a sedative hypnotic agent used for induction of anesthesia in pigs due to its rapid onset of action and short elimination time. It can also be used in coinduction protocols with midazolam or ketamine. Doses of 4-5 mg propofol / kg IV in pigs that were mildly to moderately sedated and 1-3 mg propofol / kg IV in deeply sedated pigs have been used. It does not have analgesic properties and produces cardiorespiratory depression .

Fentanyl is a short acting, full μ opioid agonist. It provides analgesia, but has respiratory and cardiovascular depressant effects (increased $PaCO_2$, bradycardia), reduces gastro-intestinal motility and patients can develop tolerance or hyperalgesia following its administration especialy at high doses (Simones et al. 2016). Intravenous administration of fentanyl at 5-30 μg/kg/h in combination with paralytic agents, ketamine, and nitrous oxide has been used successfully in cardiac surgery in swine (Pascal et al. 2015).

Benzodiazepine tranquilizers are effective sedatives and central acting miorelaxants, with minimal to negligible effect on the cardiovascular and respiratory functions. Midazolam has effects lasting less than 30 minutes and may be used on a daily basis for procedures requiring sedation (Lacoste et al. 2000).

Lidocaine, a sodium channel blocker used as local anaesthetic, can be administered as a CRI to provide analgesia (Valverde et al. 2004). It causes vasodilation, has antiarrhythmic effects and gastrointestinal prokinetic properties.

The goal of this study was to evaluate a partial intravenous anaesthetic (PIVA) protocol for Landrace pigs that would yield analgesia and miorelaxation for laparoscopic techniques.

MATERIALS AND METHODS

The study was carried out during several training session for surgeons on laparoscopic techniques between 2013 and 2015. The study received ethical approval from the Ethical committee of the University of Medicine and Pharmacology Carol Davila of Bucharest.

Sixteen pigs (weight between 15 and 30 kg, with a mean weight of 21 kg) were anaesthetized for the procedures. They were housed together 24h before the procedures with free access to water and food. Food was withheld 12h before the anaesthesia started. To minimise the stress, animals were premedicated prior to being transfered to the operating room. Animals arrived sedated and were carefully protected against hypothermia by insulating with warm water filled mattress. All the patients were premedicated with xylazine 2 mg/kg and ketamine 20 mg/kg im (in the neck area). Once the pig was profoundly sedated, vascular access was secured by placing a catheter 20 – 22G in the auricular veins. All patients were preoxygenated before induction for minimum 2 minutes. Induction was done with propofol 4 mg/kg IV up to effect. Larynx was sprayed with lidocaine 2% in order to reduce the incidence of laryngospasm (ketamine maintains this reflex). Once orotracheal intubation was achieved in sternal recumbency, the PVC endotracheal tube was connected to a circle breathing system and anaesthesia was maintained with isoflurane 1.2–1.5% in oxygen. Manual ventilation was provided when dyspnea or apnea were noted. All animals had an an intravenous infusion of Ringer lactate set at 5 ml/kg/h started immediately after the orotracheal intubation.

For the laparoscopic technique, abdominal insufflation of carbon dioxide is mandatory in order to allow proper visualization and manipulation of body tissues and instruments (Fig 1). Abdominal pressure of insufflated carbon dioxide was maintained at 6-8 mmHg.

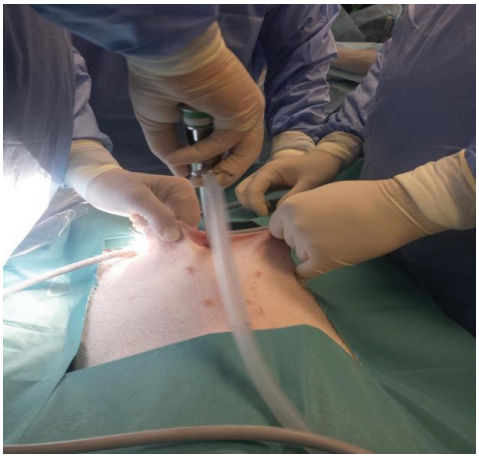

Figure 1. Abdominal insufflation with carbon dioxide

Pigs were randomly allocated to one of the following groups: MFK group (receiving midazolam 0.2 mg/kg/h, fentanyl 10 µg/kg/h and ketamine 10 µg/kg/min) (n=8) or KL group (receiving ketamine 10 µg/kg/min and lidocaine 30 µg/kg/min) (n=8).
Heart rate, respiratory rate, oscillometric blood pressure, rectal temperature and saturation of oxygen were monitored throughout the anaesthesia and recorded every 5 minutes using a vital signs monitor (Mindray MEC-1200 VET®). (Fig.2)

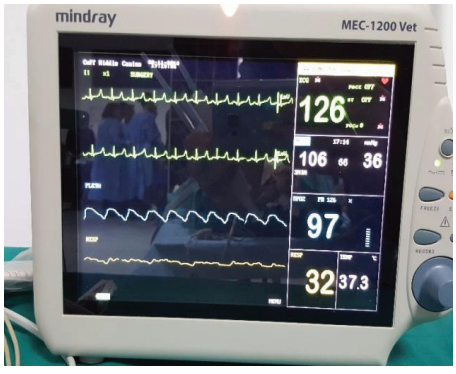

Figure 2. Vital signs monitor Mindray MEC-1200 VET®

Rescue analgesia was provided with ketamine 5 mg/kg IV if heart rate and systolic blood pressure increased by 20% compared to the baseline.
The surgeons were asked to assess subjectively the degree of miorelaxation during the procedure using a 1 to 5 scale were 1 was no miorelaxation and 5 – maximum degree of miorelaxation.
The duration of anaesthesia ranged between 210 and 240 minutes.
All patients were euthanized at the end of the procedures using 7.5% potassium chloride.
Statistical analysis used a one way ANOVA and the results are expressed in mean ± standard deviation.
A value of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSIONS

Administration of ketamine 20 mg/kg and xylazine 2 mg/kg resulted in adequate sedation of the pigs. Spontaneous breathing was maintained. Vascular access was obtained easily in the auricular vein. Pigs were transported from the housing den to the experimental lab without incidents. This was a 5 minutes distance trip. The ketamine doses reported previously for swine cover a wide interval (11 – 33 mg/kg IV or IM). We chose an intermediate dose taking into account the addition of an α2 agonist that provides sedation itself (xylazine).
After the bolus injection of propofol, followed by lidocaine sprayed on the larynx, laryngeal and pharyngeal reflexes disappeared, and all pigs were intubated without noteworthy complications. Orotracheal intubation was performed with the pig positioned in sternal recumbency, using a stylet and only after a minimum of 2 minutes facial mask preoxygenation (Fig 3).
No complications were encountered for this stage of the protocol and the intubation was achieved in less than 1 minute for all the patients.
This comes into agreement with the results reported by Theisen et al. (2009) that recommended the ventrodorsal position as the first choice for providing a smooth and fast airway in laboratory pigs.

Figure 3. Orotracheal intubation

Mean values for oscillometric blood pressure, heart rate, respiratory rate, rectal temperature and saturation of oxygen are presented in table 1.

Table 1. Mean values of physiological parameters monitored during anaesthesia for the MFK and LK groups and p values calculated between the two groups

Parameter	MFK group	LK group	P value
Sistolic blood pressure (mm Hg)	116.6	98.3	0.007
Mean blood pressure (mmHg)	82	75.6	0.055
Diastolic blood pressure (mm Hg)	61.3	52.3	0.078
Heart rate (bpm)	75.6	95.3	0.022
Respiratory rate (rpm)	5	9	0.017
Temperature (^{o}C)	35.2	35.7	0.63
Saturation of oxygen (%)	98.3	97.5	0.74

There was a significant statistical difference between the two groups for the heart rate and sistolic blood pressure (see table 1).
This parameters are commonly used as indicators of nociception during general anaesthesia. At the same time, we must not forget that they can both be influenced by the drugs administered during the anaesthetic protocol: fentanyl CRI induces bradycardia, while ketamine, midazolam and lidocaine maintain heart rate. The higher heart rate for the LK group should not be interpreted as a sign of nociception for the duration of the whole anaesthesia as this is the mean value calculated for the 210 – 240 minutes of surgical procedure. Instead, the trend in this parameters was monitored. Rescue analgesia was provided when there was a 20% increase in one of the two parameters mentioned above. No gross movement was recorded as response to surgical stimulation. The pigs in group MFK received 9 doses of rescue analgesia, while group LK received 15 ($p = 0.031$). The significant statistical difference in the requirement for rescue analgesia allows us to conclude that MFK provided better analgesia for this type of surgical procedure.
There were no significant statistical difference between the two groups regarding mean blood pressure, diastolic blood pressure, oxygen saturation and temperature values. Episodes of hypotension appeared equally in the two groups, most of the times correlated with surgical maneuvers that elicited vagal reflex. Treatment was cesation of the maneuver for a couple of minutes and if that was not enough to rstore blood pressure values, HES 6% 10 ml/kg in 10 minutes was administered. The colloid boluses were repeated as needed in order to restore a mean blood pressure over 60 mmHg.

Intermittent positive pressure ventilation was needed in 6 animals from the MFK group and in 2 animals in the LK group. This was probably due to depressant effect of fentanyl on the respiratory center, but this effects can be overcome by supplementing ventilation of the patients.
Fentanyl CRI alone or in combination with ketamine and lidocaine has been shown to have a sparing effect on the minimal alveolar concentration (MAC) of isoflurane in dogs (Aguado et al. 2011; Simoes et al. 2016). It is reasonable to assume that the same effect will be seen in swine. Kleine et al. (2015) looked at the effect of midazolam on isoflurane MAC in pigs. They found no effect of midazolam on the isoflurane MAC, but in their study midazolam was administered as a single dose and not as a continuous rate infusion. Lidocaine has a significant sparing effect on the isoflurane MAC in dogs only at higher infusion rates (100 µg/kg/min) than the one we used in our protocol (Aguado et al. 2011). We assume that for the protocols we used the major effects on

MAC would be due to fentanyl and ketamine. We did not measure the MAC in our patients, but we monitored the clinical responses to surgical stimulation and correlated them with the vaporizer setting. For the MFK group, the mean setting for the isoflurane vaporizer was 1.2% and this allowed surgery without increase in heart rate and systolic blood pressure. For the KL group, the mean value setting for the isoflurane vaporizer was 1.4%. There was a strong statistical difference between the 2 groups ($p < 0.001$). This confirms our hypothesis and comes in agreement with the studies mentioned above.

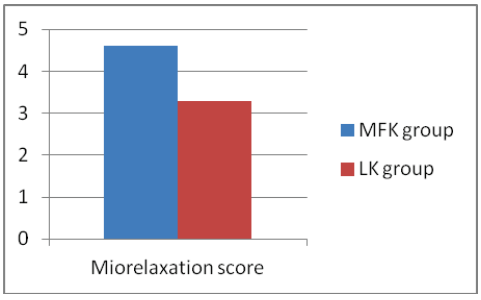

Figure 4. Miorelaxation scores subjectively assessed by the surgeons.

CRI doses for midazolam in pigs were previously reported at 1.14 to 2.71 mg/kg/h. The doses used in this study was reduced to a fifth of the minimal reported one, while ketamine and fentanyl were used at the common previously reported infusion rates (Kaiser et al., 2007). Still , MFK provided good miorelaxation (miorelaxation score 4.7) (Fig.4.). There was a significant statistical difference between the two groups ($p = 0.034$), still both protocols ensured good conditions for surgery. Other studies reported similar results, even after single intramuscular administration of midazolam-ketamine (Clutton et al. 1997).

Our study has several limitations. One is the use of oscillometric blood pressure measurement instead of the invasive arterial measurement that is considered the golden standard for this parameter. This was due to the lack of equipment in the laboratory where the study was conducted. Still, the method is accepted for routine monitoring of the blood pressure and physiological range values were determined for some breeds of swine, including after sedation with midazolam (Goodrich et al. 2001).

The second limitation of our study is the euthanasia of the patients at the end of the surgeries. This did not allow us to assess the quality of recovery and the possible occurrence of life-threatening events at this time. The recovery period is an essential time frame for any anesthesia and patient, also for experimental patients as this can interfere with the results of the experimental protocol and can introduce statistical errors in the results.

The third limitation of our study was the lack of monitoring of the end-tidal expired carbon dioxide ($EtCO_2$). This is required in order to monitor the ventilation of the patient during general anaesthesia. It is used also to indirectly monitor the cardiac function as CO_2 levels are positively correlated to the cardiac output. Also, due to abdominal insufflation of CO_2 during laparoscopic technique, hypercapnia can be encountered. A solution to this would have been the arterial blood gas analysis, but that was also not possible due to the same technical limitations of the laboratory used for the surgical training sessions.

CONCLUSIONS

MFK provided better analgesia and increased miorelaxation during laparoscopic cholecistectomy, while LK provided a better stability in terms of ventilation. Both protocols can be used in swine and ensure stable cardiovascular parameters during general anaesthesia in this species.

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COMPARISON OF BENIGN AND MALIGNANT MAMMARY TUMORS IN DOGS THROUGH RAMAN SPECTROSCOPY: TWO CLINICAL CASES

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Abstract

This paper reports on two clinical cases where Raman spectroscopy has been employed for diagnostic in dog mammary tumour surgery. The comparison between histopathology and SERS results proves that the spectral markers can be used to distinguish between malignant or normal tissue. By comparing results from histopathology and Raman spectroscopy we set up a fast and affordable diagnostic tool using the marker-like Raman bands of carotenoids and respectively interfacial water. Application of the described technique is useful in comparative studies of canine and human mammary cancer.

Keywords: *Raman spectroscopy, markers, mammary tumor, interfacial water, carotenoids*

INTRODUCTION

One of the most frequently diagnosed neoplasm in intact female dogs is the mammary tumor. Canine mammary tumors have greatly increased in recent years, thus demanding rapid diagnosis and effective treatment in order to determine the animal survival (Andrade et al., 2010).
Surgery is the basic treatment of canine mammary tumors and is the most effective for disease regional control (dos Santos Horta et al., 2014).
The aim of the surgery is the complete tumor removal while saving as much healthy tissue as possible. One of the main challenges in mastectomy is to achieve margins free of cancerous cells in order to prevent possible recurrences (Sebastian et al., 2015).
A number of methods are currently used in oncology to assess the surgical margins of tumors. Histopathology is the gold-standard method used in the margin evaluation. Its only drawback stays in being not an intraoperative procedure and consequently in its impossibility to tell in real time whether all cancerous cells have been removed at the time of surgery (Liptak, 2013).
Newly emerging intraoperative diagnostic techniques adding to histopathology encompass Raman Spectroscopy. Raman Spectra contains fingerprints of each molecule in a sample, i.e. band characteristic to their vibration modes (Kneipp et al., 2015).
The spectra results from inelastic scattering of light on the sample. Energy is exchanged between light and samples with energy and momentum conservation. Surface enhanced Raman scattering (SERS) is a special Raman technique that uses metal nanostructured surfaces to amplify the Raman signal (many orders of magnitude). The mechanism relies on surface plasmon resonance. As working principle, the system photon-bio-sample-metal gives the photon sufficient momentum for efficient interaction. Coupling to local waves (a

plasmon is a quantum or quasiparticle associated to local oscillations of charge density) in the metal-biological sample enhances the scattering cross-section thus allowing detection of even single molecules (Micsa et al., 2016). Best metals for SERS are gold and silver.

MATERIALS AND METHODS

An intact 3-year-old German Shepherd female and a sterilized 12-year-old Cocker Spaniel female, were brought in by their owners at the Faculty of Veterinary Medicine Clinic of Bucharest.

Clinical examination revealed in both cases some nodular mammary masses.

The German Shepherd, named Cora, had two tumors, one on the fourth right mammary gland and one on the fifth left mammary gland. The owner discovered the lumps but only after 3 months decided to request a physical examination.

She was also previously diagnosed with Sticker tumor and had been receiving chemotherapy treatment for it.

The Cocker Spaniel, named Kisha, had one tumor, on the second right mammary gland. Kisha was brought 4 months after the owner discovered them.

At the age of 5, she was diagnosed with pyometra and had an ovariohysterectomy.

Both females were sent for thoracic X-rays and their results were the same, their pulmonary X-rays were not showing any visible modifications (Figure 1, 2).

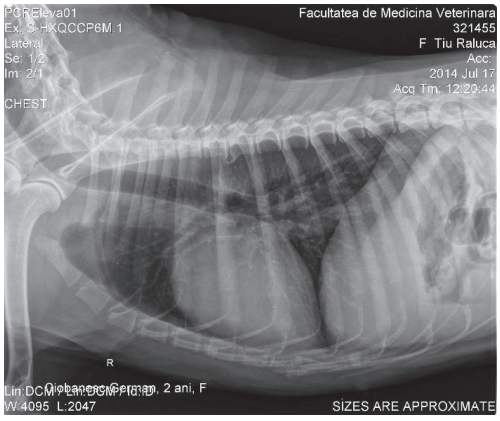

Figure 1. Cora's thoracic X-ray showed no modifications

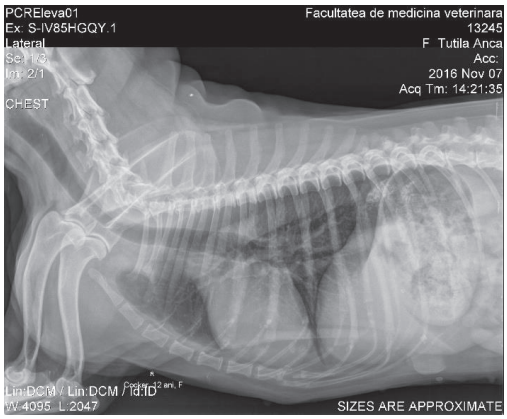

Figure 2. Kisha's thoracic X-ray showed no modifications

The biochemical parameters of both females were in normal limits.

Considering that the test results did not show any changes that could put their lives in danger if they went under anesthesia, they were scheduled for surgery.

The protocols for anesthesia and analgesia were elected in accordance with the ASA status of the patients (ASA2). Cora was premedicated with acepromazine 0,03mg/kg and ketamine 5mg/kg, and Kisha was premedicated with midazolam 0,2 mg/kg and butorphanol 0,2 mg/kg. Both dogs were induced with propofol and maintained with isoflurane gas. Butorphanol 0,2 mg/kg was used for analgesia during the surgery.

The surgeon chose for Cora to remove the last three mammary glands on both sides and for Kisha only the affected mammary gland (Figure 3, 4).

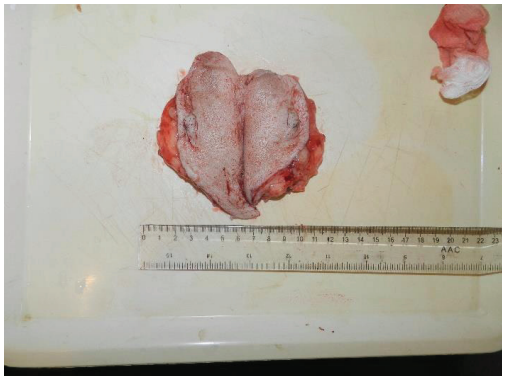

Figure 3. Part of Cora's mammary glands including the tumor

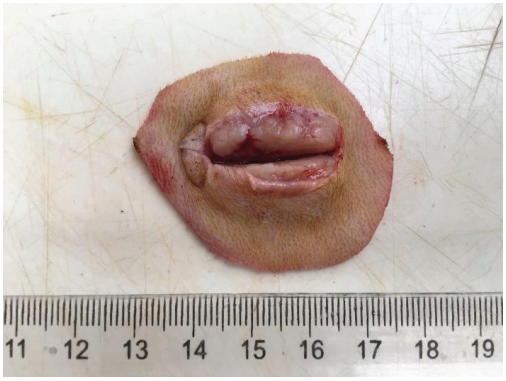

Figure 4. Kisha's mammary tumor

For the incision, a scalpel blade with a gold SERS accessory was used (Figure 5).

Figure 5. Gold SERS accessory attached to blade

The mammary tissue along with the skin were then removed using the electrocautery.
The cutaneous plane was closed by separate sutures in "U" with 2/0 Nylon.
After the mastectomy, the patients received postoperative care and medication. They were given antibiotics and anti-inflammatories. Their bodies were strapped with a piece of cotton sheet for 10 days to prevent the collection of serous fluid. After two weeks their stitches were removed.
The tumors were divided into two parts, one of which was sent for histopathological analysis and the second one was sampled for direct ex vivo (no preparation) Raman exploration.
For histopathology analysis, collected fragments from each mammary tumor were fixed in buffered 10% formalin solution for 24 h. Processing of the samples and paraffin embedding were made automatically by the tissue processor 120-3 Thermo Scientific STP. Onward, blocks were sectioned at 3 µm using Leica microtome RM 125RTS. All slides were stained with hematoxylin eosin using Thermo Scientific Microm HMS 70. The examination of the sections was made with an Olympus BX 41 microscope coupled to an Olympus DP25 video camera.
The SERS spectra were collected using a LABRAM HR 800 Horiba Jobin-Yvon spectrometer with 632nm excitation wavelength.

RESULTS AND DISCUSSION

As other research teams have found using Raman Spectroscopy in humans (Lyng et al., 2007; Surmacki et al., 2015) giving a diagnostic should need some markers (certain peaks from the Raman spectrum). Another recent study shows that a comparative analysis between humans and dogs can be useful (Birtoiu et al., 2016). Using Raman bands of carotenoids and of interfacial water as markers for benign respectively malignant tissues leads to a fast (~60 sec) and low cost diagnostic means (Birtoiu et al., 2015; 2016).
Carotenoids are exclusively present in normal tissue since they are antioxidants (substances that fight against oxidant factors like cancer cells). Their corresponding Raman bands are found in the 1000-1600 cm^{-1} spectral window (e.g. Figure 7 and Figure 9). The Raman band of interfacial water (O-H stretching) is solely found in malignant tumours. This probably shows the trend to fast multiplying of cancer cells with energy waste possibly because they need oxygen to maintain their specific redox status. Cancer cells are very rich in proteins that are hydrophilic.
The full Raman spectra of the two cases presented in this work are shown in Figure 6. Figures 7 and 8 provide a comparison of the two cases in the two regions of interest (carotenoids and interfacial water). In Figure 9 the carotenoid peak is visible, and in Figures 10 and 11, the ~3311 cm^{-1} peak corresponding to interfacial water does not appear at Cora, whereas for Kisha, the carotenoids peaks are missing and the interfacial water peak is present (Figures 12, 13, 14).

Based on this analysis we can say that Cora's tumour is benign (the carotenoid marker being observed), and Kisha's tumour is malignant (presence of interfacial water).
Histopathology analysis confirmed the results from the Raman examination.
The histopathological aspect of Cora's tumour was benign and of Kisha's tumor was malignant (Figure 15, 16).

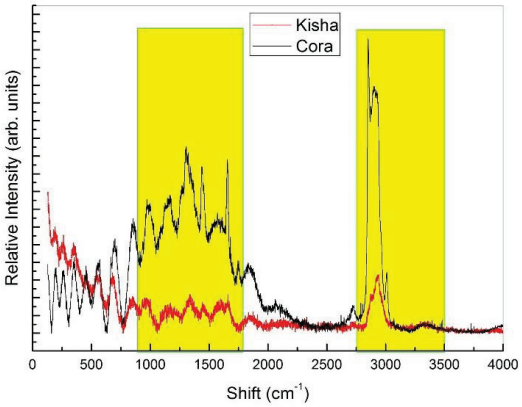

Figure 6. Full Raman Spectra for Cora and Kisha. The window 1000-1800 cm^{-1} corresponds to vibrations of carotenoids and the window 2500-3500 cm^{-1} contains vibrations of lipids and O-H.

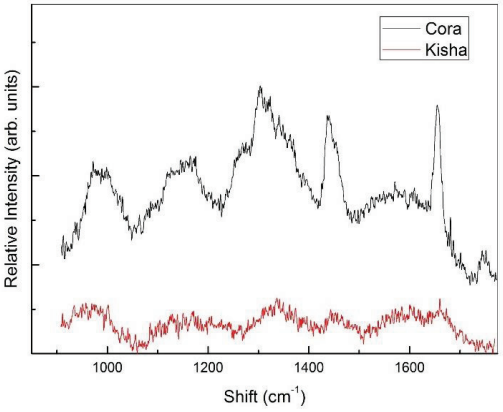

Figure 7. Raman spectra in the 1000-1600 range of both patients

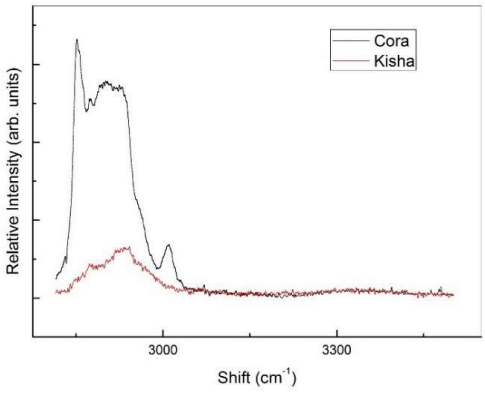

Figure 8. Raman spectra of the 3000-3500cm^{-1} region of both patients

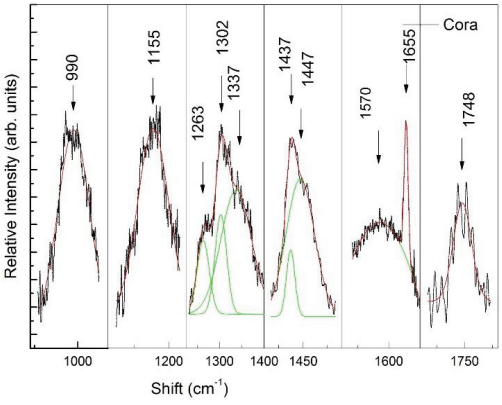

Figure 9. Deconvoluted Raman spectrum of Cora showing carotenoids at 1155cm^{-1} and 1570 cm^{-1}

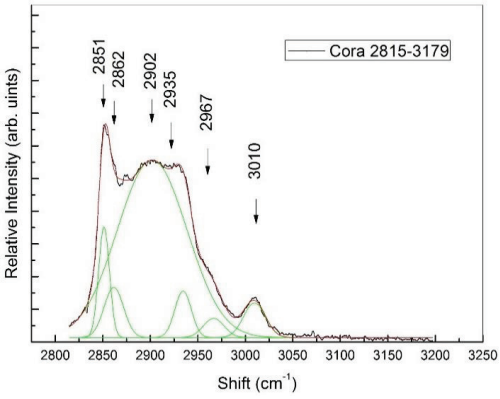

Figure 10. Deconvoluted Raman spectrum of Cora in the region 2815-3179 (lipids in normal adipose tissue)

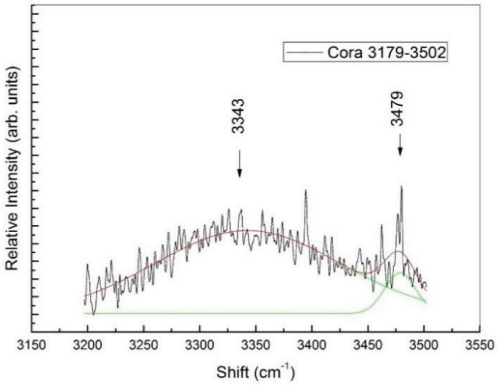

Figure 11. Deconvoluted Raman spectrum of Cora in the region 3179-3502. Benign tissue

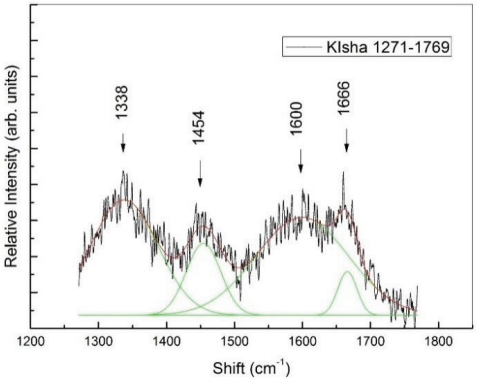

Figure 13. Raman bands of Kisha in the region1271-1769 cm^{-1}. No carotenoids. Sign of malignancy

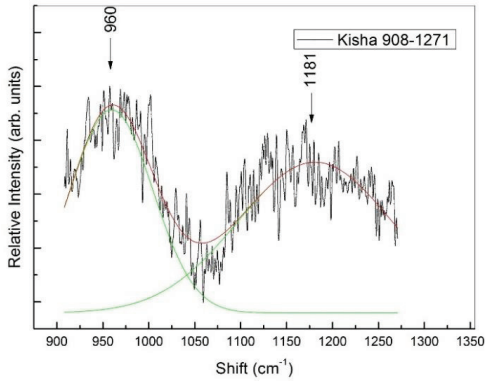

Figure 12. Raman bands of Kisha in the region 908-1271cm^{-1}. Carotenoids are absent. Sign of malignancy

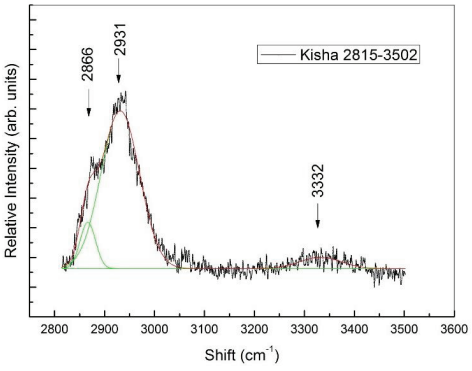

Figure 14. Deconvoluted Raman spectrum of Kisha from 2815 to 3502. 3332cm^{-1}~ interfacial water – malignancy

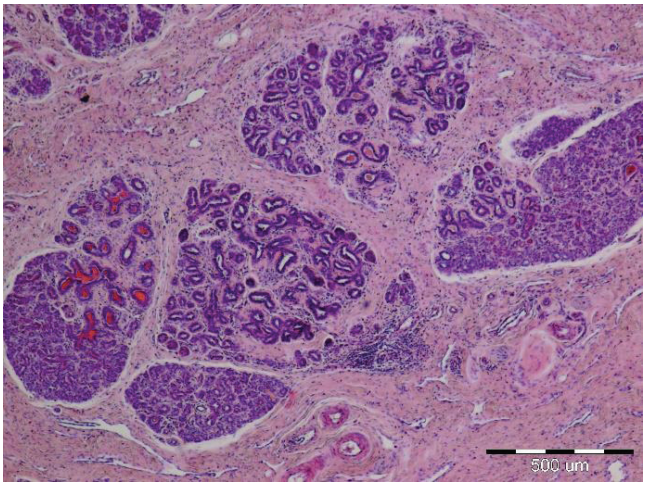

Figure 15. Histopathological aspect of Cora's tumor. Lobular hyperplasia with fibrosis, mammary gland. Increased numbers of ducts/ductules and acini per lobule. The epithelial cells exhibit no atypical changes. Note the extensive interlobular fibrosis; hematoxylin and eosin stain; objective 4x

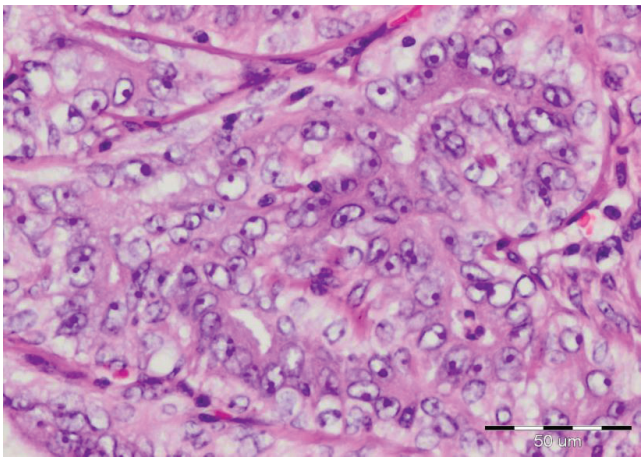

Figure 16. Histopathological aspect of Kisha's tumor. Mammary carcinoma, solid type. The neoplastic cells are arranged in solid sheets, cords or masses supported by a fine fibrovascular stroma. Neoplastic cells are polygonal to oval, with poorly demarcated cell margins and scant eosinophilic cytoplasm, moderate anisokaryosis and anisocytosis; and multiple prominent nucleoli; hematoxylin and eosin stain; objective 40x

CONCLUSION

This study on two clinical cases show a good agreement between histopathology and SERS analysis.

We set up a fast and affordable diagnostic method using the marker-like Raman bands of carotenoids and respectively interfacial water.

Visible Raman excitation wavelength (632 nm) in SERS for direct ex vivo diagnostic in dog mammary tumours is a premiere.

Owing to its non-invasiveness, high sensitivity and high specificity in fast results SERS is a useful technique to accompany histopathology.

Since SERS can give results in real time for the margin, possible recurrences may be prevented.

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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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CUTANEOUS PHAEOHYPHOMYCOSIS IN A DOG WITH COLOR DILUTION ALOPECIA - CASE REPORT

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Abstract

Phaeohyphomycoses are recognized as opportunistic fungal infections caused by several genera of melanin-pigmented moulds (dematiaceous fungi) which are ubiquitous saprophytic agents found in soil, water and decaying vegetable matter. These infections are usually acquired by direct traumatic implantation of fungal elements into tissues or by contamination of open wounds, being invariably associated with an immunosuppressive or debilitated status.
Phaeohyphomycoses are rarely reported in dogs, most appearing as focal or multifocal subcutaneous intact or ulcerated/fistulized nodules or plaques usually found in the facial area, the distal part of extremities or the tail, without any systemic signs. According to the literature data, Alternaria spp. were identified on the coat from 20-80% of healthy dogs and cats without any skin lesions.
In this paper, we have reported a case of cutaneous phaeohyphomycosis with Alternaria spp. in a 3-year-old unspayed male Cane corso dog with chronic skin lesions, not responding to antibiotherapy. The diagnosis of fungal infection was based on cytology, fungal culture and clinical response to long term oral administration of itraconazole.
In our opinion, the infection likely occurred by direct implantation into defective hairs as well as by contamination of ruptured follicular cysts with Alternaria spp.originated from skin colonization and the outdoor habitat. We also considered the inherited follicular dysplasia (color dilution alopecia) to be a promoting factor in acquisition of this opportunistic fungal infection. Finally, complete resolution of lesions under itraconazole therapy and lack of reccurence for 14 months were decisive features for diagnosis.

Key words: *alopecia, dog, hyperpigmentation, itraconazole, phaeohyphomycosis.*

INTRODUCTION

Phaeohyphomycosis (chromomycosis, eumycotic mycetoma) is an opportunistic fungal infection caused by the ubiquitous saprophytic pigmented moulds (dematiaceous fungi), often associated with immunesupperssive therapy or immunodeficient diseases.
The agents of phaeohyphomycosis in human and animals have been classified in 60 genera including more than 100 species. In dog and cat, the recognized pathogens are *Alternaria*, *Bipolaris*, *Cladophialophora* and *Curvularia* (Lloret et al., 2013; Taboada, 2016). Some of these organisms have been isolated from skin and mucosal areas of dogs with and without clinical lesions. In several studies of fungal carriage, *Alternaria spp.* were isolated on the coat from 20-80% of healthy dogs and cats without skin lesions (Dedola et al., 2010).
Most fungal infections are acquired by traumatic implantation or wound contamination, but not trough direct transmission between hosts (Lloret et al., 2013; Herráez et al., 2001). Nodules, tracts or masses in the skin or nasal mucosa are common clinical forms of phaeohyphomycosis which is more less reported in dogs than cats. Skin chronic infections are usually localized in the face, the limbs or the tail (Lloret et al., 2013; Medleau and Hnilica, 2006).
Diagnosis and treatment of most infections are quite difficult because of indistinguishable lesions from other skin disorders (bacterial pyoderma, demodicosis, dermatophytosis, foreign body/sterile granuloma, acral lick dermatitis, tumors, etc.), resistance to most antifungal medications and common recurrence after drug or surgical therapy. Itraconazole is currently the drug of choice with good results

in treatment of multiple lesions and post-surgical ablation (Medleau and Hnilica, 2006; Lloret et al., 2013).

MATERIALS AND METHODS

The case was submitted to the Department of Dermatology from Faculty of Veterinary Medicine-Bucharest, in october 2015, after previous treatments in a private clinic.

Case presentation: The patient was a 3-year-old unspayed male *Cane corso* dog of outdoor with chronic symmetrical skin lesions localized in the face and the digits showing no resolution after more series of antibiotherapy. Facial lesions appeared as focal alopecia with diffuse hyperpigmentation and fistulous tract constantly expressing small hair clumps (figure 1).

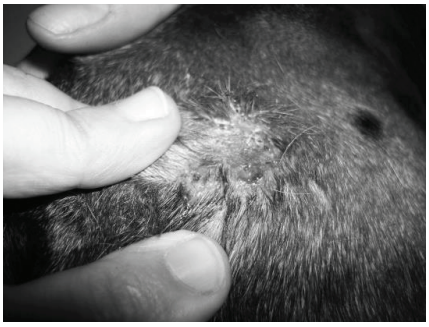

Figure 1. Facial lesion

Digital lesions were quite similar to the facial ones consisting in well-circumscribed alopecia with hyperpigmentation and hyperkeratosis (figure 2).

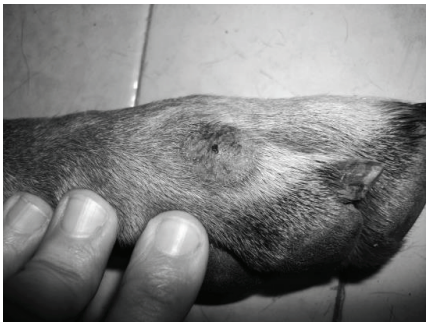

Figure 2. Digital lesion

Both lesions were nonpainful and nonpruritic. Under a more careful examination we could detected some areas of mild hair color dilution around the facial and digital lesions.

Diagnostic protocol included the following standard procedures: trichogram, cytology, bacterial and fungal cultures. Additionally, therapy monitoring represented a diagnostic-key by the resolution of lesions after long treatment with itraconazole.

Trichogram was used to visualize the hairs collected from perilesional areas on wet mounts prepared with mineral oil and lactophenol cotton blue (LPCB).

Cytology was performed on the smears made from lesional aspirates and stained by May-Grunwald Giemsa method, proving to be a useful tool in the detection of inflammatory origin of lesions, excluding neoplasia. Bacterial cultures were obtained by the inoculation of skin aspirates from facial lesions onto sheep blood agar 5% (SBA) and incubation at 37°C for 72h with the growth of non-haemolytic colonies of *Streptococcus spp.*.

For fungal cultures, skin aspirates and hairs from the edge of digital alopecic lesions were plated directly onto Sabouraud dextrose agar (SDA) containing chloramfenicol and incubated at 27°C for 21days when some woolly grey-green-coloured colonies could be seen growing over the inoculated hairs. Microscopic examination of cultures on native preps with lactophenol cotton blue (LPCB) revealed typical dematiaceous septate hyphae and brown multicellular conidia with both transverse and longitudinal septa of *Alternaria spp.*. In the direct preps from inoculated hairs we have also found a cluster of hyphae growing over an intact hair shaft.

RESULTS AND DISCUSSIONS

Skin lesions were quite irrelevant so that differential diagnosis should be made from other alopecic conditions like the bacterial pyoderma, demodicosis, dermatophytosis, nocardiosis, foreign body granuloma, acral lick dermatitis, squamous cell carcinoma.

Trichogram was the first step helping us to exclude demodicosis and dermatophytosis from the mentioned list of suspicions, but also supplied more information showing small melanin clumps distributed predominantly in the cortex beside some irregularities of hair

cuticle enabling the insertion of a brown multiseptate fungal conidia (figure 3). This pigmented conidia has been most likely implanted by trauma from the environment.

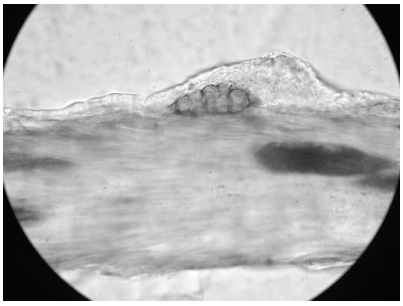

Figure 3. A brown multiseptate fungal conidia under hair cuticle; see also a melanin clump on the right (wet prep, 1000 X)

Moreover, we have considered melanin clumping and cuticle defects together with macroscopical discoloration of the coat around the lesions to be very suggestive for a follicular dysplasia known as color dilution alopecia. In this way, *Cane corso* is a known breed to have genetic predisposition to this follicular disorder (Guaguère et al., 2008). In our opinion, the lower resistance of hair shafts associated to color dilution alopecia might promote fungal inoculation especially into more exposed skin areas like the face and the limbs.

Cytology was useful to preclude skin neoplasia, demonstrating a chronic inflammatory response in both lesions. Thus, the aspirates from digital lesions evidenced a pyogranulomatous reaction comprising a mixed population of neutrophils and epithelioid macrophages satellite to hair fragments (figure 4).

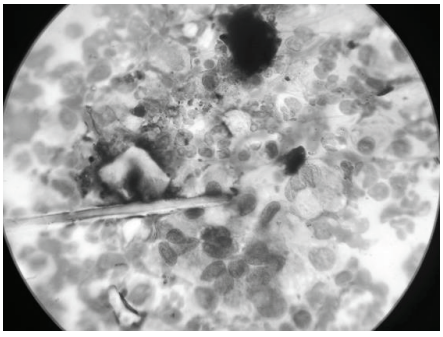

Figure 4. Pyogranulomatous reaction in digital lesions (MGG stain, 1000X)

Under a careful examination we could hardly found a fragment of melanized hyphae surrounded by degenerated neutrophils showing cariolysis (figure 5).

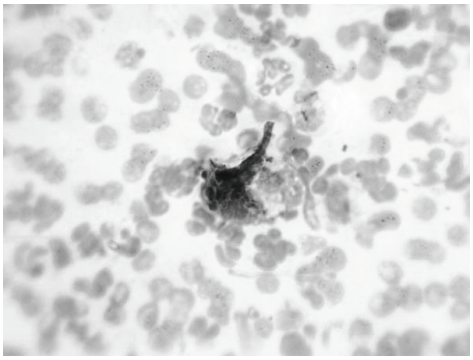

Figure 5. Melanized hyphae with satellite degenerated neutrophils (MGG stain, 1000X)

In a Gram-stained smear from digital aspirates we have also caught an ovoid dark-coloured poroconidia with lateral growing hyphae (figure 6).

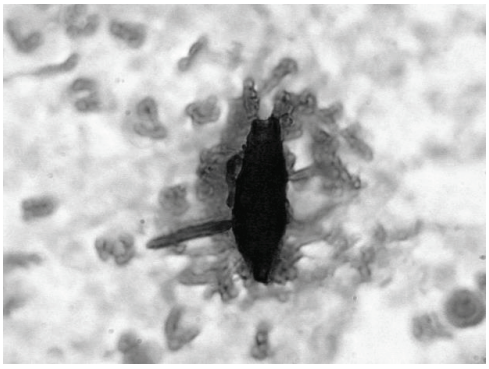

Figure 6. Dark-coloured poroconidia with lateral growing hyphae (Gram stain, 1000X)

Cytology from facial lesions were relevant for follicular cysts by the presence of abundant basophilic keratinaceous debris, numerous corneocytes and nucleated superficial keratinocytes combined with a secondary pyogranulomatous reaction just like the digital lesions.

In some fields, numerous extracellular bacterial cocci scattered in the background along with few phagocytized cocci by neutrophils could be observed indicating a pyoderma subsequently

confirmed by the isolation of *Streptococcus spp.* in the bacterial cultures. Taken together, cytology more sustained the diagnosis of primary color dilution alopecia responsible for defective hair formation and pigmentation, hair breaking, cystic accumulation of hairs and keratin into follicles and foreign body reaction with fistulization resulted from cyst rupture (Medleau and Hnilica, 2006).

Additionally, the exposure of the dog to outdoor habitat most likely promoted the secondary contamination of ruptured follicular cysts by environmental fungi.

Indeed, the isolation of *Alternaria spp.* from fungal cultures (figures 7, 8 and 9) was definitive for the diagnosis, but only in correlation with the history, clinical features and therapy response since the isolated agent is known as a resident of normal cutaneous mycobiota in dog as well as an ubiquitous contaminant.

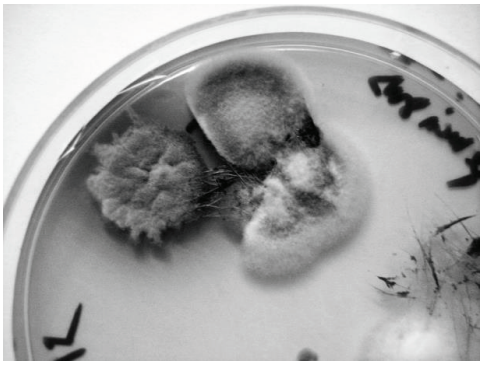

Figure 7. Fungal culture on SDA (7days of incubation)

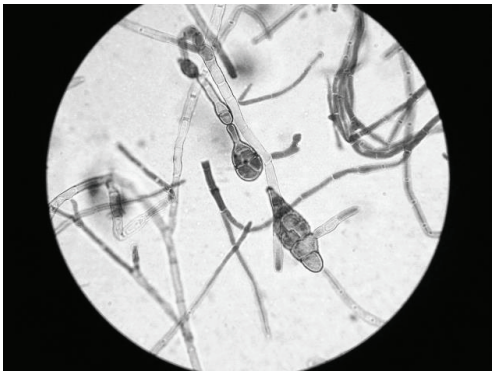

Figure 8. Culture of *Alternaria spp.*- microscopical appearance (wet prep in LPCB, 1000X)

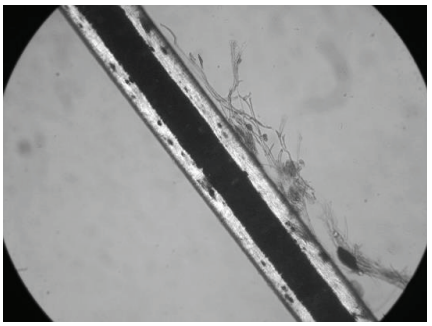

Figure 9. Fungal hyphae growing onto inoculated hairs on SDA (wet prep in LPCB, 200X)

Alternaria spp. are dematiaceous fungi with a worldwide distribution in the environment (usually in the soil and vegetal matter), most frequently isolated from mammals. They are also recognized as usual laboratory and indoors contaminants as well as the most common respiratory allergens in human (Dedola et al., 2010; Dye et al., 2009; Meason-Smith et al., 2015). A recent study about the skin fungal microbiota in dogs using molecular techniques (next-generation sequencing) demonstrated the prevalent colonization with *Alternaria* and *Cladosporium* of canine skin across all body sites and health statuses (Meason-Smith et al.,2015). The study also revealed that cutaneous mycobiota could be influenced by environmental exposure, cohabitation with other pets and skin health status. Similar fungal skin colonization were also identified in cat (Meason-Smith et al.,2017).

The isolation of a common fungal inhabitant represent a real diagnostic challenge. Dogs seem to be less susceptible to infections caused by *Alternaria spp.* than cats and human.

Clinical forms of phaeohyphomycoses range from superficial colonization to subcutaneous and systemic dissemination with encephalitis, osteomyelitis or nephritis, being not absolutely necessary a concurent immunosuppressive or debilitating disease like in human (Seyedmousavi et al., 2013; Herráez et al., 2001; Lloret et al., 2013; Taboada, 2016).

Dedola et al. (2010) isolated *Alternaria infectoria* from multiple, purulent, crusting and ulcerative skin lesions, but also from onychorrhexis and plaque-like lesions on the tongue in a dog under immunosuppressive therapy.

In human, superficial and cutaneous forms of phaeohyphomycosis are believed to result from colonization of the epidermis and hair follicles, while subcutaneous localization follows the traumatic implantation of fungus or results from the progression of superficial infections into the dermis/subcutis with induction of pyogranulomatous inflammation (Herráez et al., 2001).

In this context, the diagnosis of cutaneous phaeohyphomycosis was quite difficult due to the opportunistic nature of isolated agent. The clues for definitive diagnosis were represented by: clinical features (focal alopecia, diffuse hyperpigmentation, slow progression), the identification of pigmented fungal elements in trichoscopy and cytology, fungal cultures, lack of response to antibiotherapy and resolution of skin lesions after oral administration of itraconazole (10mg/kg) for 2 months (figure 10). It is also important to mention that no reccurence has been recorded 14 months after completion of therapy.

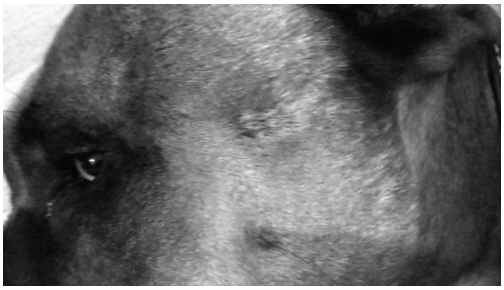

Figure 10. Clinical resolution after 2 months of itraconazole therapy

CONCLUSIONS

Phaeohyphomycoses are uncommon opportunistic infections in dog, usually occurring in pre-existing skin lesions, not always associated with an immunocompromised status.

Diagnosis of infections caused by *Alternaria spp.* represent a serious challenge since these fungi are recognized as normal residents of skin mycobiota in dog and common environmental contaminants as well.

In our case, color dilution alopecia reported in *Cane corso* breed was considered as a promoting factor in fungal infection which most likely arouse from traumatic implantation into defective hairs as well as from contamination of ruptured follicular cysts.

The complete resolution of lesions under itraconazole therapy and lack of reccurences were decisive for diagnosis.

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THERAPEUTIC MANAGEMENT OF SOME DISEASES MET IN CAGE BIRDS FROM PRIVATE COLLECTIONS

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Abstract

Currently, exotic birds have a high demand on the market; requiring care in general not too complicated, birds are a good choice for animal lovers, especially children. This paper presents the treatments carried out in diseases found in canaries, Amazon parrots, Agapornis (lovebird), parakeets, nymphs, Australian zebras. The study was partially conducted in a singing canaries farm located in Voluntari, Ilfov County; the farm includes 250 birds, grouped by age, sex and physiological status. The second part of the study was performed in Clinics of Faculty of Veterinary Medicine of Bucharest, by examining the cage birds that presented to consultation. Bacterial diseases were the most commonly found in canaries - a respiratory infection and 3 cases of bacterial enteritis. Antibiotherapy with Adeno-Coli-Mix product (amoxicillin, colistin sulphate, nitrofurazone) has been successfully used in canaries diagnosed with colibacilosis. In the 2 cases of cage birds diagnosed with enteritis, Enroxil product was used. Traumatic disorders caused by accidents inside the cage disappeared after implementing measures of birds' insulation into smaller spaces in order to avoid additional effort and drug therapy with Metacam. Stress disorders and nutritional deficiencies were represented by feathers consumption, massive moulting and a case of stress adaptation; therapy aimed at correcting the diet and supplementation with vitamin products.

Key words*: cage birds, canaries, enteritis, traumatic disorders, nutrition.*

INTRODUCTION

Currently, exotic birds have a high demand on the market; speaking or singing, beautiful or intelligent, requiring care in general not too complicated, birds are a good choice for animal lovers, especially children.

Birds have specific needs regarding food, environmental conditions and care, and failure to comply with these needs can lead to medical problems of varying intensities (Fowler and Miller, 2003; Girling, 2013; Mayer and Donnely, 2013; Paunescu, 2011).

As low the bird would be, its life and health cannot be measured in money, but in its owner's affection.

The Law for the Protection of Animals says in an article: *the purpose of this law is to establish people's responsibility for their animal as a fellow, whose life must be protected* (Carpenter and Marion, 2013).

Diseases encountered in all species of birds, as many are really researched, would fill thousands of pages; therefore, this paper presents the treatments carried out in diseases found in canaries, Amazon parrots, Agapornis (lovebird), parakeets, nymphs, Australian zebras.

MATERIALS AND METHODS

The study was partially conducted in a singing canaries farm located in Voluntari, Ilfov County; the farm includes 250 birds, grouped by age, sex and physiological status; all birds are marked with a ring around the leg and disinfested regularly (disinfestation of the environment is made with Duramitex Plus and the individual disinfestation is made with Foractil spray for birds) (Ghinăraru, 2016).

The second part of the study was performed in Clinics of Faculty of Veterinary Medicine of

Bucharest, by examining the cage birds that presented to consultation (Ghinăraru, 2016).

RESULTS AND DISCUSSIONS

The diseases found in the Voluntari farm were:

- *colibacillosis* - manifested in general without affecting the general condition, by the presence of green-gray diarrheic faeces. Dissemination of infection was achieved by faeces, both in birds in collective aviaries and individual cages, due to the bird's behaviour to scrape the litter. The treatment was implemented in the form of medicated water, using the product Adeno-Coli-Mix (amoxicillin, colistin sulphate and nitrofurazone), at a dose of 5 g/2 litres of water daily, for 5 days. As a support medication, Detoxicum product was used at a dose of 4 g/litre of water, 5 days (this product is a herbal supplement, with role in sustaining hepatic and digestive functions).
- *fractures and luxations* –were encountered particularly by catching one's legs between the bars of the cage or in fighting between males. The birds were placed in a small cage, for limiting the motion, in a quiet and sheltered spot; medicinal treatment consisted of the administration of anti-inflammatory drugs – Metacam – 0.05 ml diluted in 0.5 ml saline solution; it was administered 0.1 ml orally/day, with a syringe without a needle, until the completion of the entire quantity. In fractures, after cleaning the place, layered bandages were used, which ensure good fixing and do not have hard edges that could injure the bird; these bandages were maintained for 10 - 15 days;
- *moulting* (massive) – normally, feathers fall gradually, their absence is not seen on the body of the bird, but in massive moulting stopping or retarding the growth of new feathers occurs, the birds showing large areas of bare skin. The treatment consisted of birds' isolation in quiet location, dietary supplementation with niger seeds, Oropharma Muta-Vit (1 g/100 ml drinking water) and vitamin A (5 – 10 drops in drinking water) (Ghinăraru, 2016).

The birds examined in the Clinics of Faculty of Veterinary Medicine of Bucharest are presented in Table 1.

Table 1. Birds examined in the Clinics of Faculty of Veterinary Medicine of Bucharest

No.	Species	No. of cases	Clinical signs	Diagnostic	Treatment	Evolution
1.	Amazon parrot	1	Circular movement, head left on one side, lack of appetite	Vestibular syndrome	Enroxil 0.01 ml orally/day, 5 days Dexamethasone 0.01 ml orally/day, 3 days Betaserc ¼ cp pulverized, orally Duphalyte 2-3 drops/day, orally	Favourable Vertigo disappeared after 3 days Treatment was continued for another 4 days
2.	Agapornis (lovebird)	3	1). Slightly messy around the beak, present appetite but with empty throat, 42ºC	Suspicion of cryptosporidiosis	Enroxil 15 mg/kg orally, 4 days (0.2 ml Enroxil diluted with 0.8 ml saline solution, from which 0.1 ml were administered daily) Berforvel 0.1 ml/day, 4 days	Unknown
			2,3). Pair with faecal consistency slightly modified, greenish	Enteritis	Enroxil 4 days Duphalyte 10-15 drops/100 ml drinking water, 4 days	Favourable Faeces returned to normal after 2 days of treatment
3.	Parakeets	4	1). Left leg slightly deflected laterally; without signs of inflammation	Suspicion of luxation	Metacam 0.005 ml orally/day, 4 days Mixture of Duphalyte 5 ml, Glucose 5% 5 ml, vitamin C 0.5 ml administered in drinking water – 1 ml/day, 5 days	Favourable It was recommended to continue treatment for another 3 days

			2). A pair – the female is pecking male's feathers frequently, especially those on the head and chest	Feathers eating	Selevit-E and vitamin AD_3, a few drops in drinking water for 7 days, then once every two weeks	Favourable The female is pecking less the male, it no longer consumes feathers
			3). General condition is changed, it presents obvious respiratory effort, closed eyes, nostrils slightly deformed	Respiratory infection	Enroxil 5 days A teaspoon of honey mixed with drinking water	The bird did not survive (it died after 2 days of treatment)
			4). Normal plumage, elevated heart rate	Accommodation stress	Duphalyte 2-3 drops in drinking water, with the recommendation to keep the bird in a quiet location to accommodate the new environment	Favourable
4.	Nymph	1	Sad, ruffled feathers, agglutinated, dirty feathers around the cloaca	Enteritis	Enroxil 1 ml/200 ml drinking water, 4 days Multivitaminico alimentary supplement, 12-16 drops in 200 ml water	Normal faeces after 4 days of treatment; antibiotic continued for another 2 days and vitamin complex for another 10 days
5.	Australian zebra	1	Abdominal decubitus, legs wide apart (penguin position)	Suspicion of egg retention	Maintaining at heat, application of vegetal oil around the cloaca	The bird has not expelled the egg during the examination; the owner did not return
6.	Canary	1	Avoids the support on the leg caught earlier between the cage bars. Appetite present	Contusion	Metacam 0.005 ml/orally/day, 4 days The bird was isolated in a smaller cage to avoid extra effort	Inflammatory signs disappeared, the bird regained the support in the left leg

CONCLUSIONS

The most commonly diseases found in canaries were bacterial diseases - a respiratory infection and 3 cases of bacterial enteritis. Antibiotherapy with Adeno-Coli-Mix product (amoxicillin, colistin sulphate, nitrofuran) has been successfully used in canaries diagnosed with colibacilosis.

In the 2 cases of cage birds diagnosed with enteritis, Enroxil product was used with a demonstrated usefulness of support medication with Duphalyte, Multivitaminico, Detoxicum products throughout the period of antibiotic therapy. Birds treated had favourable evolution.

Traumatic disorders disappeared after birds' insulation and drug therapy with Metacam.

In case of fracture, fixation of the member with a bandage determined a partially or totally recovery of the affected limb functionality.

Stress disorders and nutritional deficiencies were represented by feathers consumption, massive moulting and a case of stress adaptation.

The results of treatment with vitamin products – Oropharma Muta-Vit, vitamin A, Selevit E and AD_3, Duphalyte were a success.

In the case of a vestibular syndrome, the evolution was favourable after treatment with Enroxil, Dexamethasone, Betaserc, Duphalyte.

The efficacy of treatment in birds (and other animals of course) depends heavily on the promptness and accuracy of medication implementation, and the strict compliance with the hygiene and microclimate conditions necessary for each species.

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THE PREVALENCE OF HEMATURIA IN DOGS AND CATS

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Abstract

This paper briefly reviews the basic definitions of hematuria, the common causes and the prevalence of this simptom on dogs and cats. This study was conducted in the Clinic of Faculty of Veterinary Medicine in the period between 1.10. 2016 – 1.02.2017 using IDEXX UA dipstick, IDEXX VetLab UA, Refractometer RHCN-200ATC and Grant Bio PCV-2400 Combined Centrifuge. Data collected recorded 65 animals, with felines obtaining a precentage of 42% and canine 28% in regard of hematuria prevlance by species. In case of hematuria prevalence based on gender we registered 62% males and 38% females. In hematuria prevalence by age we listed 32% of animales cu age range between 6-10 years, 31% with 1-5 years, and also 31% over 10 years, and 6% less than a year. From a total of 51 urinary dipstick who tested positive, we discovered on the examination of urine sediment that 46 samples confirms, and 5 samples offered a false - positive. Of the 71 samples analyzed 76% were within the macroscopic hematuria and 24% to microscopic hematuria, and 28% of the samples showed no hematuria. As a conlcusion hematuria is a common finding of urological pathology and it is important to known the risk factors of every species.

Key words: *hematuria, prevalence, gross hematuria, microhematuria.*

INTRODUCTION

Hematuria is defined as the presence of five or more RBCs per high-power (40x) field in a fresh, centrifuged specimens obtained by either manual compression of the bladder catheterisation, or cystocentesis (Reine at al., 2005).
Healthy animals can excrete as many as 3 red blood cells per high-power field (Chew, 2011).
Hematuria is commonly subdivided according to its type as macroscopic hematuria (i.e., visible, named gross hematuria) and microscopic hematuria (Figure 1, Figure 3) (i.e., nonvisible detected with a microscope), time of micturition (initial, terminal or total), duration (intermittent or persistent) and the clinical form - symptomatic or asymptomatic (Costache, 2005).
Initial hematuria indicates the origin in urethra or prostate (males), total hematuria – in bladder, ureter, and kidneys, and terminal hematuria, bladder or prostate (Mazhari, 2002) (Figure 2).
Gross hematuria is suspected when urine is has an abnormal color, usually red or tea-colored. In evaluating gross hematuria, it is important to confirm the presence of RBCs by microscopy (Massengill, 2008)
Microhematuria is often detected during investigation of symptoms such as dysuria, urinary frequency, or flank pain (Massengill, 2008).

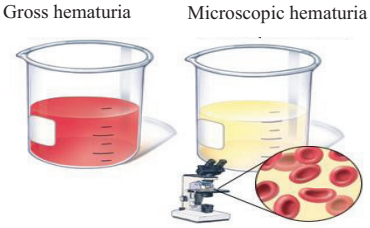

Figure 1. Gross hematuria vs. Microhematuria
https://www.drugs.com/health-guide/hematuria.html

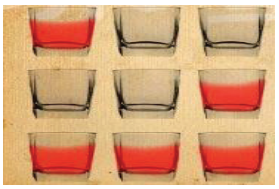

Figure 2. Hematuria. Time of micturition
http://www.kidneyservicechina.com/pkd-symptoms/4325.html

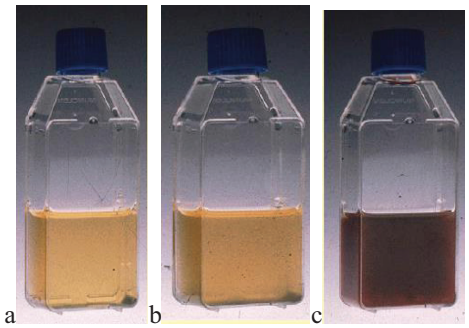

Figure 3. **a** Normal urine; **b** microscopic hematuria (+++); **c** macroscopic hematuria (Barrat, 2007).

"Dipstick hematuria" and "dipstick microhematuria" is a nondiagnostic screening test offering a positive result due to oxidation of a test-strip reagent (Rao and Jones, 2008).
This paper briefly reviews the basic definitions of hematuria, the common causes and the prevalence of this simptom on dogs and cats.
There are many causes of hematuria, and is a priority in the medical act for the doctor to discover the origin of hematuria by establishing whether its nature is renal or non-renal (Table 1).

Table nr. 1 Causes of hematuria

Intrinsic Renal Disease	***Non-Renal Disease***
Glomerulonephropathy	Ureteric (stones,tumor)
Cystic disease	Bladder
Renal tumors Interstitial disease Interstitial nephritis Papillary necrosis/analgesics Stones or crystals Acute infection	- tumors - stones - Cystitis) Prostate (carcinoma - Prostatitis)
Atrioventricular malformation	Urethral lesions

after (Fine, 2002)

Diagnosis of hematuria can be pronounced easily by using urine strips which evaluates for pyuria, proteinuria, heme positivity, and urinary concentrating defects (Table 2) and using microscopic examination of urine sediment by evaluating for white blood cells and clumps, RBC morphology, crystals, and casts. Crystalluria can be determined by calcium oxalate, calcium phosphate, uric acid, or cystine crystals. Hypercalciuria is the most common cause of crystalluria (Massengill, 2008).
A reagent strip, also called a dipstick, is a narrow strip of plastic with small pads attached to it. Each pad contains reagents for a different reaction, thus allowing for the simultaneous determination of several tests. The colors generated on each reagent pad vary according to the concentration of the analyte present. Colors generated by each pad are visually compared against a range of colors on brandspecific color charts (Bataille et al., 2016).
Urine dipstick is used to test for the peroxidase activity of erythrocytes (Robert, 2007).

Table 2. Substances tested for by urine dipsticks

Commonly assessed Less commonly assessed	**Commonly assessed Less commonly assessed**
Blood Ketones	Blood Ketones
Protein Urobilinogen	Protein Urobilinogen
Glucose Bilirubin	Glucose Bilirubin
Leukocyte esterase Specific gravity	Leukocyte esterase Specific gravity

after (Barrat, 2007)

Urine culture is performed in cases that have clinical symptoms or laboratory evidence (pyuria, hematuria, bacteriuria, positive nitrites) of a urinary tract infection (Bataille et al., 2016).
Also in the assessment of patients that express hematuria, complementary tests should be performed such as blood pressure, evaluation of renal functional parameters and proteinuria (Van Der Molen et al., 2012).
If the test is positive for hematuria unaccompanied by urine abnormality it is necessary to determine serum creatinine. If it has a high value more tests will be done. The normal values of serum creatinine indicates the need for an ultrasound (Fine, 2002).

MATERIALS AND METHODS

This study was conducted in the Clinic of Faculty of Veterinary Medicine in the period between 1.10.2016 – 1.02.2017.

In order to establish the prevalence of hematuria in dogs and cats, fresh midstream urine samples (5-10 mL) were obtained from animals with urologic signs.
We evaluated 71 samples, 27 from feline and 18 from canine, 40 being males and 25 females, with age range between 4.5 months and 17 years.The urine samples were first evaluated macroscopically, and its color and clarity were recorded; than we used IDEXX UA dipstick for detecting the blood in IDEXX VetLab UA Analyzer and Clinical Refractometer RHCN-200ATC for measuring the urine specific gravity.
The change in the color were noted an compared with standard provided. After the macroscopic evaluation the samples were prepared for urine sediment examination by centrifugation for 10 minutes at 6000x in a Grant Bio PCV-2400 Combined Centrifuge. The supernatant fluid was decanted and a drop of sediment was transferred to a glass slide and a coverslip was placed on the specimen. The slides were examined at 400 magnification for red blood cells.
Hematuria was considered to be present at five or more RBCs per high-power (40x) field.

RESULTS AND DISCUSSIONS

In the study prevalence of hematuria on dogs and cats, we have taken in consideration parametres that influence the result and conclusion, such as species, age and gender.
In regard of hematuria prevalence survey considering species, our result was that 65 animals participated, and felines obtained a precentage of 42% and canine 28%.
Giving this result and from the diagnosis established, the felines are more prone to express hematuria, being liable to develop FUS (feline urological syndrome), urinary lithiasis and cystitis (Figure 4).
In case of hematuria prevalence based on gender we recorded a significant proportion of males which have registered a percentage of 62% followed by 38% females.
Based on the diagnosis discovered the percentage 62 % males with hematuria is slightly predictable being well known the frequency of urological pathology in males due to anatomical features of lower urinary tract (Figure 5).
The males who participated in this survey most were diagnosticated with urinary lithiasis and FUS.

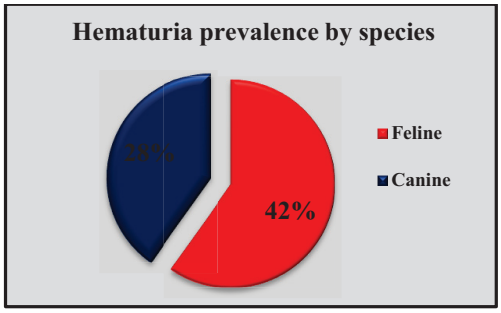

Figure 4. Hematuria prevalence by species (Original)

Females who manifested hematuria were diagnosticated majority of them with cystitis, microlithiasis and just one case with glomerulonephritis and FUS.

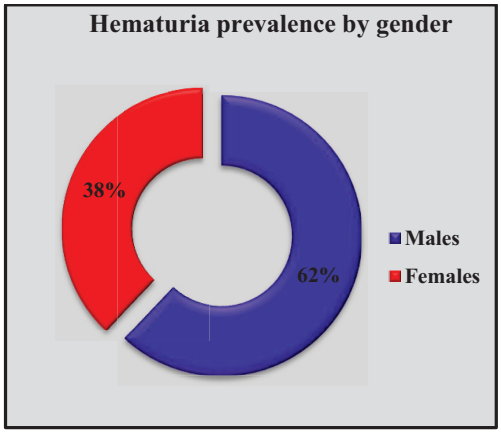

Figure 5. Hematuria prevalence by gender (Original)

Hematuria was encountred in different ages of the subjects, in a prevalence of 32% of animales cu age range between 6-10 years, 31% with 1-5 years, and also 31% over 10 years, and 6% less than a year (Figure 6).
Other studies have concluded that risk-factors for stone formation, are age, gender and breed, together with influences such as geographical location, presence of UTI and dietary history (Syme, 2012).
Cystine urolithiasis occurs preponderantly (98%) in male dogs and are not common in very young dogs but tend to occur in middle-age (Syme, 2012).

The prevalence of cystine calculi is highly dependent on geographical location, with a higher reported prevalence in dogs in Europe than dogs from the USA (Syme, 2012).

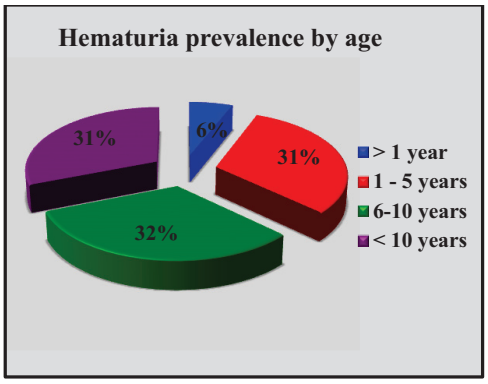

Figure 6. Hematuria prevalence by age (Original)

We evaluated 65 animals from which we colected 71 samples on a period of 5 months; the result we obtained using urinary dipstick IDEXX UA, was 51 tested positive for RBC/ hemoglobin/ myoglobin - urine is discolored but no RBC are noted on microscopic examination (Figure 7).

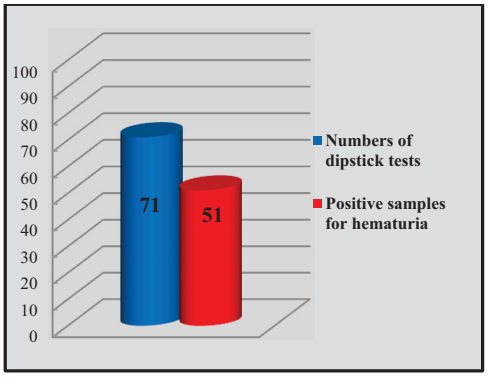

Figure 7. Numbers of dipstick test and positive samples for hematuria (Original)

It is been noted that microscopic hematuria is present when more than five red blood cells (RBCs)/high power field are found (Fine, 2002). Dipstick testing is the initial test for detecting hematuria. It is very sensitive and will pick up one to two RBCs/hpf (Fine, 2002).
Dipstick testing will register positive in a urine that has microscopic hematuria allowed to stand for too long (*i.e.,* with hemolyzed RBCs) in spite of few or no red cells being seen on the film. In some cases, dipstick tests provides a false negative or false positive (Choi, 2003).
Table 3 summarizes the main causes in appearance of urine strips errors (Fine, 2002).

Table 3. Reasons in appearance of urine strips errors*

Reasons blood shows on dipstick urinalysis but not on microscopic exam (false positive)	Reasons blood doesn't show on dipstick urinalysis but is present on microscopic exam (false negative)
Exercise	Captopril Vitamin C
Dehydration	Acidic urine (pH <1.5)
Hemoglobin (part of red blood cell) Myoglobin (break down product of red blood cell)	Concentrated urine Protein in the urine dipstick exposed to air

after (O'Brien et al., 2014).

The protocol in the diagnosis of hematuria includes first testing with urinary strip and if it is positive urine should be sent for urinalysis. If the patient has more than five RBCs/hpf, microscopic hematuria is present.
The American Urologic Association declares "evaluation should be based solely on findings from microscopic examination of urine sediment and not on a dipstick reading." (O'Brien et al., 2014).
From a total of 51 urinary dipstick who tested positive, we discovered on the examination of urine sediment that 46 samples confirms the presence of more five red blood cells (RBCs)/high power field, and 5 samples offered a false - positive (Figure 8).
Positive dipstick for hematuria with negative microscopy are often due to false negative microscopy. With bright field microscopy, negative results may occur as a result of spontaneous lysis of red cells or by failure to detect 'ghost' forms (Choi, 2003).

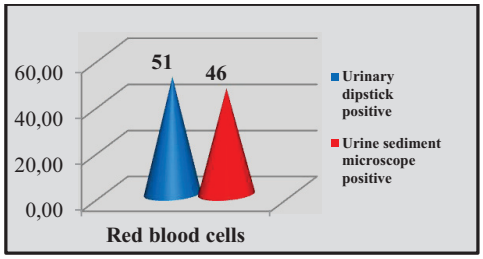

Figure 8. Urine dipstick positive vs. Urine sediment microscope positive (Original)

Of the 71 samples analyzed it was determined that 76% were within the macroscopic hematuria and 24% to microscopic hematuria, and 28% of the samples showed no hematuria (Figure 9).

In assessing gross hematuria, it is important to verify the presence of RBCs by microscopy. After centrifugation of the urine, the finding of red urinary sediment with a positive strip test for hemoglobin it suggest hematuria, whereas red supernatant with negative dipstick testing for hemoglobin is indicative of myoglobinuria, hemoglobinuria, or other causes of discolored urine (Veerreddy, 2013).

The overwhelming result in which macrohematuria is triple to microscopic hematuria is probably due to the fact that most of the diagnosis established implies o advanced pathological disorder in which bleeding is a common sign .

Concerning the micturition moment, terminal hematuria records a higher proportion, being an element suggesting the involvement of the lower urinary tract.

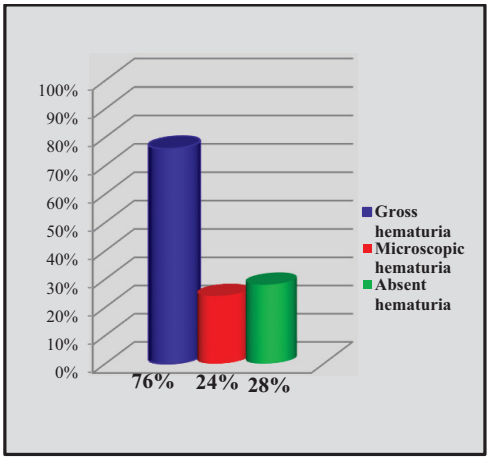

Figure 9. Gross hematuria and microhematuria (Original)

CONCLUSIONS

Hematuria is a common finding of urological pathology as we discovered in our study. Hematuria in dogs and cats has a wide differential diagnosis, with different therapeutic approaches compared to human patients.

It is important that in the first evaluation of the animals the clinician takes account of the risk factors of every species.

Knowing that felines with male gender are prone to develop urological disorders that express macroscopic hematuria such as FUS, urolithiasis and cystitis, can help the clinician to pursue a reduced number of methods of investigation offering a fast diagnosis.

More often than not, owners, demand an immediate diagnosis, particularly when there is macroscopic hematuria due to the impact of blood in the urine that alarms them.

A simple and practical approach to the animal who presents hematuria should consists in a fewer invasive studies, a less costly evaluation, and a accurate diagnosis.

Our results suggest that asymptomatic microscopic hematuria in dogs and cats is rarely associated with clinically significant disease of the urinary tract, but is mandatory to keep under observations the animals.

Gross hematuria is more commonly and is associated with urinary tract disease, such that a thorough evaluation for determination of the bleeding origin is justified and recommended.

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HEMODIALYSIS IN VETERINARY MEDICINE: REVIEW

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Abstract

Hemodialysis is used for the management of acute and chronic renal failure that is refractory to conventional medical therapy. For the moment, there are two types of hemodialysis: intermittent and continuous hemodialysis. Intermittent hemodialysis (IHD) is a renal replacement therapy that is defined by short and efficient hemodialysis sessions with the goal of removing endogenous or exogenous toxins from the bloodstream. IHD is indicated in cases of acute azotemia, electrolyte abnormalities or acidosis unresponsive to medical management. Continuous renal replacement therapy (CRRT) is a continuous process and, once treatment begins, therapy continues until renal function returns or the patient is transitioned to intermittent dialysis. The most common indication for CRRT is the treatment of acute kidney injury (AKI) in cases in which renal function is expected to return in the near future or for patients who are to be transitioned to IHD. Vascular access is the first and most basic requirement of successful extracorporeal renal replacement therapy (ERRT) and usually the jugular vein is used. Another vascular access consists of arteriovenous (AV) fistula or graft and it is the preferred access in patients with chronic hemodialysis. The ERRT catheter should be used only for ERRT procedures and handled only by ERRT personnel. When patients undergo IHD, their blood is removed from their bodies and run through an extracorporeal circuit. The blood is exposed to foreign material that may activate the clotting cascade. Therefore, anticoagulant therapy is often required during a dialysis treatment and special equipment is necessary for monitoring the level of anticoagulation. Complications of IHD have been widely reported and include hypotension and hypovolemia, vascular access problems and neurologic, respiratory, hematologic and gastrointestinal complications. The most significant complications of CRRT is coagulation. Despite appropriate heparin management, clotting of the CRRT circuit is inevitable.

Key words: *hemodialysis, acute kidney injury, central venous catheter, anticoagulation, complications*

INTRODUCTION

Dialysis is the process of separating a substance with colloidal dispersion from particles with molecular dispersion, based on the membrane properties to retain only the colloidal particles. This is used for artificial kidney function replacement. The kidneys role is to filter waste and toxins from the blood, concentrate or dilute urine, prevent dehydration or overhydration, regulate blood pressure and produce certain hormones.

Hemodialysis is a therapeutic procedure that uses the extracorporeal circulation of a patient's blood to ameliorate the azotemia, fluid overload, electrolyte and acid-base abnormalities characteristic of the uremic syndrome. Hemodialysis is used for the management of acute and chronic renal failure that is refractory to conventional medical therapy. Additional applications include acute intoxications (e.g. ethylene glycol poisoning) and preoperative conditioning of renal transplant recipients. Hemodialysis is a technically demanding procedure that requires an extensive array of sophisticated delivery equipment and specifically trained and dedicated staff to perform, monitor and ensure the integrity and safety of the procedure in critically ill patients (Elliott, 2000).

DOG HEMODIALYSIS HISTORY

The first hemodialysis was performed experimentally on dogs in 1913 by Abel, Rowntree and Turner on experimental dogs with an "artificial kidney" composed of celloidin tubes, the predecessor of the modem hollow fiber dialyzer (Cowgill and Langston, 1996). Its use in dogs began in 1960 and continued by the early 1970's; these efforts were directed to therapeutic applications. By the early 80's vascular access technique used was the Quinton-Scribner modified method: arteriovenous shunts (AV), but in the past 30 years, the use of double lumen central venous

catheters have revolutionized the technique of hemodialysis on dogs, mostly because of its anatomical features (Cowgill, 2013). John Jacob Abel directed arterial blood from animal patients, mixed it with an anticoagulant, passed it through a device that divided the blood into straw-like semipermeable membranes that were suspended in fluid and then directed the blood back to the patient and demonstrated that the subject's blood could be altered by changing the composition of the fluid. The first clinical program dedicated to providing hemodialysis services for dogs was established in 1990 at the University of California-Davis. Technological advances of today's systems have established efficacy, tolerance and safety in animals undergoing hemodialysis (Cowgill, 2013).
In Romania, the first hemodialysis performed successfully in dogs occurred in the Faculty of Veterinary Medicine's Clinic in February 2014.

HEMODIALYSIS TYPES

At the moment, there are two forms of hemodialysis: intermittent hemodialysis and continuous hemodialysis.
Intermittent hemodialysis (IHD) is a renal replacement therapy that is defined by short and efficient hemodialysis sessions with the goal of removing endogenous or exogenous toxins from the bloodstream. Common indications for IHD include drug or toxin ingestion, acute or acute-on-chronic kidney injury and chronic kidney disease (CKD).
Sessions can be performed once, as is common with toxin ingestion, or can be repeated daily or every other day for several days or longer, as is often done for acute kidney injury (AKI). Sessions can be conducted 2 or 3 times a week for the duration of the patient's life, as may be selected for CKD. Sessions are traditionally 1 to 6 hours in length, but can be longer, depending on the stability of the patient and efficiency of the session. IHD is designed as a more efficient modality than continuous renal replacement therapy (CRRT), meaning that IHD sessions remove small dialyzable molecules (blood urea nitrogen [BUN], creatinine, phosphorus, electrolytes, and certain drugs and toxins) from the bloodstream more rapidly than CRRT. Between treatments (the interdialysis period), these dialyzable molecules may again increase in the bloodstream (Bloom and Labato, 2011).
Continuous renal replacement therapy (CRRT) is a more recently developed blood purification modality. CRRT is a continuous process, and once treatment begins, therapy continues until renal function returns or the patient is transitioned to intermittent dialysis. CRRT is similar to IHD because patient blood is divided into thousands of straw like semipermeable membranes contained within a dialyzer; however, whereas IHD is primarily a diffusive therapy, CRRT uses diffusion, convection and, to a lesser extent, adhesion. CRRT has several significant advantages compared with IHD. The slow and gradual nature of the technique provides better control of electrolytes and acid-base balance (Bellomo et al., 1995). The continuous operation more closely approximates the functioning of a normal kidney (Clark et al., 1994). The goal of IHD is to make dramatic changes in a patient's uremic, acid-base and fluid status over short periods using diffusion; therefore, significant quantities of pure dialysate must be produced onsite. This technique requires a sizeable investment in the purchase and maintenance of specialized water treatment facilities (Langston, 2002).

PRINCIPLES OF HEMODIALYSIS

The main forces used during HD are diffusion, convection and adsorption. In HD, diffusion is the most prevalent force for exchange of solutes and fluids; convection and adsorption generally play a minor role. During diffusion, solutes move from areas of high to low concentration. In their moves, solutes leave the blood or dialysate fluid compartment in which they had been dissolved, cross the dialysis membrane and enter the opposite fluid compartment. Blood solutes such as BUN, creatinine, and electrolytes diffuse across the semipermeable dialyzer membrane into dialysate, which is discarded. Solutes in high concentration in dialysate, such as bicarbonate and selected electrolytes, may diffuse across the dialyzer membrane according to their concentration gradient into blood. Diffusion is best at removing molecules with low molecular weight from the blood, including BUN and creatinine, sodium, potassium, phosphorus and

magnesium. Blood traveling in semipermeable membranes of the dialyzer is exposed to positive transmembrane pressure, which pushes fluid (ultrafiltrate) and dissolved solutes out of blood, across the dialyzer membrane and into the dialysate, which is discarded. Convection, a prevalent force in CRRT but not in IHD, is best in removing molecules with low and middle molecular weight from the blood. Middle molecules include many inflammatory mediators, as well as uremic toxins (Yeun and Depner, 2000).

INDICATIONS FOR IHD AND CRRT

IHD may be appropriate when medical management fails to achieve a positive outcome. Therefore, IHD is indicated in cases of significant or rising azotemia, electrolyte abnormalities or acidosis unresponsive to medical management. IHD is also indicated in cases of oliguria and anuria in the lack of response of appropriate medical management. IHD is commonly used in the management of humans with CKD (Yeun and Depner, 2000). IHD is an uncommon but available therapy for management of CKD in veterinary patients. Indications for IHD in patients with CKD include reduction of chronic progressive azotemia, hyperkalemia and fluid overload, as well as stabilization before renal transplantation. IHD may become part of a routine treatment of patients with systemic inflammatory response syndrome, sepsis or other severe inflammatory conditions via filtration and removal of inflammatory mediators, or fluid overload and congestive heart failure via ultrafiltration and removal of excess intravascular fluid volume as well as apheresis (Groman, 2010).

The most common indication for CRRT is the treatment of acute kidney injury (AKI) in cases in which renal function is expected to return in the near future or for patients who are to be transitioned to IHD. CRRT is used for patients with leptospirosis, tumor lysis syndrome, heatstroke, pre- and postsurgical support of ureteral obstructions, as well as aminoglycoside and melamine toxicities (Johnson and Simmons, 2006). Recent studies showed that hemodialysis can work wonders in propylene glycol intoxications on dogs, if ethanol 20% in bolus in alternated with CRRT (Claus et al., 2011). CRRT has also been used to treat patients with diuretic-resistant congestive heart failure; however, this treatment has not yet been evaluated in companion animals (Clark and Ronco, 2004). Toxin ingestion is a common cause of acute renal failure (ARF) in dogs (Stanley and Langston, 2008). Raisins and grapes have been reported recently as an underlying etiology for ARF in dogs, although the underlying mechanism of nephrotoxicity remains unknown (Penny et al., 2003; Eubig et al., 2005).

BLOOD FLOW RATE AND LENGTH AND FREQUENCY OF HEMODIALYSIS SESSIONS

One way to calculate desired efficiency of the initial session based on severity of uremia is via the urea reduction ratio (URR) to determine the volume of blood that needs to be processed through the dialyzer to achieve a certain percent reduction in BUN level (Langston et al., 2010). URR and the corresponding blood flow rate (L/kg body weight) needed to achieve that particular URR have been determined for dogs and cats using empirical data from the Companion Animal Hemodialysis Unit at the Veterinary Medical Teaching Hospital at the University of California-Davis. In IHD, blood flow rate is the primary determinant of small molecule clearance, including BUN and potassium clearance. Therefore, one way to begin a dialysis prescription is to determine a desired URR, determine the volume of blood per kilogram body weight the machine must process to achieve that desired URR, and determine the desired length of the session, which is often 1.5 to 2 hours for the first session, 3 hours for the second session, and 4 hours (cats) to 5 hours (dogs) for the third or fourth sessions. (Langston, 2002). Using the patient's body weight, desired blood volume to be processed, and desired length of the session, you can set your blood flow rate in mL/kg/min accordingly. Blood flow rate is often set low at the start of the session and is slowly increased to the prescribed blood flow rate in the first 30 minutes of the session, to avoid hypotension or nausea (Elliott, 2000). Cowgill (2008) and Elliott (2008) recommend blood flow rates as

low as 1 to 2 mL/kg/min for animals with predialysis BUN level greater than 180 mg/dL.
For IHD of patients with CKD, sessions are performed 2 or preferably 3 times per week, with twice-weekly sessions appropriate only for those patients with sufficient residual renal function to avoid significant rebound solute accumulation between dialysis sessions.
Blood flow rate targets can be set at 15 to 25 mL/kg/min if thepatient's starting BUN level and vascular access can tolerate this high rate. Targets for length of session are 4 hours in cats and 5 hours in dogs, again, if well tolerated by the patient (Langston, 2002; Cowgill, 2008). Cowgill (2008) and Elliott (2008) recommend prolonged, slow treatment sessions of up to 8 hours for small patients with severe uremia (BUN level >250 mg/dL), using blood flow rates less than 2 mL/kg/min. For acute and chronic IHD, longer sessions may be both feasible and desirable, depending on treatment goals. Between dialysis sessions, solutes do reaccumulate. Therefore, bloodwork must always be taken at the start and end of the dialysis session and between dialysis sessions, so that appropriate dialysis prescriptions and interdialysis treatments are optimized for each individual patient.

VENOUS ACCESS AND CENTRAL VENOUS CATHETERS (CVC)

Vascular access is the first and most basic requirement of successful extracorporeal renal replacement therapy (ERRT). An adequately functioning dialysis catheter allows for smooth and efficient patient management. Various materials can be used to make a catheter that is minimally thrombogenic, flexible, and nonirritating to the vessel wall. (Chalhoub et al., 2011). To allow simultaneous removal and return of blood, a dialysis catheter has 2 lumens. Although catheters are placed in a central vein, the lumen that provides blood egress from the body is generally referred to as the arterial port or access port and the lumen that provides blood return to the body is termed the venous port or return port. The arterial lumen is usually shorter than the venous return lumen to avoid uptake of blood returning from the dialyzer (access recirculation), which would decrease the efficiency of treatment. In some situations, 2 single-lumen catheters are placed in separate vessels or in the same vessel to provide blood egress and return. In lumens with a single opening (at the tip or a side port), partial occlusion from thrombosis or a fibrin sheath can decrease catheter function to the point of it being unable to provide adequate dialysis. The risk of complete occlusion is lessened by having multiple ports. If the ports are positioned circumferentially around the catheter, even if the vessel wall is drawn against the ports on one side of the catheter, blood flow can continue on the opposite side. If the side ports are small, blood preferentially flows through the tip, making the side ports superfluous. If the side ports are large, they weaken the catheter, and increase the amount of heparin that diffuses out of the catheter between dialysis treatments (Depner, 2001).
Temporary catheters should more precisely be called nontunneled, noncuffed catheters. Depending on the type, a temporary catheter may function for up to 4 weeks. In most cases, a temporary catheter is the appropriate choice unless there is suspicion of preexisting chronic kidney disease and the owners are interested in chronic dialysis (Chalhoub et al., 2011).
Permanent hemodialysis catheters have an external cuff which is usually made of Dacron. The catheter is placed with a portion in a subcutaneous pocket, which separates the site where the catheter exits the skin from the site where the catheter enters the vessel by several centimeters. Permanent catheters may have the ends of the lumens separated, so that the intravenous portion acts like 2 separate catheters placed in the same vein. By having separated tips, side ports can be placed circumferentially on each lumen, and the increased flexibility of the tips and their movement with each cardiac cycle may help decrease fibrin sheath formation (Depner, 2001).
Another vascular access consists of arteriovenous (AV) fistula or graft is the preferred access in patients receiving chronic hemodialysis. An artery is surgically anastomosed to a vein with a section of autologous vein or synthetic graft (typically PTFE). Within approximately one month, endothelial cells line the graft, and the endothelial cells of the autologous vein segment take on characteristics of arterial endothelium instead of venous. The

graft/fistula is then accessed by percutaneous puncture of the arterial and venous segments with large - gauge needles at each dialysis treatment. Between treatments, no anticoagulant is needed because blood is continually flowing through the graft/fistula. A model of AV fistula has been developed for canine hemodialysis, and a brachial-cephalic access could be considered for dogs receiving chronic dialysis (Adin et al. 2002).

CATHETER FLOW CHARACTERISTICS

Because flow is proportional to catheter diameter and inversely proportional to catheter length, it is desirable to select the largest diameter catheter that can be placed. Minor changes in catheter diameter cause very large changes in flow, based on the Poiseuille equation:

$$Q_b = \frac{(KxPxD^4)}{(LxV)}$$

where Q_b is blood flow; K, a proportionality constant; P, the change in pressure; D, the luminal diameter; L, the catheter length and V, the blood viscosity. A 19% increase in catheter diameter doubles the blood flow; a 50% increase causes a fivefold increase in blood flow (Depner, 2001).

CATHETER CARE AND MANAGEMENT

The ERRT catheter should be used only for ERRT procedures and handled only by ERRT personnel. At each ERRT treatment, the exit site should be inspected and cleaned with antiseptic solution. When the ERRT catheter is accessed at the beginning and end of each treatment or at any other time, the catheter ports should receive an aseptic scrub for 3 to 5 minutes. When not in use, the catheter is bandaged in place and completely covered (Chalhoub et al., 2011).

CATHETER PERFORMANCE

Catheter function can decrease over time if thrombosis or stenosis occurs gradually, or performance can decline abruptly. A simple way of monitoring function at each dialysis treatment is to record the blood speed when the pressure in the arterial chamber (prepump) is -200 mm Hg. A gradual decline in the blood speed at a standardized pressure predicts catheter malfunction. The arterial pressure should be maintained above -200 to -250 mm Hg, because at more negative values, the pump speed indicated on the machine is probably higher than the actual blood flow. Catheter design should focus on lowering flow resistance by increasing internal diameter rather than by shortening length (Depner, 2001).

EXTRACORPOREAL REMOVAL OF DRUGS AND TOXINS

The type of extracorporeal therapy used can greatly affect the extent of drug and toxin removal. The available modalities include intermittent hemodialysis and three types of continuous renal replacement therapies (CRRTs). Intermittent hemodialysis is primarily a diffusive process, whereas CRRT uses a combination of diffusion, convection, and adsorption. The continuous modalities include continuous venovenous hemofiltration (CVVH), a purely convective modality; continuous venovenous hemodialysis (CVVHD), a diffusive modality; and continuous venovenous hemodiafiltration (CVVHDF), which combines the aspects of both convection and diffusion (Bugge, 2001).

Systemic pH levels, body fluid composition, tissue perfusion, residual renal function, and contribution of non-renal routes of elimination can affect clearance (Bouman, 2008). Clearance describes the theoretical volume of blood fromwhich a solute is removed per unit time (Goodman and Goldfarb, 2006). A patient's native clearance depends on the ability of that solute to pass across the glomerular basement membrane; it may be affected by tubular secretion or reabsorption and is a function of the molecular weight, charge, and urine flow rate (Bayliss, 2010; Choi et al., 2009).

ANTICOAGULATION

The most common anticoagulant used in veterinary IHD is unfractionated heparin. Another method of anticoagulation is regional anticoagulation with citrate. Citrate is infused into the patient's blood as it enters the

extracorporeal circuit, and chelates calcium in the blood rendering it incapable of clotting. To prevent the patient from becoming hypocalcemic, calcium is restored as an infusion. Citrate is not used as often in IHD as in continuous therapies, but it may be useful in certain patients. When using citrate regional anticoagulation, the level of anticoagulation is assessed by measuring the ionized calcium concentration in the extracorporeal circuit. Once in the body, citrate is converted into bicarbonate; therefore, a chemistry analyzer capable of measuring both ionized calcium and pH becomes essential (Poeppel et al., 2011).

During intermittent hemodialysis, the patient's blood is exposed to many substances, including the dialysis catheter, blood tubing, chambers and headers, and the large surface area of the dialyzer membrane. These surfaces exhibit variable degrees of thrombogenicity (Suranyi and Chow, 2010). In order to deliver a safe and effective dialysis treatment, an appropriate level of anticoagulation must be achieved to prevent thrombosis of the extracorporeal circuit without causing excessive bleeding in the patient. Careful monitoring of the extractorporeal circuit during dialysis may provide many indicators of potential clotting problems. The simplest method of evaluation is visual inspection. Very dark blood within the circuit, streaks within the dialyzer or the presence of fibrin on the walls of the arterial or venous chambers may indicate clotting and should be further evaluated by flushing the circuit with saline while temporarily occluding the arterial blood line. (Ross, 2011).

In veterinary medicine, anticoagulation in routine intermittent hemodialysis typically consists of the systemic administration of a standard dose of heparin (10–50 U/kg) as a bolus 5 minutes before starting the dialysis treatment. Adequate anticoagulation is then maintained with a continuous infusion of heparin (10–50 U/kg/h) into the arterial limb of the circuit. The heparin infusion or bolus administration may be discontinued up to 30 minutes before the end of the treatment or continued throughout the treatment, depending on the patient's bleeding risk and the degree of clotting in the extracorporeal circuit (Ross, 2011).

In some patients presented for hemodialysis, systemic anticoagulation may be contraindicated. Patients who have recently (<48 hours) undergone surgery, biopsy or some other invasive procedure or patients with gastrointestinal hemorrhage, possible cranial trauma, pulmonary contusions or any evidence of active bleeding should not receive systemic anticoagulation because of the risk of inducing or exacerbating bleeding.

EQUIPMENT

There are several basic types of dialysis machines. In general, dialysis machines are designed to be used either for intermittent hemodialysis (IHD) or for continuous renal replacement therapy (CRRT). Regardless of the model or manufacturer, all modern IHD machines have certain common characteristics. First, they all contain a display screen, which may be a touch screen on newer models. This screen displays the current dialysis treatment mode, all options available in that mode, treatment parameters, alarm conditions and any necessary instructions. During the dialysis treatment, the screen also displays treatment status (ie, time left, amount of fluid removed, catheter pressures, and so forth). The electrolyte solution is a highly concentrated salt solution containing sodium, chloride, glucose and other components as desired (potassium, calcium, magnesium). The machine operator sets the desired sodium concentration of the dialysate (within the limits of the machine of between 130 and 155 mEq/L) based on patient parameters. The sodium concentration can be readily adjusted to avoid large or rapid changes in the patient's serum sodium concentration, thus avoiding dramatic fluid shifts. Bicarbonate is incorporated separately because bicarbonate and calcium from the electrolyte concentrate are incompatible in a concentrated form without inducing precipitation, thus allowing the bicarbonate concentration to be adjusted independently from sodium concentration, generally within the range of 25 to 40 mEq/L. (Poeppel et al., 2011).

A well-maintained water treatment system is essential to provide a safe hemodialysis treatment. The patient is exposed to roughly 20 gallons (76 L) of water in an average dialysis treatment, so even trace amounts of impurities can have detrimental effects (Ward, 2002; Van

Stone, 2004; Ward, 2008). Newer dialysis machines are capable of producing "ultrapure" dialysate. The dialysate made from purified water is filtered through a special membrane before it is passed into the dialyzer. Water and electrolytes are able to pass through the membrane, but any bacterial contaminants are excluded. The ultrapure dialysate is then delivered to the dialyzer that contains the patient's blood (Bommer and Jaber, 2006).

Because CRRT is intended to be provided over a longer treatment period (ie, 24 hours a day compared with 4–5 hours a day for IHD), a slower blood flow rate is generally selected. In addition, clearance with CRRT is influenced more by effluent rate than by blood flow rate, making a rapid blood flow rate unnecessary in most cases. Because the CRRT machines do not prepare dialysate, there is no need for internal conductivity meters. At the end of treatment, all of the fluid bags and blood lines are discarded, so there is no need for disinfecting or cleaning cycles for CRRT machines. One additional piece of equipment that should not be overlooked is a heat source. Patients undergoing IHD tend to be hypothermic due to their azotemia and hypotension. The temperature of the dialysate can be adjusted so that the patient's blood can be warmed (or cooled) as it passes through the dialyzer. Circulating water heating pads provide some (Poeppel et al., 2011).

COMPLICATIONS

Complications of IHD have been widely reported, and include hypotension and hypovolemia; problems with vascular access; and neurologic, respiratory, hematologic, and gastrointestinal complications (Cowgill and Elliott, 2000; Elliott, 2000). Hypotension and hypovolemia occur during IHD sessions as a result of ultrafiltration and large extracorporeal blood volumes and can persist during or between sessions as a result of blood loss (from bleeding secondary to uremic ulceration, overheparinization, coagulopathy or blood loss secondary to filter or line clotting in which not all extracorporeal blood volume can be returned to the patient). Approximately 50% of IHD cases have problems with hypotension and hypovolemia (Langston et al., 1997; Langston 2000). Respiratory signs can occur in IHD patients as a result of underlying disease, complications or IHD or both. Respiratory complications include uremic pneumonitis and pulmonary hemorrhage, pleural effusion and pulmonary edema, hypoxemia, hypoventilation, and pulmonary thromboembolism (PTE) (Elliott, 2000). Hypoxemia and hypoventilation can be caused by ventilatory failure in the critically ill or neurologically impaired patient; whereas hypoxemia can be caused by diffusion failure as a result of pulmonary hemorrhage, pneumonitis, infectious pneumonia, or edema, or ventilation-perfusion mismatch caused by PTE (West, 2008). Hematologic complications including anemia, thrombocytopenia, and leucopenia are also common in patients with IHD. Again, these complications can be caused by primary disease; anemia is a common sequel of CKD. Anemia and thrombocytopenia result from coagulopathy and vasculitis common with systemic inflammatory response syndrome and leucopenia can result from infectious or inflammatory processes. Thrombocytopenia can occur secondary to contact activation with the dialysis membrane, and promotion of the coagulation cascade as a result of disease-specific or iatrogenic coagulopathy, whereas leucopenia can occur transiently as a result of white blood cell interaction with the dialysis membrane (Elliott, 2000). Gastrointestinal complications such as nausea, vomiting and inappetance are common in uremic animals and can also be a complication of dialysis-induced hypotension, DDS, dialysate contaminants and incompatible blood transfusion reactions (Elliott, 2000; Fischer et al., 2004; Ross, 2010).

The most significant complications of CRRT involve coagulation. Despite appropriate heparin management, clotting of the CRRT circuit is inevitable. Hypotension is another potential complication. Although the cause of the blood pressure drop at the start of therapy is likely to be multifactorial, the amount of blood needed to fill the CRRT circuit is at least partly the reason (Sulowicz and Radziszewski, 2006).

Regarding the catheter complications, clothing is one of the most common complications. Despite using the least thrombogenic materials possible, hemodialysis catheters have a high rate of thrombosis. Both ports of the catheter should be flushed with saline or heparinised

saline after every use (approximately 10–12 mL for a large catheter, 3 to 6 mL for smaller catheters) to prevent intraluminal thrombosis (Beathard, 2001). Infections are the most dangerous catheter complication in hemodialysis patients and they are most probably the predominant cause of morbidity (Himmelfarb, et al., 2005). Instillation of antimicrobial solutions, such as citrate or heparin combined with an antibiotic, may reduce the risk of bacteremia (Donlan, 2001).

CONCLUSIONS

IHD is a useful and feasible modality to improve outcome in dogs and cats with kidney injury that do not respond adequately to medical management. The decision to pursue hemodialysis in patients with acute or acute-on-chronic kidney injury should be made as quickly as possible to improve the likelihood of a successful outcome. IHD requires thorough understanding of renal physiology, as well as the principles and machinery involved in dialysis.

CRRT is a relatively new extracorporeal blood purification modality for the treatment of AKI, fluid overload and toxin exposure. Although CRRT has both therapeutic and operational advantages compared with IHD, its intensive nature and the need for specialized 24-hour care will likely limit the availability of this modality to a small number of referral institutions.

Monitoring catheter performance should be a routine part of dialysis patient care. Dual-lumen catheters are the most commonly used method of vascular access for extracorporeal renal replacement therapy. They are fairly quick to place but require meticulous care for optimal function. The most common complications are thrombosis and infection.

Several methods to prevent extracorporeal circuit clotting during hemodialysis have been used, but unfractionated heparin remains the mainstay of anticoagulant therapy in both human and veterinary intermittent hemodialysis.

There are several different machines available for the performance of renal replacement therapy in veterinary medicine. Extracorporeal renal replacement therapies (IHD and CRRT) involve dedicated personnel who are familiar with the operations and maintenance of the equipment.

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CONGENITAL PERITONEOPERICARDIAL DIAPHRAGMATIC HERNIA (PPDH) IN A MIXED BREED DOG

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Abstract

Congenital pericardial diseases are rare in dogs and cats and most part of them were reported as incidental findings on radiography or necropsy examinations. In this case report, a definitive diagnosis of congenital peritoneo-pericardial diaphragmatic hernia (PPDH) in a three year old male mixed breed dog (Canis lupus familiaris) is described. The dog was referred in relatively shock status with a history of anorexia, dyspnea, cough, abdominal distension, progressive weight loss, but died after conservative treatment was applied. At necropsy examination, a cranial displacement of abdominal viscera (portion of right hepatic lobe) into the pericardial sac was observed, while the pleural space was intact. Supplementary, another dog from the same nest/littermates (3-year-old female), which died in identical conditions was submitted for post-mortem investigations and showed no congenital abnormalities.

Keywords: *PPDH, congenital diaphragmatic hernia, pericardial disease*

INTRODUCTION

Peritoneopericardial diaphragmatic hernia is a common congenital abnormality which involve the pericardium of dogs and cats. These congenital abnormalities were reported as incidental radiographic findings while evaluating other problems or at necropsy examination (Keirandish et al. 2014, Statz et al. 2007). PPDHs occurs as a result of an embryonic development defect of the dorsolateral *septum transversum* in the so-called sternocostal triangle structure. Persistent communication between the peritoneal and pericardial cavities allows the abdominal organs to herniate into the pericardial sac without involving the pleural space (Berry et al. 1990, Evans et al. 1980, Keirandish et al. 2014, Nelson et al. 2014). Other congenital anomalies such as umbilical hernias (most frequent), hydrocephalus, sternal defects, cranial midline abdominal hernias, abnormal swirling of hair on the ventral abdomen, intra-cardiac defects can be associated with this anomaly (Bellah et. al. 1989, Evans et al. 1980, Neiger 1996, Hunt et al. 2003, Statz et al. 2007, Wright et al. 1987). The initial onset of clinical signs associated with PPDH can occur at any age (ages between 4 weeks and 15 years have been reported). The majority of cases are diagnosed during the first 4 years of life. In some animals, clinical signs never develop. Males appears to be more affected than females. Literature mentions Weimaraners among the dog breeds that show a predilection for this disorder, as PPDH with a percentage of 0.5% of their congenital cardiac diseases (Evans et al. 1980, Nelson et al. 2014). A similar percentage of feline peritoneo-pericardial diaphragmatic hernia is frequently identified in Persian cats, whose pattern of inheritance is consistent with an autosomal recessive trait (Dêbiak et. al. 2009, Neiger 1996).

MATERIAL AND METHODS

A mixed-breed dog (3-years-old male of *Canis lupus familiaris*) weighting around 12 kg, body lenght of 45 cm was submitted for postmortem

investigations: necropsy and histopatological examinations.

RESULTS AND DISCUSSIONS

Postmortem changes prove a period of approximately 24±8 hours after the installation of death. To sustain this observation specific postmortem changes appear, as corneal dehydration and hemoglobin imbibition. General appearance attests a good maintenance.

The pericardial sac was severely deformed with a marked increased volume and presenting a breach in the intimate contact with the diaphragm in the form of hernial ring about 2 cm width, through which a portion of the diaphragm conjunctly with about 35% of the right medial lobe of liver (Fig. 1).

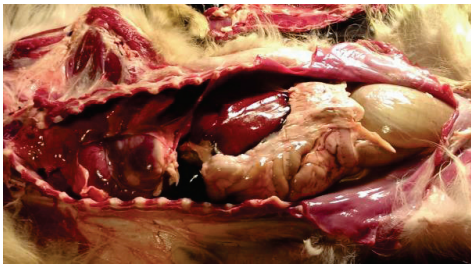

Fig. 1 – Abnormal topography of liver, mixed breed dog, 3 years

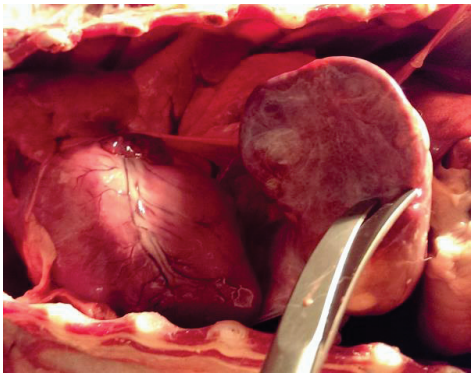

Fig. 2 – Section in the pericardial sac with heart and portion of liver

The diaphragmatic membrane hernias's portion was intact, engaging with it a liver lobe which is strangled by the hernial ring described above. The part of liver spotted in pericardial cavity with the diaphragm shows a whitish pink color, suggesting an increased fibrosis consistency and presenting a visible stricture that demarcate the affected portion of adjacent liver tissue (Fig. 3).

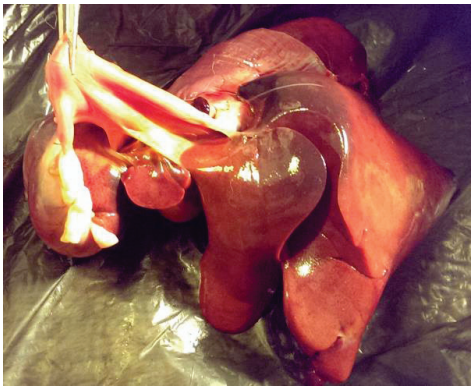

Fig. 3. - Part of right medial lobe liver spotted in pericardial cavity

The hepatic fragment mentioned and the diaphragm hernias portion showed no adherence to adjacent tissues (Fig. 4).

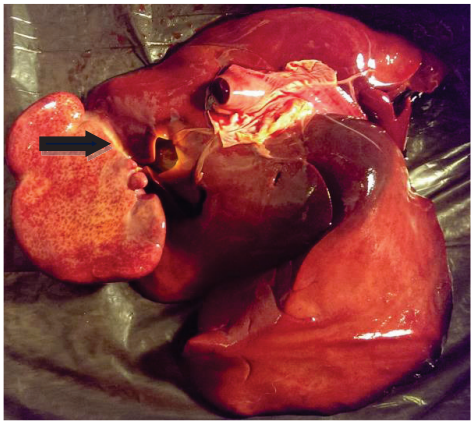

Fig. 4. Increased hepatic fibrosis and ring of strangulation (black arrow)

The right atrium presented an increased volume (partial atrial dilatation),the left ventricle being reduced. Endocardium presented thickening of right atrioventricular valve with corresponding features for vegetative endocarditis and left ventricular hypertrophy.

The lesion from the lungs was represented by incipient pulmonary oedema (frothy exsudate was present in the trachea and bronchi but not in the nasal cavity).

The spleen presented lack of normal tissue continuity (fragmented) with marked areas of fibrosis, suggesting an previous injury/trauma. The urinary bladder showed a moderate amount of urine (aprox. 100 ml) and insignificant quantity of yellow-orange dry, putty-like material. The stomach presented scant red fluid and no distension. Postmortem discoloration occurred in intestine. The kidneys were normally situated and the capsules striped easily revealed a smooth surface. The corticomedullary demarcation was obscured by congestion.

Histopathology examination:

Lungs presented diffuse distension of alveolar and interstitial capillaries. The alveolar space was occupied by protein-like material mixed with red blood cells (hemorrhages and oedema). The interstitial space was increased by moderate mononuclear infiltrate, also discreet in some areas. The bronchioloalveolar space was frequently blocked by mucus, desquamated cells and inflammatory cells (neutrophils and macrophages).

Liver: The hepatic parenchyma entrapped in the pericardial sac presented abundant connective tissue especially in the porto-biliary space. Additionally, fibrosis area were associated with marked bile duct hyperplasia. The spleen keeps histological architecture with a clear demarcation between the red and the white pulp.

The most severe lesions identified in gross and histological examination were identified in respiratory system expressed as interstitial pneumonia and oedema, the other injuries being discrete changes, secondary to respiratory causes and agonic state. The congenital anomaly, incidentally detected, caused functional disorders of cardiac activity without major pathological implications. The lesions described in lung may be the result of infectious disease (Distemper/ Kennel cough). The other dog from the same nest which was examinated had the same macroscopical lesions excepting the peritoneopericardial diaphragmatic hernia. As with other diaphragmatic herniations, clinical signs of affected animals may vary from none to severe respiratory impairment, depending on herniated organs and degree of organ damage. The liver and gall bladder are herniated most frequently, followed by small intestine, spleen and stomach. (Ettinger et. al.2017). Peritoneopericardial diaphragmatic hernias are rare but should be included as a differential diagnosis in dogs with enlarged cardiac silhouette. The main tool for diagnosis of congenital diaphragmatic hernia is radiography. In this case the injuries provoked by congenital abnormality didn't cause the death of the studied animal. The case reports available in the literature indicate that the intensity of clinical symptoms vary according to size of diaphragm defect, as well as the kind and volume of the herniated organs (Dêbiak et al. 2009).

CONCLUSIONS

Peritoneopericardial diaphragmatic hernia in dogs can be an incidental finding during necropsy examination.

Depending on the volume of the herniated organ can be appreciated if it is a cause of death. The fragment of liver surprised in the pericardial sac presented ischemia with degeneration and secondary fibrosis. The cause of death in the examinated dog was respiratory insufficiency.

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ANIMAL PRODUCTION, PUBLIC HEALTH AND FOOD QUALITY CONTROL

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ANTIMICROBIAL EFFECT OF COMMERCIAL MANUKA HONEY AND CONVENTIONAL LOCAL HONEY AGAINST GRAM-NEGATIVE AND GRAM-POSITIVE BACTERIA

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Abstract

Introduction: Due to the escalating antimicrobial resistance of numerous frequent pathogens, research on natural antimicrobial compounds is intensively published. One of the most in trend, yet controversial antibacterial natural products, is the Manuka honey. Manuka honey, produced from the Manuka (Leptospermum scoparium or Leptospermum polygafolium) tree, contains a unique antimicrobial factor (Unique Manuka Factor, UMF), which is absent in other types of honey.
Aims: Commercial Manuka honey was investigated for assessment of antimicrobial effect against different Gram-negative and Gram-positive bacteria.
Materials and Methods: Two types of Manuka honey with different UMF and one local polyfloral honey were assessed for antimicrobial activity against Staphylococcus sp., Streptococcus sp., Listeria sp., E coli, Salmonella sp. Pure broth cultures were pour plated on agar and incubated. Each type of honey was spotted in a marked place on the agar, pending the examination of inhibition areas after another incubation period.
Results: Commercial Manuka honey has antimicrobial activity for Staphylococcus sp., E.coli and Salmonella sp., but not for Streptococcus sp. and Listeria sp. No antimicrobial effect was noticed for regular polyfloral honey.
Conclusion: Using the described method, Manuka honey revealed an antimicrobial effect against Staphylococcus sp., E.coli and Salmonella sp., the intensity of which was directly proportional with UMF.

Key words*: antimicrobial effect, Manuka honey, Unique Manuka Factor .*

INTRODUCTION

Manuka honey, produced from the Manuka (Leptospermum scoparium) tree contains a unique antimicrobial factor (Unique Manuka Factor, UMF), which is absent in other types of honey (Molan and Russel, 1988). The escalating microbial resistance of various pathogens is keeping research concerning natural antimicrobials in the spotlight. The antimicrobial effect of pure New Zealand, Manuka honey was revealed *in vitro* against some pathogenic bacteria (Sherlock *et al.*, 2010; Lin *et al.,* 2010). Nevertheless, in public opinion, the antibacterial properties of commercial Manuka honey are highly controversial (Niko, 2009).
Honey has been known for centuries for its beneficial effects over the human organism, being used as a palliative treatment of various diseases and lesions, such as wounds, mycotic (fungal) infections, eczema, skin infections, ulcers etc. The literature indicates that the antibacterial properties of honey are generally mostly due to hydrogen peroxide content, and to a specific high osmolarity (80% w/v sugar) (Alvarez-Suarez J.M. et al., 2010). Some authors indicate that the antibacterial properties of honey are the result of a complex of synergic factors, such as phenolic compounds, hydrogen peroxide, pH and osmolarity (Alvarez-Suarez J.M. et al., 2014).
Manuka honey has been standing out since the 80's, for its particular, higher antibacterial effect, compared to conventional honey. Dr. Peter Molan from University Waikato of New Zealand was the first who proved by inactivating the peroxidase activity in Manuka honey, that it exhibits an intense antibacterial activity, heat and light resistant, called non

peroxide activity (Molan P.C. et al., 1988). The non peroxide activity was later attributed to certain organic chemical compounds, namely the 1,2-dicarbonyl compounds; the list of 1,2-dicarbonyl compounds was later narrowed down to methylglyoxal (MGO), which was awarded with all the credit for the antibacterial superiority of Manuka honey in comparison with conventional honey (Adam C.J. et al., 2008; Mavric E., et al., 2008). The non peroxide activity is undoubtedly related to the methyglyoxal (MGO) concentration. The MGO comes from the decomposing of dihydroxyacetone, a compound which is found in high concentrations in the nectar of *Leptospermum scoparium* flowers). MGO concentration in Manuka honey is measured in ppm, while the non peroxide activity (NPA) is measured as percent phenolic equivalent, and not by MGO concentration. Molan P. (2008) showed that the antibacterial properties of Manuka honey does not depend directly of its MGO content, revealing the following equation which explains the nature of the MGO-UMF relationship: Antibacterial activity = [0,0275 x MGO (mg/kg)] + 7,826. Therefore, there is a certain synergy in MGO's action when found in honey, proven by comparison with MGO in water solutions. The synergy proves that the pronounced antibacterial activity of Manuka honey is not exclusively due to MGO, but is also related to other factors, such as the nectar quantity of honey.

MATERIALS AND METHODS

We investigated the antimicrobial effect of different types of commercial Manuka honey available in para-pharmaceutical stores in Bucharest, against different Gram-negative and Gram-positive bacteria.

Three types of honey were used in this experiment in order to compare results: Manuka honey UMF 10, Manuka honey UMF 15 and local polyfloral honey. Pure bacterial cultures of *Staphylococcus sp*., *Streptococcus sp*., *Listeria sp*., *E coli*, *Salmonella sp*. were grown in nutrient broth. All 18-24 hours old broth cultures were transferred on nutrient agar, by pour plating procedure. After drying the agar surface, a loop full of each type of honey was spotted in a marked place, pending 24 hours incubation at 37°C (European Committee on Antimicrobial Susceptibility Testing, 2012). Controls were prepared with seeded agar without honey and were incubated in the same conditions. After the incubation interval, all Petri dishes were examined for clear areas of bacterial inhibition.

RESULTS AND DISCUSSIONS

All controls grew noticeable, relatively uniform cultures after the incubation end due time. No clear areas were noticed for the polyfloral honey plates. Following the investigation of the antibacterial properties of various types of commercial Manuka honey towards some Gram negative and Gram positive bacteria, there is noticeable antimicrobial activity of the tested samples of Manuka honey against *Staphylococcus sp*., *E.coli* and *Salmonella sp*. No antimicrobial activity was seen for *Streptococcus sp.* and *Listeria sp.* No antimicrobial effect of the conventional honey used as control, was noticed for the above mentioned bacteria species.

Following the comparative assessment of UMF 10 and UMF 15 Manuka honey (fig. 1, 2 and 3), the inhibition area was greater for UMF 15 than UMF 10 Manuka honey (5 and 2 mm for Staphylococcus -fig. 1, 4 and 3 mm for E.coli - fig.2, 6 and 5 mm for Salmonella sp., respectively- fig.3). (according to the method described by the European Committee on Antimicrobial Susceptibility Testing, 2012).

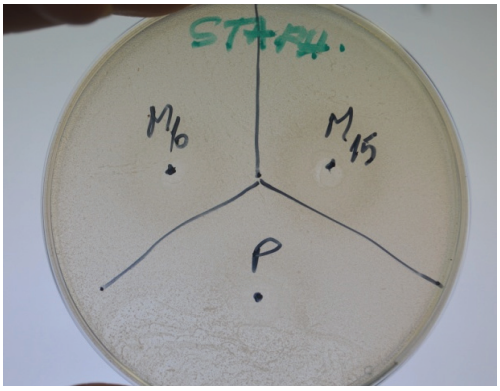

Figure 1. The antimicrobial activity of three types of honey: UMF10 Manuka honey (M_{10}), UMF15 Manuka honey (M_{15}) and polyfloral honey (P), against *Staphylococcus sp.*

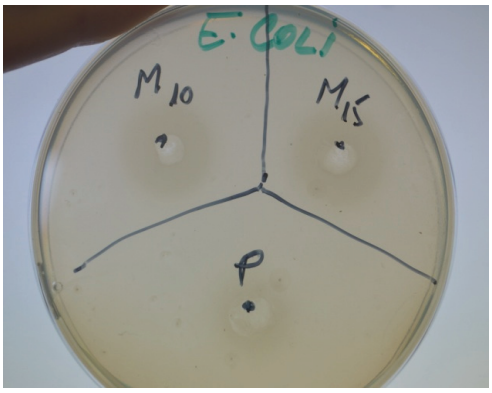

Figure 2. The antimicrobial activity of three types of honey: UMF10 Manuka honey (M_{10}), UMF15 Manuka honey (M_{15}) and polyfloral honey (P), against *Escherichia coli*

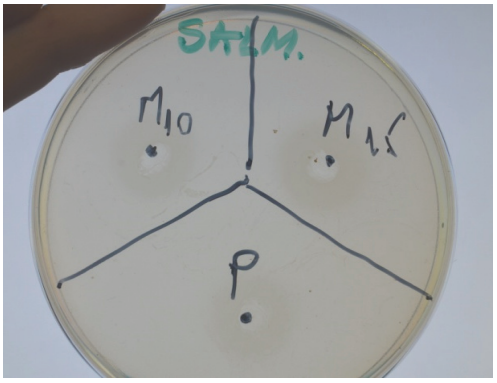

Figure 3. The antimicrobial activity of three types of honey: UMF10 Manuka honey (M_{10}), UMF15 Manuka honey (M_{15}) and polyfloral honey (P), against *Salmonella sp*

Many authors communicated noticeable antimicrobial effect of Manuka honey against various pathogens, but there are insufficient studies comparing different UMF Manuka honeys, amongst each other as well as with other types of honey, in terms of antimicrobial activity. Similar marked differences of antimicrobial activity intensity between Manuka honey and other types of honey were also communicated by other authors, such as Willix (1992), or Sherlock (2010).

Nevertheless, aside this study, there were no comparisons made between samples of Manuka honey with different UMF values and conventional honey. Moreover, most authors used original Manuka honey, tested for its content of antimicrobial compound, while regular commercial Manuka honey, was used in this study.

While the peroxidase activity varies within large limits considering the various honey assortments, and with harvesting time, heat and light exposure, the non peroxide activity is constant and stable in time. This is probably the reason why the non peroxide antibacterial activity of Manuka honey is more intense than the regular peroxide activity in conventional honey.

Nevertheless, there are studies which indicate significant antibacterial activity for certain conventional honey assortments. For example, the minimum inhibitory concentration, MIC (defined as the lowest honey concentration at which there is an inhibition of the visible growth of the organisms on the Petri dish) communicated by Bourabah A. et al. (2014), for conventional Algerian honey, over some bacterial strains of goat mastitis milk, was between 11-14%, which is comparable to Manuka honey MIC values (6-25% v/v) over various bacterial pathogens.

There are insufficient studies indicating a comparative evaluation of the antibacterial properties of Manuka honey and the antibacterial properties of different conventional honey assortments (Tan H.T. et al., 2009; Shahina S. et al., 2013). This is the reason why, the relevance of results concerning this issue may become questionable.

Using conventional honey in research activities that focus on Manuka honey would be extremely valuable, as conventional honey is a control sample group that might help with the relevance of results; in addition, such a control group might be useful in eliminating the suspicions upon the Manuka honey authenticity.

Moreover, such an approach would reduce the consumers' reluctance for Manuka honey (Niko K., 2009), which leads to a higher preference for local, conventional honey, in detriment of exotic Manuka honey, despite the valuable properties of the latter.

Therefore, the cultural aspect of honey consumption would be more easily separated from the medicinal properties and indications of Manuka honey.

CONCLUSIONS

Commercial Manuka honey has antimicrobial activity for *Staphylococcus sp*., *E.coli* and *Salmonella sp*., but not for *Streptococcus sp.* and *Listeria sp.* The intensity of this effect was directly proportional with the UMF. No antimicrobial effect was noticed for regular polyfloral honey.

The differences existing between the synergy of various honey assortments, with different floral sources, remain unexplained, as a current subject of tremendous scientific interest. Therefore, the research aiming the evaluation of the antibacterial properties of Manuka honey should include honey with different UMF values, along with the comparison with conventional honey, as control.

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ORGANIC AGRICULTURE – THE GUARANTEE OFFOOD SAFETY AND POPULATION HEALTH

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Abstract
The correlation between nutrition and the population health in the context of increasing the range of food products provided to the population, and quantifying their metabolic effects with frequent episodes of illness due to consumption of impropers food products, while have led to increase the population requirements for purchase of food with high food safety guarantees. Lately we remark from the consumers' part, that they provide a greater degree of confidence to organic products, in the detriment of conventional food. It seems that the high incidence of cancers, the increasing resistance to germs at treatments and more aggressive forms of manifestation of certain diseases, it could be reduced to extinction in the case of using organic food. Food safety has as main objective to guarantee the lack of harmfulness, toxic character and those factors which could cause illness to the consumers. By promoting certain processing technologies, along with no use of pesticides and genetically modified organisms or banning prophylactic antibiotic administration and hormonal preparations as growth promoters, the organic farming can supply food products to high standards of food safety.

Key words: *food products, food safety, organic agriculture, population health.*

INTRODUCTION

The nourishment represents for the population a permanent action factor, which determines the deployment of metabolic processes. It also maintaining the body homeostasis depends on the character of nutrition, influencing the human system functions, through enzymatic and hormonal factors (Ilie, 2007).

The quality of raw materials, unprocessed, is essential to the safety and quality of the finished product. Therefore, is necessary to a systematic approach along the whole food production chain, to prevent the food contamination and to identifying some possible risks associated with food production.

Providing food for the population into continuous growing and obtaining the varieties with high productivity, resistant to diseases and pests, are just some of the motivations for use in conventional agriculture of the fertilizers, the genetically modified organisms or a „modern" technology from land management (Tăpăloagă, 2014).

The damage of these actions are extremely serious, both for the environment: starting from reducing soil fertility, increasing the danger of erosion, changing the biotic balance, and for the population to, as a result of penetration of these toxins in the atmosphere, soils, surface water and foodstuffs, through residual doses of plant and animal products.

The risk food become contaminated exists throughout the food chain (Ilie, 2013). In general, food safety is threatened by the factors that fall into three categories. The first category is the most dangerous and it is represented by the biological contaminations which include bacteria, fungi, viruses or parasites that may contaminate raw materials and finished products. Chemical contaminants are the second category and include the chemicals from the environment, veterinary drug residues, heavy metals or other residues that reach accidentally in food, during the processes that involve agriculture practices or animal breeding and raising poultry, processing, transport or packaging food products. The third category includes the foreign bodies, metal fragments, broken glass, plastics accidentally reached in foods and that affects a limited number of customers, most frequently without endangering their lives (ILSI, 2011).

Till recently, the use of synthetic chemicals, including pesticides, it was a common practice. The use of pesticides, including insecticides,

fungicides, herbicides, rodenticides, in order to protect the crops against the pests, significantly reduced the losses, increasing the crop yields (vegetables and fruits) and protecting animals and humans to certain diseases (Carvalho, 2006). Today the use of these compounds is prohibited in countries with organic farming. The danger is still represented by the residues of these chemicals released into the environment that can contaminate food.

MATERIALS AND METHODS

1. Food safety

The concept of „food security" it's difficult to insure, of the one part due to diversify the range food products varieties, and on the other way, due to more intense atmospheric pollution, by emanations of toxic gases, by the specific activityes from industry and agriculture (Ilie, 2007). The harmful elements are increasingly numerous, requiring the enlargement of the area from the laboratory determinations and establishing new techniques, more efficient and faster.

Food safety concept used today includes the whole food chain intended for consumption by animals or humans. This is based on a series of regulations by which are assigned attributions and responsibilities of all those engaged in food chain, starting from livestock farmers, to raw material suppliers, including the processors and up to traders who must ensure the wholesomeness and quality of food purchased by the buyer (Ilie, 2013). Through this monitoring of all stages of food production, can be evaluated the risks of food chain, which may have direct or indirect effect on food safety provided, including animal welfare and plant health (Hansen et al., 2002).

2. Population health

Obtaining and providing increased amounts of the foodstuffs unfortunately is not synonymous with a high quality of their. The pollution of soils and water with heavy metals, nitrates, nitrites, hormones and bacterial toxins it led to the emergence and evolution of fulminant diseases such as autoimmune diseases, nutrition disorders and cancer (Carvalho, 2006).

The people's worries are supported by a series of events, such as: the pressence of Salmonella in meat and eggs, the identification of Listeria monocytogenes in milk and milk products, discovery animals with BSE, high levels of pesticides, antibiotics or additives in different foods or selling non-organic products as organic products (Hansen et al., 2002).

In most cases, the consumer is attracted to food that „looks good" from point of view of packaging and form of presentation, on the second place being the label which shows the safety and nutritional value of the product concerned.

3. Organic agriculture

Industrial agriculture began to be replaced with organic farming, which has as objectives firstly preserving the integrity of the biosphere and obtaining of food products by the rational exploitation of existing resources in the agro-ecosystem. This will ensure a high level of current population health and future generations, as a direct consequence of the consumption of a superior quality and salubrity food in a healthy environment (Rundgren, 2006).

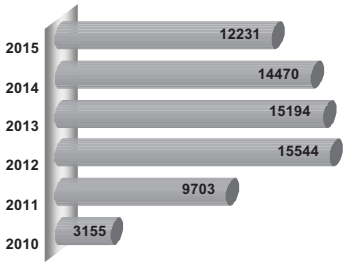

Figure 1. The number of operators certified in organic agriculture (http://www.madr.ro/ro/agricultura-ecologica)

Conventional agriculture has as main objective obtaining of quantities of increasingly food, even if this involves the use of technologies and substances which have a fully unclear effect in long term. The organic agriculture is considered an alternative to conventional agriculture systems (Tocan, 2013), who gathering every year increasingly more operators to be certified in this area, as can be seen from the Figure 1.

Although different countries use different terms such as organic, biological or ecological, they have the same meaning and designate one and the same, relying on the principles and practices of Standards International Federation

of Organic Agriculture Movement (IFOAM). They understand that can be obtained sufficient quantities of high quality food by using natural systems which must preserve the plant and animal diversity and not unilateral production increase by destroying of others (Scialabba, 2007).

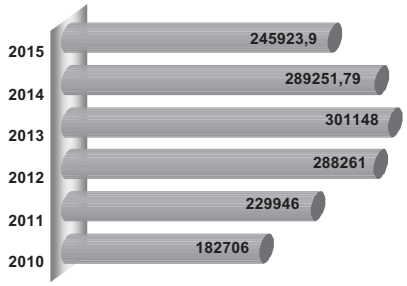

Figure 2. The total area under organic agriculture (ha) (http://www.madr.ro/ro/agricultura-ecologica)

An alternative for increasing the food production could be represented by expanding the cultivated areas, as can be seen from Figure 2. But this creates two additional problems, namely: increasing the consumption of water required for irrigation and increased need for farmers to work these new surfaces. Considering that the water resources per capita decreases from year to year, it requires the use of plant varieties resistant to environmental conditions of the region.

RESULTS AND DISCUSSIONS

The increasing of soil fertility can be done through proper management on crops rotation and using of manure, using the dung, to the detriment of using fertilizers, pesticides or growth stimulants. As the ground level there are more plants and living beings, the soil biological value is higher.

Livestock farming of productive, in intensive systems, using modern breeding biotechnique, associated with modern systems processing, they have improve people's living conditions through access to a wide range of food products of great diversity (Tăpăloagă et al., 2016).

In recent years they have taken a special scale animal welfare issues. Organic principles and regulations require that animals be applied to human treatment that does not cause pain, being forbidden to generate suffering by ill-treatment. Also, they are encouraged the husbandry practices and exploitation of breeds adapted to local operating conditions, resistant to disease, which benefit from microclimate conditions, comfort and organic feed to exploit their productions at maximum capacity, without using growth stimulants or GMOs.

Concepts like „sustainable development"or "sustainability" they are still widely discussed subjects in order to establish of priority of ensuring environmental quality and increase the food production (Davidson, 2005). It must be considered that in order not to endanger future generations it is necessary to achieve well-being of present population with environmental protection and rational use of natural resources (Scialabba, 2007).

According to a FAO Organic Agriculture and Food Security report on organic farms (2006), the stability of organic agro ecosystems is sustained by increasing soil organic matter and microbial biomass (Chaoui and Sorensen, 2008).

An increasing number of scientific studies have shown that agriculture is one of the main factors that play a role in climate change. The organic agriculture contributes to reducing emissions of greenhouse gases, global warming or acid rain (Hansen et al., 2002).

The organic agriculture is based on principles, which is based on best practices designed to minimize human impact on the environment (Gonciarov, Neagu and Tăpăloagă, 2014).

The specific practices of organic agriculture include: prohibiting the use of pesticides, synthetic chemical fertilizers, antibiotics, growth stimulators and genetically modified organisms. It encouraged the use of plant varieties resistant to pests and diseases, adapted to local conditions, growth animals in open spaces and feeding with organic feed or crops rotation for efficient and at the same time renews the soil.

CONCLUSIONS

The role of organic agriculture is to produce safe food, fresh, higher quality, with minimal intervention by a human using local resource.

Using the best performing varieties, resistant to disease and enabling higher yields, it may be a more prudent contributing to food security.

Maintaining animal health status of the premises for obtaining foodstuffs of high nutritional value, which have very low production associated risks of diseases associated with food consumption among consumers.

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COMPARATIVE STUDY ON YIELD QUALITY OF GRAIN LEGUMES PROMOTED BY ORGANIC AGRICULTURE

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Abstract

This paper presents the results of the research on the yield quality of some grain legumes (lentil, faba bean, chickpea, fenugreek, mung bean and adzuki bean) promoted by organic agriculture.
In order to determine the quality of grain legumes, there were analysed several physical indicators, such as the thousand grain weigh (TGW), the hectolitre mass (MH), the moisture content (%) and chemical indicators, such as the protein content (% d.m.), the carbohydrates content (%), the fat content (% d.m.), the ash content (%). The seeds energetic value (Kcal.%) was calculated as well.
In order to determine the TGW, 8 *samples of 100* seeds *were weighed, while to determine the MH, the Hectolitre Measuring System - Chondrometer, with a capacity of 0.5 l, was used. The following methods were used for the determination of the biochemical compounds of seeds: for carbohydrates - the Bertrand method; for proteins - the Kjeldahl method; for fats - the Soxhlet method and for ash - the Spectrophotometer method.*
On average, the chemical composition of these crops was the following: for lentil - 22.50% proteins, 2.81% fats, 63.56% carbohydrates, 3.95% ash, and an energetic value of 358.47 kcal.; for faba bean - 21.60% proteins, 4.45% fats, 63.90% carbohydrates, 5.90% ash, and an energetic value of 366.77 kcal; for chickpea - 21.19% proteins, 4.30% fats, 56.17% carbohydrates, 3,31% ash, and an energetic value of 356.16 kcal; for feenugreek - 21.24% proteins, 4.66% fats, 63.81% carbohydrates, 5.71% ash, and an energetic value of 360.58 kcal.; for mung bean - 23.23% proteins, 2.08% fats, 68.09% carbohydrates, 3.88% ash, and an energetic value of 362.89 kcal.; for adzuki bean- 21.9% proteins, 2.6% fats, 69.3%carbohydrates, 4.1% ash, and an energetic value of 361.14 kcal.
The nutritional value results for these grain legumes highlighted the very special role that they should play in the development of biodiversity as well as in the diversification of human and animal feeding.

Key words*: chemical composition, grain legumes, nutritional value.*

INTRODUCTION

Grain legumes (or pulses) are important food crops that can play a major role in addressing global food security and environmental challenges and they contribute as well in healthy diets (www.fao.org/fsnforum/activities/discussions/pulses).
Grain legumes are a vital food resource that helps meet food requirements in human diets in different parts of the world (Roman et al., 2011).
Grain legume seeds are excellent sources of proteins, vitamins, minerals, fibres and polyunsaturated fatty acids (Tharanathan, 2003; Bouchenak & Lamri-Senhadji, 2013).
Depending on species, the protein represents between 20 and 40% of the grain mass (Roman et al., 2011).
In general, the grain legumes are rich in lysine but poor in methionine content, thereby complementing the reverse amino acid pattern found in cereals (Hymowitz, 1990).
Legumes are normally consumed after processing, which not only improves palatability of foods but also increases the bioavailability of nutrients, by inactivating trypsin and growth inhibitors and hemagglutinins (Tharanathan, 2003).

Leguminous plants can be used in animal feed, green or silage, alone or in mixtures. The by-products of legumes (stems, leaves and husks) resulted after threshing, have a high protein content (8-14%) which is exceeding 10 times the protein content of cereal straws (0.7-1.3%) making them suitable for feed purposes (Roman et al., 2011).
Additionally, all grain legumes fix their own nitrogen, thereby reducing, in many situations, the cost of nitrogen inputs by farmers (Hymowitz, 1990).

MATERIALS AND METHODS

The aim of the paper is to present the results of the research conducted in 2016 on the quality of some grain legumes promoted by organic agriculture.
The biological material used in the research came from agricultural product markets in Romania, Greece, Turkey, France and Slovenia and from the UASVMB Biobase field.
The studied species were the following: lentil (*Lens culinaris*), faba bean (*Vicia faba*), chickpea (*Cicer arietinum*), fenugreek (*Trigonella foenum-grecum*), mung bean (*Vigna radiata*) and adzuki bean (*Phaseolus angularis*).

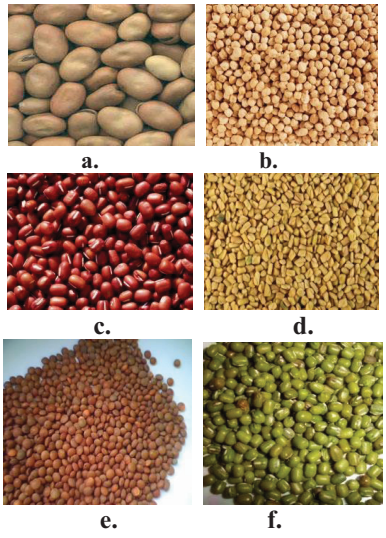

Figure 1. Biological material used in the research a- faba bean, b- chickpea, c- adzuki bean, d- fenugreek, e- lentil, f- mung bean

The determinations were performed in laboratory and targeted the physical quality parameters (thousand grain weight, hectolitre mass and moisture) as well as the chemical parameters (carbohydrates, protein, fats and ash contents).
The TGW was determined by weighing 8 samples of 100 seeds, while MH by using the Hectolitre Measuring System - Chondrometer with a capacity of 0.5 l. In order to determine the biochemical compounds, chemistry laboratory methods were used: for carbohydrates - the Bertrand method; for proteins - the Kjeldahl method; for fats - the Soxhlet method; for ash - the spectrophotometer method.
The determination of the energetic value involved highlighting the relationship between the necessary daily nutrients and the contribution of these substances per product unit (usually 100 g).
The formula for calculating the energetic value of grain legumes seeds (kcal) was:
Energetic value = % proteins x 4.1 + % fats x 9.3 + % carbohydrates x 4.1

RESULTS AND DISCUSSIONS

Physical quality parameters. The data from Table 1 show that for lentil, the thousand grain weight was on average of 67.83 g, the hectolitre mass - 86.58 kg/hl and the moisture content of the seeds - 12.20%. It can be observed that Turkey variety registered the lowest mass per storage volume (85.98 kg/hl), this amount being correlated with small TGW and moisture values of seeds.
For faba bean seeds, TGW was on average of 510 g, hectolitre mass - 80.8 Kg/hl and moisture content - 13%. It should be noted that for all the physical parameters analysed there were very small differences between varieties.
Chickpea seeds had the following values for the physical parameters: TGW - 370.75 g, hectolitre mass - 79.53 kg/hl, moisture content - 12.80%.
The thousand grain weight for fenugreek seeds was on average of 13.10 g, the highest value of 14.90 g being determined for Greece variety seeds and the smallest value (11.89 g), for Turkey variety. It can be observed that the Greece variety registered the highest moisture

and hectolitre mass values, i.e. 12.80% moisture and 97.7 kg/hl MH.

The mung bean seeds registered on average the following values for the physical indicators: 158.53 g for the thousand grain weight (TGW), 77.4 kg/hl for the hectolitre mass and 13.20% for the moisture content.

For adzuki bean seeds, the thousand grain weight registered values ranging from 135.9 g to 142.3 g, the average value being of 130.1 g. The hectolitre mass was on average of 76.2 kg/hl, with variation between 75.9 kg/hl for Slovenia variety and 76.4 kg/hl for Greece variety.

Table 1. Physical quality parameters of grain legumes

Species	Variety	TGW (g)	MH (Kg/hl)	Moisture (%)
Lentil	France	67.86	86.37	12.20
	Slovenia	68.23	87.40	12.50
	Turkey	67.40	85.98	11.90
	Average	**67.83**	**86.58**	**12.20**
Faba bean	UASVM Biobase	511.0	79.9	12.96
	Greece	509.0	80.7	13.01
	Turkey	510.0	81.8	13.04
	Average	**510.0**	**80.8**	**13.00**
Chickpea	UASVM-Biobase	358.93	77.9	12.80
	Slovenia	363.31	78.8	12.78
	Greece	400.67	81.7	12.83
	Turkey	360.10	78.1	12.81
	Average	**370.75**	**79.53**	**12.80**
Fenugreek	Slovenia	12.52	86.1	11.09
	Greece	14.90	87.7	11.13
	Turkey	11.89	86.0	11.07
	Average	**13.10**	**86.6**	**11.10**
Mung bean	France	162.96	76.6	13.33
	Slovenia	154.11	77.2	13.07
	Average	**158.53**	**77.4**	**13.20**
Adzuki bean	Slovenia	135.9	75.9	12.90
	Greece	142.3	76.4	12.50
	Average	**139.1**	**76.2**	**12.70**

Biochemical parameters. In the analysis of the grains legumes chemical composition, the highest protein content values were registered for faba bean seeds (27.07%), followed by mung bean seeds with 23.23% protein. The lowest values were registered for chickpea seeds i.e. 21.19%. Lentil seeds had a medium protein content of 22.50% (table 1).

The fat content of the studied species ranged from 1.90% to 4.30%. Higher fat content was observed at chickpea species (4.30%) and faba bean species (3.35%).

The lowest values were registered for lentil, fenugreek, mung bean and adzuki bean seeds with 2.81%, 2.66%, 2.08% and 1.90% fat content.

Table 2. Chemical composition of grain legumes (% d.m.)

Species	Variety	Quality parameters*			
		P (% d.m.)	F (% d.m.)	C (% d.m.)	M (% d.m.)
Lentil	France	22.50	2.87	58.10	4.00
	Slovenia	22.85	2.95	58.98	3.94
	Turkey	22.23	2.61	58.61	3.91
	Average	**22.50**	**2.81**	**58.56**	**3.95**
Faba bean	UASVM Biobase	26.65	3.40	53.90	3.85
	Grece	27.35	3.65	54.98	3.94
	Turkey	27.23	3.31	54.81	3.91
	Average	**27.07**	**3.45**	**54.56**	**3.90**
Chickpea	UASVM Biobase	21.23	4.31	56.20	3.41
	Slovenia	21.15	4.25	55.98	3.20
	Greece	21.18	4.34	56.32	3.32
	Turkey	21.19	4.30	56.17	3.31
	Average	**21.19**	**4.30**	**56.17**	**3.31**
Fenugreek	Slovenia	21.30	2.65	60.83	4.69
	Greece	21.17	2.71	60.79	4.74
	Turkey	21.24	2.62	60.81	4.71
	Average	**21.24**	**2.66**	**60.81**	**4.71**
Mung bean	France	23.30	2.10	60.10	3.93
	Slovenia	23.17	2.01	59.90	3.84
	Average	**23.23**	**2.08**	**60.00**	**3.88**
Adzuki bean	Slovenia	22.30	2.00	62.50	4.25
	Greece	21.50	1.80	62.10	3.95
	Average	**21.90**	**1.90**	**62.30**	**4.10**

*Note: P (% d.m.) - protein content; F (% d.m.) - fat content; G (% d.m.); M - moisture (% d.m.)

Regarding carbohydrates, higher contents (over 62.30%) were observed at adzuki bean seeds. Mung bean and fenugreek had a carbohydrate content which ranged between 60.81% and 60.00%, compared to 56.17% and 54.56% at chickpea and faba bean seeds.

The ash content had values ranging from 3.31% for chickpea seeds to 4.71% for fenugreek seeds.

Mung bean, faba bean and lentil seeds had a medium ash content of 3.90%, 3.95% and 3.88%.

The energetic values of grain legumes (figure 2) ranged from 356.16 kcal at chickpea to 366.66 kcal at faba bean.

Mung bean and fenugreek seeds had medium energetic values of 360.58 and 361.14 kcal.

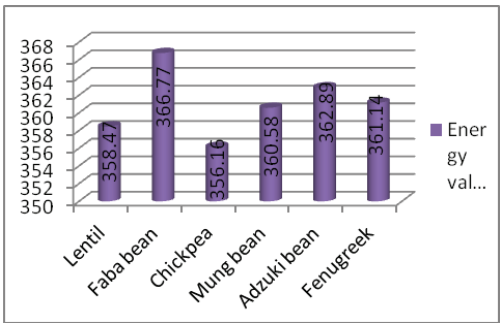

Figure 2. Energetic value of grain legumes

CONCLUSIONS

The research on the yield quality of these grain legumes highlighted the very special role that they should play in the development of biodiversity as well as in the diversification of human and animal feeding.

The highest protein content values were registered for faba bean seeds (27.07%) and the lowest values for chickpea seeds, i.e. 21.19%.

The fat content of the studied species ranged from 1.90% at adzuki bean to 4.30% at chickpea.

Regarding the carbohydrates, higher contents (over 62.30%) were observed at adzuki bean seeds, followed by mung bean and fenugreek with a carbohydrate content that ranged from 60.81% to 60.00%.

The nutritional value of grain legumes seeds was as follows: 358.47 kcal/100 g at lentil, 366.77 kcal/100 g at faba bean, 356.16 kcal/100 g at chickpea, 360.58 kcal/100g for fenugreek, 362.89 kcal/100 g at mung bean and 361.14 kcal/100 g at adzuki bean.

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DETERMINATION OF ANTIBIOTIC RESIDUES IN HONEY USING DIFFUSIMETRIC METHODS

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Abstract

One of the major risks resulting from the consumption of honey is the presence of drug residues, especially antibiotics, because they are widely used for treating various diseases. The study aimed to evaluate antibiotic residues in honey, in terms of quality and quantity, by diffusimetric methods. Thus, the studied antibiotics were oxytetracycline and streptomycin, and their action was studied using three bacterial strains, namely Bacillus subtilis, Staphylococcus aureus ATCC 6538 and Staphylococcus aureus ATCC 25923. S. aureus ATCC 25923 and S. aureus ATCC 6538 strains proved to be very sensitive to oxytetracycline and streptomycin. Bacillus subtilis showed no zone of inhibition for the 4 concentrations of oxytetracycline and for all 7 concentrations of streptomycin, indicating the high degree of resistance of the bacteria to these antibiotics. The analysis of honey samples contaminated with oxytetracycline and streptomycin showed inhibition zones with radius segments that were not strictly directly proportional to the antibiotic's concentration. In this respect, the tests carried out revealed the presence of inhibition zones even around the negative control. Both as such and diluted, honey caused the inhibition of bacterial growth, inhibition zones being directly proportional to the percentage of honey. In view of the fact that the honey itself possesses antibacterial properties, testing of honey samples in order to identify antibiotic residues cannot be achieved by microbiological methods, since there is a risk of obtaining false-positive reactions.

Key words*: antimicrobials, residues, honey, oxytetracycline, streptomycin.*

INTRODUCTION

For humans, nutrition is a factor with permanent action, which determines the conduct of metabolic processes, as food is the source and moderator of exchange processes. Also, the character of nutrition affects the system's functions, particularly enzymatic and hormonal factors, in order to maintain body homeostasis (Shils and Shike, 2006).

Food safety has become increasingly more difficult to obtain, both because of the wide range of foods, and also because of the air pollution that does not cease to intensify through the toxic gases, industry and agriculture (Nestle, 2013; Sun et al., 2017).

One of the major risks resulting from the consumption of honey is the presence of drug residues, especially antibiotics, because they are widely used for treating various diseases.

Because antibiotic residues present in food affect human consumers causing various problems such as antimicrobial resistance, immunoreactivity phenomena, imbalances at intestinal level, and toxicity, antibiotics were banned from administration to animals in order to stimulate productions, and, if used to combat certain diseases, a withdrawal period was imposed to allow the elimination of residues from the body (Beilke and Fritz, 2016; Solomon et al., 2006).

Maintaining under control of this chemical risk and keeping the hygienic quality of food were carried out by establishing maximum residue limits meant to keep the residues of antibiotics at a level that can not affect the human body and thus, methods by which they are determined both quantitatively and qualitatively were established. In general, the methods

used for this purpose are complex and require appropriate equipments.
The aim of this paper was to conduct a microbiological study for the qualitative and quantitative determination of antibiotics residues in known concentrations and to assess the effectiveness of these methods for determining antibiotics residues in honey.

MATERIALS AND METHODS

The study aimed to evaluate antibiotic residues in honey, in terms of quality and quantity, by diffusimetric methods. The aim was to determine the effectiveness of the method for two antibiotics present in different known concentrations in honey samples contaminated in the lab. Thus, the studied antibiotics were oxytetracycline and streptomycin, and their action was studied using three bacterial strains, namely *Bacillus subtilis*, *Staphylococcus aureus* ATCC 6538 and *Staphylococcus aureus* ATCC 25923. Therefore, it was targeted a possible relationship of proportionality between the concentration of the antibiotic and the area of the inhibition zone obtained.
To accurately observe the correlation between the antibiotic concentration and the inhibition zone, honey samples were contaminated with the two antibiotics in known concentrations (Table 1). It should be noted that, under the Regulation (EU) No. 37/2010 of the European Commission on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin, at present, the presence of antibiotic residues is no longer permitted in honey (virtually no maximum residue limits exist), but supervision in this area should continue as streptomycin and oxytetracycline were the most commonly used antibiotics for treating bacterial diseases in bees (Bargańska et al., 2011; Galarini et al., 2015; Kaufmann et al., 2003; Korkmaz et al., 2017).
Thus, the honey was contaminated with oxytetracycline in concentrations between 6400 µg/kg – 100 mg/kg and streptomycin in concentrations between 6400 µg/kg – 100 µg/kg (Table 1).
Inoculation of Petri plates was done as follows: a sterile cotton swab was immersed in the bacterial suspension, removing the excess liquid by pressing it to the inner wall of the tube, after which it was rubbed uniformly over the entire surface of the plate by making parallel moves and by turning the plate by 60 degrees.
With a pipette tip, wells were made in the inoculated culture medium (agar), the plates being ready for pipetting honey samples. Next, the contaminated honey samples were pipetted in the wells, and also the negative control (there was used a 1:1 mixture of honey and saline solution).
All Petri plates were incubated for 24 hours at a temperature of 37°C without special atmospheric conditions.

RESULTS AND DISCUSSIONS

After the incubation of the Petri plates for 24 hours, the cultures of *Bacillus subtilis*, *Staphylococcus aureus* ATCC 6538 and *Staphylococcus aureus* ATCC 25923 developed uniformly over the entire surface of the agar, with the exception of the zones where the wells were made and contaminated honey samples were pipetted (in the case of some antibiotic concentrations). The zones of inhibition were measured with a ruler, noting the radius segment of each area.
Staphylococcus aureus ATCC 25923 strain proved to be very sensitive to oxytetracycline and streptomycin, which caused the formation of inhibition zones approximately directly proportional to the antibiotic's concentration for all concentrations, including the negative control (Figures 1-4, Tables 2-3).

Table 1. Maximum admitted residue limits and concentrations of antibiotics accomplished experimentally in honey samples

Sample	Antibiotic	Maximum residue limits (µg/kg)	Used concentrations (µg/kg)
Honey	Oxytetracycline	- (0)	100; 200; 400; 800; 1600; 3200; 6400
	Streptomycin	- (0)	100; 200; 400; 800; 1600; 3200; 6400

Figure 1. Inhibitions zones for oxytetracycline (100 µg/kg, 200 µg/kg, and 400 µg/kg concentrations) and negative control (*S. aureus ATCC 25923*)

Figure 2. Inhibitions zones for oxytetracycline (800 µg/kg, 1600 µg/kg, 3200 µg/kg, and 6400 µg/kg concentrations) (*S. aureus ATCC 25923*)

Figure 3. Inhibitions zones for streptomycin (100 µg/kg, 200 µg/kg, and 400 µg/kg concentrations) and negative control (*S. aureus ATCC 25923*)

Figure 4. Inhibitions zones for streptomycin (800 µg/kg, 1600 µg/kg, 3200 µg/kg, and 6400 µg/kg concentrations) (*S. aureus ATCC 25923*)

Also, the strain of *Staphylococcus aureus* ATCC 6538 turned out to be very sensitive to the two antibiotics, as evidenced by the inhibition zones present for all of the concentrations used, including the negative control (Figures 5-8, Tables 2-3).

Figure 5. Inhibitions zones for oxytetracycline (100 µg/kg, 200 µg/kg, and 400 µg/kg concentrations) and negative control (*S. aureus ATCC 6538*)

Figure 6. Inhibitions zones for oxytetracycline (800 µg/kg, 1600 µg/kg, 3200 µg/kg, and 6400 µg/kg concentrations) (*S. aureus ATCC 6538*)

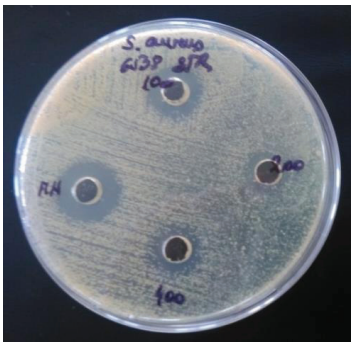

Figure 7. Inhibitions zones for streptomycin (100 µg/kg, 200 µg/kg, and 400 µg/kg concentrations) and negative control (*S. aureus ATCC 6538)*

Figure 8. Inhibitions zones for streptomycin (800 µg/kg, 1600 µg/kg, 3200 µg/kg, and 6400 µg/kg concentrations) (*S. aureus ATCC 6538*)

The plates inoculated with *Bacillus subtilis* showed no zones of inhibition for oxytetracycline in concentration of 100 µg/kg, 200 µg/kg, 400 µg/kg, 800 µg/kg and for the negative control, but only for 1600 µg/kg, 3200 µg/kg, and 6400 µg/kg concentrations. Moreover, the culture of *Bacillus subtilis* showed no zone of inhibition for the 7 concentrations of streptomycin, indicating the high degree of resistance of the bacteria to this antibiotic (Figures 9-12; Tables 2-3). Thus, it was concluded that the strain is not suitable for the determination of streptomycin residues by diffusimetric methods, since it does not develop inhibition zones for the concentrations of interest in the food industry.

Figure 9. Inhibitions zones for oxytetracycline (100 µg/kg, 200 µg/kg, and 400 µg/kg concentrations) and negative control (*B. subtillis*)

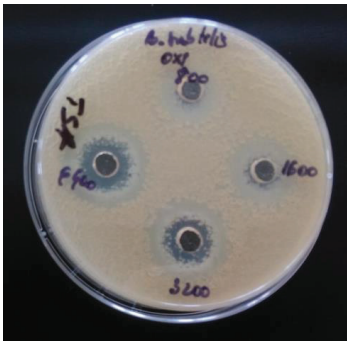

Figure 10. Inhibitions zones for oxytetracycline (800 µg/kg, 1600 µg/kg, 3200 µg/kg, and 6400 µg/kg concentrations) (*B. subtillis*)

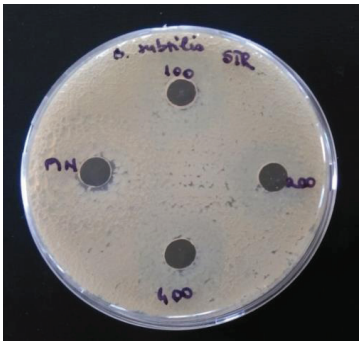

Figure 11. Inhibitions zones for streptomycin (100 µg/kg, 200 µg/kg, and 400 µg/kg concentrations) and negative control (*B. subtilis*)

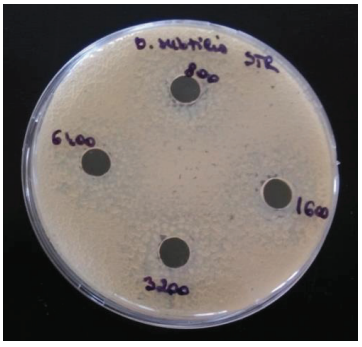

Figure 12. Inhibitions zones for streptomycin (800 µg/kg, 1600 µg/kg, 3200 µg/kg, and 6400 µg/kg concentrations) (*B. subtilis*)

Table 2. Radius segments of the inhibition zones caused by oxytetracycline

Concentration (µg/kg)	The radius segments of the inhibition zone (mm)		
	S. aureus ATCC 25923	*S. aureus ATCC 6538*	*B. subtilis*
Negative control	5	4	0
100	3	4	0
200	4	5	0
400	5	5	0
800	7	6	0
1600	5	6	1
3200	8	7	3
6400	4	7	4

Table 3. Radius segments of the inhibition zones caused by streptomycin

Concentration (µg/kg)	The radius segments of the inhibition zone (mm)		
	S. aureus ATCC 25923	*S. aureus ATCC 6538*	*B. subtilis*
Negative control	5	6	0
100	4	4	0
200	3	3	0
400	5	3	0
800	6	4	0
1600	6	4	0
3200	6	2	0
6400	6	5	0

The conflicting results obtained from the analyzes of honey samples contaminated with antibiotics, respectively obtaining inhibition zones with radius segments that were not directly proportional to the concentration of antibiotic (in some cases) and even obtaining inhibition zones around the negative control, led to the suspicion that honey itself could have an antibacterial effect against studied bacterial strains.

To highlight the potential bacteriostatic action of honey, it was tested on the *Staphylococcus aureus* ATCC 6538 strain, both as such and in the form of binary dilutions (2^{-1}, 2^{-2}, 2^{-3}).

The radius segments of the inhibition zones caused by honey are shown in Table 4.

Table 4. Radius segments of inhibition areas caused by honey

Dilution	Radius segment of inhibition area (mm)
2^{0} (M)	17
2^{-1}	13
2^{-2}	4
2^{-3}	2

Thus, it was found that honey, even at 2^{-3} dilution, possesses antibacterial properties (Figure 13). In this situation, the results obtained after testing samples of honey contaminated with different antibiotic concentrations were not considered correct, being highly influenced by the antibacterial properties of honey.

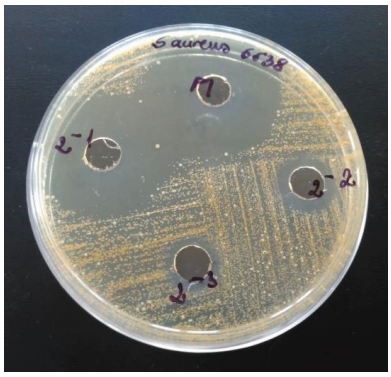

Figure 13. Areas of inhibitionfor honey – dilutions 2^0 (M), 2^{-1}, 2^{-2}, and 2^{-3} (*S. aureus ATCC 6538)*

CONCLUSIONS

The analysis of honey samples contaminated with oxytetracycline and streptomycin showed inhibition zones with radius segments that were not strictly directly proportional to the antibiotic's concentration.

In this respect, the tests carried out revealed the presence of inhibition zones even around the negative control.

Both as such and diluted, honey caused the inhibition of bacterial growth, inhibition zones being directly proportional to the percentage of honey.

In view of the fact that the honey itself possesses antibacterial properties, testing of honey samples in order to identify antibiotic residues cannot be achieved by microbiological methods, since there is a risk of obtaining false-positive reactions.

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SWINE, A NEGLECTED SPECIES BY ANIMAL HUSBANDRY RESEARCH IN ROMANIA

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Abstract

In the last times pork production was discredited since it was considered concurring humans in food resources and provoking blood vessels inconvenient to consumers. With 2016 Food Day, FAO launched the call:" The climate is changing. Food production must to". The present paper argues for the opportunities pork production brings in delaying the global heating of the Earth, at least. At it is known the Earth temperature at the atmosphere level is due to the concentration of the gases having "greenhouse effect".
These gases are water vapors, carbon dioxide, methane, CFCs and nitrogen protoxide. Water vapors are at saturated concentration and can't act in global heating. CFCs are technical gases and have no relation with food production. CO_2 is produced by all live beings. CH_4 is result of anaerobic fermentation present in herbivorous animal digestion. Hogs and birds produce very small quantities of CH_4.The last mentioned gas results from manure fermentation and can be input in soil as fertilizer. On the other hand swine as genetic species is able to perform in different husbandry systems living outdoor or even in natural environment where they get their feed. The present paper suggests some themes concerning pork production that gives possibilities to reduce methane production from farm animal husbandry and fossil fuel consumption.

Key words: *greenhouse effect, pork production, pig farming systems.*

INTRODUCTION

There is no doubt Earth planet's temperature is increasing. "Climate is changing; agriculture and food must to", says FAO for the Food Day of the 2016 year.
At the same time the number of people on the planet has passed over 7.3 inhabitants. Food security is in danger. Animal farming has to react in helping life sustainability (Paraschivescu M. Th. et al. 2014).
Causes of Earth's heating aren't completely known. But one known cause is the increasing concentration of greenhouse gases in the planet atmosphere (Paraschivescu M. Th. et al. 2009). As the main greenhouse gases are considered: water vapors (H_2O), Carbon dioxide (CO_2), Methane (CH_4), Chlorofluorocarbons gases (CFC) and Nitrogen protoxide (N_2O).
Water vapors are at saturation level so their concentration can't increase. Chlorofluorcarbons are freezing industry gases involved in food storage not in food production (Sandu Mariana, 2016). Nitrogen protoxide results from animal manure by mineralization of urine. But manure, before attaining this stage of degradation can be used as valuable organic fertilizer of soil.
The remaining two gazes: carbon dioxide and methane are involved in the live beings' metabolism. CO_2 + H_2O are the mineral compounds which green plants use to synthesize organic substances in the presence of chlorophyll pigment during the day light. These way green plants are decreasing the CO_2 concentration in the atmosphere, no matter the same plants are emitting some quantities of CO2 all the day and along night. At the same time green plants aren't emitting any quantities of CH_4.
On the same land surface the green plant species have different capacity of fixing C in their bodies related to the present quantity of chlorophyll resulting from the depth of the

green layer on the land surface. For these reason forests are most efficient actor in taking CO_2 out of atmosphere. On contrary animal organisms don't fix any atmospheric CO_2 (Tufescu V., M. Tufescu,1988).

Concerning CH_4 emission animal organisms express different statuses depending of their nutrition type. Herbivorous species, since using vegetable feed only, have in their digestive tract a special fermentation compartment which is the rumen in ruminants or caecum in non-ruminant herbivorous and rodents (Paraschivescu M. Th. et al. 2014). There takes place an anaerobic fermentation producing high quantities of methane emitted by ruminants' trough eructation and by non-ruminant herbivorous through flatuses. Omnivorous species, the ones using both vegetable and animal feeds have also a caecum but it is relatively smaller and their digestion is mostly chemical. Par consequence they produce lesser quantities of methane (Paraschivescu Maria and al., 2001). The carnivorous, eating almost meat, are emitting even less methane, most of it with the expiratory air.

Another source of atmospheric CO_2 resulting out of animal production is burning fossil fuel to obtain the needed energy to conditioning the inner environment of animal housing.

We can say with regard to the impact of animal farming on the natural medium surrounding more pressure results from herbivorous species whose digestion tract has an anaerobic fermentation compartment and from poultry production requiring ventilation and heating of birds' houses. The second of the above servitudes is pretended to pigs grown in industrial technological systems, too.

Pork has been blamed, up to not long ago, for its high content of fat which was considered a danger for the blood vessels sanity of the consumers. Now it was demonstrated the cholesterol formatted from pig fat acids is needed and not a dangerous one.

Pigs were also blamed eating concentrate feed that is a costly one and might be food for humans, as well.

Since these sanogenezis arguments are dismounted pork production deserves to be considered among solutions helping to delay the actual planet global heating by reducing the methane emission from the animal production enterprises (Paraschivescu M. Th. et al. 2014). On the other hand there is one economic reason in this respect, too. In pork production one kg of body gain needs 3 kg of combined food (93% Dry Matter) to be obtained while it is necessary to feed 7 kg of DM to get 1 kg of body gain in cattle. In sheep and goats the need of feed is even more, since these animals are smaller and have a higher basal metabolism.

There are other economic reasons to develop pork production, as well. The first one is due to the fact that pigs have an everyday continue production. Before puberty they put body gain. After puberty gilts are producing piglets and continue to grow. Further prolificacy of sows is increasing in the new generation cycles and their live weight becomes greater in the next two or three years.

The second one is that pregnancy period length in swine is short (less than 4 month) and the piglets' suckling, as well (about 4 weeks). It is easy to receive twice litters, from one sow within one year (Paraschivescu Maria and al., 2001). Precocity in swine is a good one. Sexual maturity is expressed in the first year of life. It is also possible to have populations with sexual maturity before 6 month of age and the first parturition in the first year of life.

One other important trait in swine species is the modest requirements for the housing comfort along their life time. Pigs don't need special bed for their rest. They can rest on soft or on hard floor, as well. The main difficulty resumes to the request of piglets for a rather high temperature in their first week of life (up to 33^0 C in the first three days). During the rest of their life pigs can live even in rough, natural, medium.

Also it could be added the scientifically progresses in reducing the production cost of pork by better feed conversion due to less fat content of pig carcasses getting a decrease in the variable input costs. There are also hopes in reducing the fixed costs of pork production by outdoor housing of pigs and to increase the labor productivity by better management and feeding. These explain why pork covers 60% of the world consume of meat.

Such themes deserve to be discussed as targets of current research in pork production industry.

RESULTS AND DISSCUSSIONS

After privatization in order to obtain leaner meat hybridization procedure similar with the one practiced in poultry production, but using differently selected maternal and paternal lines, has been promoted. The same goal was intended with combined feed receipts having severe estimated content of essential amino acids.

The obtained results were placed and used instead of the former industrial system of pork production in continuous flow. Research units were privatized and other pork production systems have been neglected. Research units were converted in commercial units to reduce production costs and scientifically management was received from the furnishing the hybrid commercial pigs firms. The hopes for more efficient production of pork didn't confirm. Pork production decreased tremendously in Romania.

Before privatization in the pork production of Romania a pyramidal scheme of breeds' improvement was applied. In top of the pyramid there were elite farms with maternal breeds improved for fertility and paternal breeds improved for higher daily body gain, in both cases by grading up selection, in *inbreeding*. As maternal breeds Large White and Danish Landrace were used. As paternal breeds American Duroc and Hampshire were submitted to the grading up process for lean meat and higher daily gain. Later the Romanian research officer Liviu Beris succeeded in creating a synthetic breed, that he called Peris 345, a massive type of pig with very high performances of body daily gain and large muscles (Beris L., I. Petcu, 1974). He used the idea of synthetic lines promoted, those times, in the former Democratic German Republic.

Under the pyramid top multiplication farms were placed. These farms received gilts and young boars from the elite farms, bred them in closed reproduction or by crossing maternal breeds or the paternal breeds in-between sold piglets of both sexes to the commercial units where by cross breeding of fertile sows with lean meat boars produced the commercial pigs for the slaughter houses. Out of this schedule some crossbreeding schemes have resulted.

This project which we might call "Pyramidal Crossbreeding Scheme" was very efficient. The pork production of Romania for the inner market and for export was impressive (Beris L., I. Petcu, 1974). There was also a pork production for countrymen families' subsistence, including the annual traditional Christmas pig of at least 3000000 country side families.

After 1990 by liquidation of the state agriculture enterprises and of collective agriculture units as privatizing way of agriculture the former project of pork production has been abandoned. In the industrial continuous flow pork production system imported hybridcommercial pigs were brought instead. The pork production for the market vertiginously declined in Romania.

The causes of declining of the former pork production after 1990 weren't objective. There were mistakes of the successive incompetent Romanian governments and the ignorance or maladroit of advisory teams paid by EU to help our economy which acted for the outside of Romania interests.

The greatest mistake of those times in this field was the privatization of the pork production research units. Now we have no research unit for pork production. All genetic material is imported with the sanitary risk of contamination of the local livestock and only the intensive industrial pork production for the market is under the public authorities' attention. There are some research themes concerning pig feeding still in course aiming to improve receipts of feed supplements or of combined feed, only.

The difference between producing pork by *hybridization* and by *pyramidal crossbreeding schemes* refers mostly to artificial biodiversity inside the genetic species of swine. Hybridization claims for two genealogic lines one maternal of high fertility and one paternal presenting thick muscles and controlled microclimate, meanwhile the pyramidal crossbreeding scheme accepts many fertile maternal breeds and at least 2 or three paternal breeds suitable in different environment conditions (Beris L., I. Petcu, 1974). The maternal breeds all of them selected for prolificacy at the firth farrowing might differ by their body type concerning the frame, the size or by the body daily gain. The two last of these traits are related with the animals' precocity, what means earlier term of puberty, gilts getting able to be

fertilized before 6 month of age and having their first litter before 1 year of age. In the former pyramidal crossbreeding scheme the grading up selection for prolificacy associated to large size of the body started with the Large White breed for a tall frame and with the Danish Landrace breed for a long lines frame allowing more number of teats.

To have smaller size sows' selection was directed to the sexual precocity as the first selection criterion and prolificacy as the second one. The initial genetic material had to be the local Basna breed that was expressing these traits. Basna pigs were of middle size and of short body type, having a black robe with a white belt around the shoulders like in Hampshire or Saddleback breeds.

As paternal breed it was better to call and to dispose of boars from the Perish 345 breedince it had very large muscle mass and very high daily gain performances. Pietrain or Belgium Landrace boars could also be used. But it would be better to breed the paternal boars, doesn't matter the breed, in own small farms with closed reproduction, to avoid their transport on long distances and, as prophylaxis measure against contagious diseases by avoiding frequent introduction of new animals in pig herds. The Perish 345 and Belgium Landrace boars have the advantage of being free of the recessive gene of exudative myositis impelling on the maturation of the meat. There are rumours Suisse disposes of a free of this gene Pietrain population.

To this sophisticated biodiversity we have to add the still existing in Romania some rear local breeds as Mangalitsa (disposing of a thick layer of under skin fat wanted in minced meat industry and Swamp Pig able to live in natural medium

Biodiversity of artificial populations inside genetic species offers the opportunity of combining more traits in the crossing process offering a diversity of wares for the market and, what is more important, gives permission to locate pork production in peculiar environments with different resources and adequate husbandry systems.

Actual targets in pork production research might be breeding for biodiversity, implementtation of organic pork production, reduction of conventional energy and increased labor force productivity.

Breeding for Biodiversity

Let the private enterprises to continue their business. Thus it will be possible to compare breeding swine for hybrids with the pyramidal crossbreeding scheme for lean pork production scheme which must be remounted.

In this case, for the biodiversity purpose, it is necessary to preserve and select for fertility the two maternal races above mentioned Large White and Danish Landrace. Each breed has to have a clearly stabled body type able to sustain metabolism ensuring excreted net energy delivery to sustain their fertility. The project has to find out the most efficient program of multiplicative reproduction to receive the most fertile mothers for the commercial crossbred pigs. The first target of research in this field is to clarify the effect of age upon the cost of one weaned piglet due to the size of successive litters and to the number of births to have from one sow during her reproductive life.

Concerning the paternal side the Perish 345 population has to be saved and conserved in closed reproduction. It is of great interest to establish the minimal number of families in a herd and the way of eliminating feeble families and of accepting new better families. For competition the import and adoption of a Suisse Pietrain population, free of the exudative myositis gene is indicated. Projecting of a smallest farm to keeping a paternal population in sustainable inbreeding state seems to be of great importance for such schemes. Of course one or two commercial pork production units of satisfactory size have to be implemented in the research net for such schemes.

As biodiversity goal in maternal breed adding precocity to the fertility would be of interest. That means to have a maternalbreed of smaller body size, whose gilts entered puberty before 6 month of age. Virtues of such kind of size and precocity are less variable cost for sows' feeding and the possibility of a diversified and changeable reproduction plan within the year. Such scheme gives the possibility to use the farrowing boxes two times a year at fixed terms, in the spring and in the autumn, to offer hogs of about 25 kg to be further grown for the traditional Christmas pork by the interested people. Such type of units is able to integrate grading up selection in the maternal breed with the crossbreeding to parental breed boars to

dispose of different kinds of commercial animals. Such farm can sell hogs (20 kg-25 kg) to be grown further by clients or to sell finished pigs for slaughter (100 kg-110 kg). Great advantages could be received out of this peculiar technology if reproduction plan implements the so called "one birth gilt" breeding.
Resulting advantages are:
Better outputs due to the fact that to the value of the weaned piglets' the value of body gain of mothers during the pregnancy can to be added.
High selection intensity by self-performance test for prolificacy (size of the litters) is allowed.
Lower conventional energy needs in the pig houses is required since farrowing is placed outside of the cold season.
Much technical independence is offered since all the breeding schemes might be promoted in the same yard.
Surest prophylaxis against contagious diseases is guaranteed since the mentioned independence allows closed breeding.
Good start in this respect could be enjoyed since this trait is met in the gene pool of the Basna breed and is no need to induce the trait along many generations.
For biodiversity reason Mangalitza breed has to be studied, as well. Mangalitza pigs have a very thick under skin fat layer containing plenty of unsaturated fat acids. Volatile fat acids giving pleasant taste to Mangalitza pork are also present. For this reason food preparations from mixt minced meat contain in their composition Mangalitza fat. In addition Mangalitza pigs have a firm sturdiness against frost and even to sun rays being suitable for outdoor pork production.
With the same target acclimatization of any fat and prolificacy chinese breed would be tried, this way avoiding to import continuously live animals with dangerous antigens. Severe measures of quarantine must be officially promoted and supervised when importing pigs from Asia. Do not forget that located pigs in Romania have no antibodies for Asiatic diseases.
Now we know nothing about pigs grown in the country side families for their subsistence. It is of scientific interest to know if there are differences among the kind of pigs from plains, from mountains and from along the Danube or in the Danube's Delta.

Pork production husbandry systems

As it was said in the introduction of the present paper industrial pork production is one of the ways Carbon from the fossil fuel gets in atmosphere as CO_2. Much conventional energy is used to substitute labour force, to ensure a sanitary microclimate inside the pig houses, for cleaning and disinfection of pig boxes and equipment. Reducing conventional energy consumption will act not only for natural medium protection but also for less production costs of the pork.
But food security of humans is helped also if other feedstuff than concentrates feeds is used for pigs' nutrition, of the kind humans can't eat. Such circumstance is related to pork production system as well. We don't know anything about the kinds of husbandry systems practiced in small pig production enterprises in this country.
Safety food is other requirement of these days. Here is the question of food additives involved, not only of toxins or pathogenic agents. In principle the most natural is the best safety food and market offers better prices for the organic food than for products resulted from chemically treated cultured.
Let us see now what pork production systems are or were practised in Romania and which are their virtues and servitudes from the point of view of the former enlisted criteria.
The criteria are considered starting with the one of least human intervention in the running on of the system (the natural system) and going to the most artificialized one (the permanent flow industrial system).
In such order they will be:
• The natural system, met in the Danube Delta;
• The extensive system, practiced in the high hills of the Northern part of Romania;
• The familial, adopted for subsistence by mostly of families living in the country side and villages;
• The changeable flow production system, which allows changing the farrowing program of the saw herds;
• The continuous flow production system, which is the most artificialized.
• The outdoor production system, which declines using of pig houses, except farrowing boxes.

The natural system

We met such pork production system in the Danube Delta of Romania. There are three distinct zones in the Danube Delta:

a) The reservation zone where no lucrative human activity is legally permitted, but where herds of cattle or studs of horses are living like wild, stray animals, some of them being harvested by hunting;

b) The non-residential zone, without permanent inhabitants, but where some economic activities dedicated to using local resources as fishing, navigation control or periodical biomass collection is running;

c) The populated zone with small villages, some agriculture and subsistence animal husbandry.

The natural system of pork production is present in the non-residential zone of the Delta. In the neighborhood of existing facilities for fishing activity or other purposes some pens are done and let opened. At these pens pigs are coming together for rest. From them pigs live stray to any part around. They are not afraid of people as the cattle in the reservation Delta are. Colour of hair or skin of pigs is of all kinds we know: white, black, red, white, white - black spotted, red – white spotted, black with white belt around shoulders or even gray. Many new born piglets are of reddish color with whiter strips along the body like in wild species boar. All pigs, doesn't matter their color, had in common big head with strong and long jawbones. Pigs were taking all their feed out of the Delta ground and land. No chemicals were involved. No fossil fuel was wasted. It is the most possible organic feeding.

The need for research concerns natural land characteristics provable to pigs' location, the usual category structure of a swain flock in natural environment and the best unit measuring the size of herds, the minimal surface needed per flock unit, the minimal distance between pig herds, pigs protection against predators, the sanity surveillance status of animals and the mild, least disturbing method of harvesting the pork production to be used.

The extensive system

May be this kind of system is the oldest one and originates in keeping 2 – 3 sows at the sheepfold in order to eat the whey or other remainders from cheese preparation. Then, such sows and their piglets being free went grazing together with the sheep and entered in forest where they founded more fruits and roots than on pastures. Later when human communities became sedentary the ones disposing of herbivorous animals and pastures in the neighborhood of falling list forests let their pigs to go "grazing". So pigs entered forests eat acorns or beechnuts and other fruits. To the evening all animals return to their yards where, eventually, their feeding was completed. Now this system is still practiced in the hamlets from forested hill parts of the Northern Romania as subsistence pork production. There is not too much research to be done in this field except how to use the system for commercial organic pork production.

The familial system

The *familial* system is spread in less industrialized countries whose agriculture furnishes more vegetable wares. The goal of pork production is mostly to nourish the owner's family and may the occasionally engaged labor force. In Romania there is the tradition of having a pig for the Christmas fest and as source of meat preparations for the winter. The number of pigs slaughtered for the Christmas Eve must be around 3000000 heads. This pork production is completely neglected by the research.

Usually 1 or 2 pigs, preferably males, are kept by a family and fed with kitchen remainders and with own produced cereals (corn, barley, wheat, oats, sorghum or other) or residues from the vegetable oil production industry. Where and when the hogs (young pigs) are purchased God knows. Nothing is organized. It is not possible to say something about breeds. There is much to do apart research units in this field, and we have no one research of this kind. The main questions concern the seasonality of reproduction keeping two parturitions per sow per year, the controlled artificial biodiversity of Christmas pigs, efficient feeding of pigs using local resources and the needed additives to correct them, cheap solutions to housing and watering pigs, sanitary protection of livestock and so on. As it is evidently all the above requirements are questions of services which must fi answered by research units.

The controlled changeable flow system

That is a new proposed system intended to answer the above mentioned questions.

In principle it supposes to have a pavilionar housing system with, preferable, 6 houses one for different 6 families intended to preserve the genetic variability of the maternal population of the system. Best are 3 walls houses opened in front where the automat feeding facilities are fixed on a low fence, along one external alee.

Each pavilion has to be divided, let's say, into 21 rectangular boxes 1, 2 m large and 4, 0 m long in depth, from the front to the back of the house. Out of the total 21 boxes, 3 boxes are designated to 3 senior sows who are mothers of 15 their daughters housed in other 15 boxes. The remaining 2 boxes have to receive, one of them the male piglets of mother sows and the other one to receive the female offspring of the same mothers, when they are weaned. These offsprings are grown there up to puberty when daughters are moved in the before mentioned 15 boxes and the sons are selected for inbreeding or soled. The daughters are mated by crossbreeding to produce commercial kinds of pigs. When they finish suckling their first offspring the young sows (daughters) are sold or slaughtered. The piglets remain in boxes up to 20 – 25 kg live weight when they are sold to the interested country side families. Daughters are used for single parturition only, excepting the selected ones to become mothers. The hind part of the boxes is built as a ditch 20 cm deep and 60 cm vide to serve for the mechanical evacuation of the manure. Special attention must be given to the environmental temperature in the first week of live. For this reason each box, excepting the 2 designated to the offspring of mother saws, must dispose of a nest that can be wormed up to 30^0 C in the 3 days of litters' life.

The continuous flow system

This is the intensive industrial pork production for the market. The main trait of the system is to allow equal distribution of pork production along the year. Research must find out the way of keeping this trait in more complex units that have its own reproduction sector to obtain the commercial piglets by hybridization. The question of artificial insemination can't be excluded in order to stop new animals to enter the houses. Reducing the risk of infectious contamination of pig herds is a permanent task especially in units concentrating large amount of livestock.

The Intensive Outdoor Pig Production System

The system is, may be, the most sophisticated one and intends to reduce the fix costs of pork production by eliminating needs of buildings and of energy for good microclimate but keeping the strict rules of industrial programs. Detailed information in this field is given by Keith Thornton in his book "Outdoor pig production", reprinted by Farming Press in 1993.

The Intensive Outdoor Pig Production System is a novelty for the Romanian research in pig meat production. Extracting ideas from Thorton's book main tasks in this field are to find the acceptable traits of land and ground to locate such units (Thorntorn K.,1993).

The land has to be plain and the ground light. Clay presents the risk of retaining rain water and formatting mud. Facilities referring to electric energy and running water access is necessary. Most attention is paid to the breeding stock of the units. Sows and gilts are housed in distinct sectors. Each category must dispose of three kinds of boxes or pens: for mating, for in pig sows and for suckling sows. Pens of this last sector are provided with huts for farrowing acting as shelters for the sucking piglets. Weaners are collected at 3 or 4 weeks of age and moved indoor to be grown. It is not very clear why? The number of boxes (pens) in each sector is established in relation to the total surface at the unit's disposal, the number of sows, and the culling rata of sows (to count the needed number of gilts), the grouping interval of animals (shorter if number of sows is large), the ground characteristics and others. Convenient large tracks for tractors or other vehicles have to ensure good access to boxes. The best fences are the electrical ones with two strips of wire (at 20 cm and respectively 60 cm from the soil) fixed at wooden posts placed 8 – 10 m distance one to the other. Wooden hurdles are less resistant and more difficult to be moved when the place of the unit is changed.

Changing the place of the units gives the advantage of including some fertilized land in the general rotation cropping system of the farm. Thus no land is taken out from vegetable production of Agriculture. Next advantage is

reducing costs of shelters. In the outdoor intensive pig production there are needs for some cheap wire fences, genuine water troughs and small huts instead of expensive large buildings with roof and walls, windows, doors and concrete drives. Thorton says: Outdoor pigs' welfare is better than indoor (Thorntorn K.,1993).

But there are also some vices of outdoor pig production. One of them is decreased conversion of ingested energy to net energy of the biological production because of more het increment outdoor. There is considerable wastage of feed stolen by birds and vermin or gone with the wind. The last wastage can be avoided by formatting the combined feed as nuts or creep. Protection of piglets against predators has to be considered too. Difficulties are met in grouping sows of uniform size to fit the live weight of boars. Difficulties are met in depicting barren sows in the paddocks of in pig sows, what induces the need to use catch boars. Nevertheless advantages of outdoor pig production are net superior to disadvantages.

CONCLUSIONS

Menace of human food security caused by the global heating of the Earth and the climate changes might be tempered in the field of animal production by decreasing CH_4 emission through increasing pork production instead of producing beef or mouton.

Concerning pork production itself reducing conventional energy consumption for micro-climate control inside pig houses has to be considered as a positive measure, too.

New pork production systems as the natural system, the controlled changeable flow production system or the outdoor pork production allow sever reduction of energy consumption needs.

Such systems allow also to produce organic meat that benefits of better prices on the market, especially now when certainly was stabled that pig meat is a safety food.

Animals' health protection has to be improved all over the country in any husbandry system, including the intensive, industrial one.

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COMPARATIVE STUDY OF ACIDIFYING ACTIVITY OF SOME STARTER CULTURES USED IN DAIRY INDUSTRY

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Abstract

Four starter cultures used in dairy industry were investigated in this study for their acidifying activity. Two of them are mesophilic cultures R-703 and R-708 and the other two are thermophilic cultures FRC-60 and FRC-75. These cultures were inoculated in sterile cow milk and incubated at 32°C (mesophilic cultures) and at 45°C (thermophilic cultures). The pH value was determined every 2 hours. The acidification profile of all cultures was similar, but the termophilic cultures produced a faster acidification (after 4 hours), than the mesophilic cultures (after 6 to 8 hours). After 10 hours of incubation the pH value of the medium for all studied cultures was stabilized around 4.5.

Key words*: starter cultures, thermophilic, mezophilic.*

INTRODUCTION

Starter culture used in dairy industry is defined as an active microbial preparation deliberately added to initiate desirable changes during preparation of fermented products (Hati S. 2016). A starter culture can provide particular characteristics in a more controlled and predicttable fermentation (Huttkins R. 2006). The primary function of lactic starters is the conversion of lactose to lactic acid, but other functions of starter cultures may include the following: flavour, and alcohol production, proteolytic and lipolytic activities and inhibition of undesirable organisms (Dobrea M. 2008, Hati S. 2016). The lactic acid is responsible for the texture development of fermented milk products and contributes to the overall flavour, enhancing preservation boundaries (Bhattacharay S. 2010, Dobrea M. 2008).

MATERIALS AND METHODS

Two kinds of lyophilised, multiple starter cultures, were used: mesophilic cultures R-703, R-708 and thermophilic cultures FRC-60 and FRC-75. The starter cultures were inoculated in cow's milk with 3.5% fat content (autoclaved at 120°C for 15 minutes). The inoculation rate was 0.1 UI/l milk. The incubation temperature was 32°C for mesophilic cultures and 42°C for thermophilic cultures, for 14 hours. The cultures were examined for purity, and every 2 hours samples for pH determination were taken.

RESULTS AND DISCUSSIONS

The examination for purity of starter cultures showed that all cultures were pure.

The bacterial composition of mesophilic cultures was as follows: *Lactococcus lactis subsp. lactis, Lactococcus lactis subsp. cremoris* and flavoring bacteria *Lactococcus lactis subspecia lactis biovar. diacetilactis* and *Leuconostoc mesenteroides subsp. cremoris.* The thermophilic cultures included streptococci (*Streptococcus thermophilus)* and lactobacilli *(Lactobacillus acidophilus, Lactobacillus helveticus and Lactobacillus delbrueckii subsp. bulgaricus).*

The results regarding pH values of mesophilic cultures were noted in table 1 and fig. 1.

After analyzing the acidification curve of R-703 starter culture results that in the first 4 hours of incubation at 32°C, pH decreased by 0.22 units. An intense decrease of this value was recorded after 6 hours (1.15), and 8 hours of incubation (1.95). After 10 hours of

incubation the pH value was stabilized at 4.5. The acidification curve of culture R-708 shows that in the first 4 hours of incubation at 32°C, pH decreased by 0.27 units. As in the case of culture R-703, a significant decrease of this value is recorded after 6 hours (1.45) and 8 hours of incubation (2.05). After 10 h of incubation the pH value was stabilized at 4.5. The pH values of thermophilic cultures are included in table 2 and fig.2.

Table 1. The pH values of mesophilic cultures (incubated at 32°C)

Determining number	Time (hours)	pH values R-703	pH values R-708
1	0	6.65	6.65
2	2	6.5	6.48
3	4	6.43	6.38
4	6	5.5	5.2
5	8	4.7	4.6
6	10	4.5	4.5
7	12	4.5	4.5
8	14	4.5	4.5

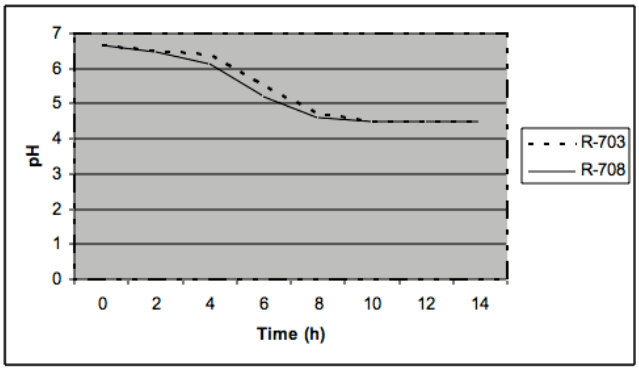

Fig.1. The dynamic of pH of mesophilic cultures

Table 2. The pH values of thermophilic cultures (incubated at 42°C)

Determining number	Timp (hours)	pH values FRC-60	pH values FRC-75
1	0	6.65	6.65
2	2	6.55	6.55
3	4	6.28	6.1
4	6	5.74	5.32
5	8	4.85	4.75
6	10	4.55	4.52
7	12	4.5	4.5
8	14	4.5	4.49

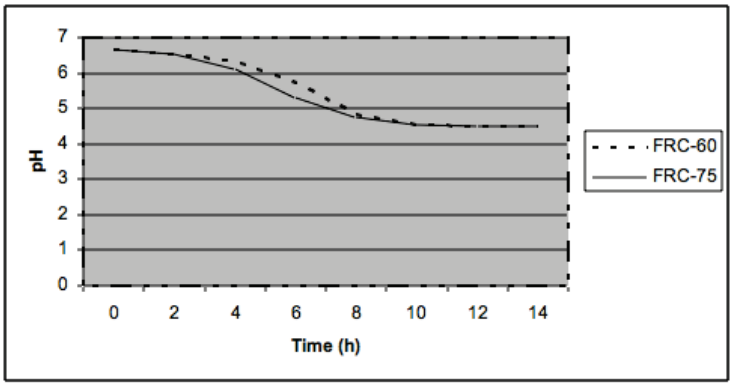

Fig. 2. The dynamic of pH of thermophilic cultures

Examining the acidification curve of culture FRC-60 it seemed that in the first 2 hours of incubation at 42°C, pH value decreased by 0.1 units. A stronger decline of this value is recorded after 4 hours (0.37), 6 hours (0.91) and 8 hours of incubation (1.80).

The acidification curve of culture FRC-75 showed that in the first 2 hours of incubation at 42°C, pH value decreased by 0.1 units. An intense decrease of this value was recorded after 4h (0.55), 6h (1.33) and 8 hours of incubation (1.9). After 12 hours pH value was stabilized to 4.5 for both thermophilic cultures.

Examination of acidification curves of starter cultures R 703, R-708, FRC-60 and FRC-75 showed that the fermentative profile of those four cultures is similar. However, if for the mesophilic starter cultures R 703 and R-708 the most significant changes in pH values were recorded after 6 and 8 hours of incubation at 32ºC, in the case of thermophilic starter cultures, after 4 hours of incubation at 42ºC, we found significant pH decreases, which continued up to 10 hours of incubation.

pH values remained constant to 4.5 after 10h of incubation of mesophilic cultures and 12 hours in case of thermophilic cultures.

CONCLUSIONS

All starter cultures showed a lower acidify-cation during the first 2 hours of incubation.

The most important decreasing of pH values were recorded after 6 and 8 hours of incubation for mesophilic starter cultures R-703 and R-708, whereas thermophilic cultures have induced significant acidification after 4 hours and continued to decline the pH up to 8 hours of incubation.

3. The constant pH value of 4.5 was reached after 10 hours of incubation for the mesophilic cultures and after 12 hours of incubation for the thermophilic cultures.

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"IN VITRO" STUDIES ON USING NATURAL ESSENTIAL OILS IN TREATMENT OF NOSEMOSIS IN HONEY BEES: DETERMINATION OF THE THERAPEUTIC DOSE

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Abstract
Essentisl oils have been assigned with special properties over the time. They are used much in herbal medicine, but also in apiculture, mainly due to their emollient, calming, carminative, antispasmodic, and mainly antiseptic properties. In order to determine the therapeutic dose to be used in treatment of nosemosis in bee colonies, it has been tested the product Suppressor 1, a mixture of medicinal herbs and etheric oils obtained from melliferous plants, dissolved in ethylic alcohol 96°. The studies were carried out in laboratory conditions with microclimate parameters being permanently monitored. Five experimental modules with natural infection with Nosema spp. were organized for testing the product, together with three control ones (one positive and two negative controls, respectively); each module consisted in two groups, with at least 100 bees/group (106 - 187 bees). The bee groups were kept in wooden cages equipped with glass, mesh ventilation and nutrition systems. The tested product was used in different concentrations, from 1 to 50 ml /litre in sugar syrup, administered ad libitum. In order to determine the therapeutic dose, the mortality rate, at various intervals (24, 48, 72, 96 hours, and then at 8, 16 and 26 days, p.t.), was registered. The best efficacy was obtained for the concentration of 5 ml of product/l syrup; this was considered the therapeutic dose. The tested product was not proved to affect the viability and vitality of bees. These results will be the base for further field studies of the product, in order to provide alternative solutions for control of honey bee's diseases using natural products and to avoid drug residues in apiculture products.

Key-words*: honeybees, Nosema spp., plant extract, etheric oils, therapeutic dose.*

INTRODUCTION

Honey bee colonies grow exponentially in early spring and can use efficiently their first harvests. One of the limiting factors in the maximum development of the bee colony, particularlry for the European honey bee (*Apis mellifera*) is represented by infection with *Nosema spp*, a microsporidian parasite (Microspora: Microsporidida).

Nosema apis, the historical microsporidian parasite of European honey bees, can decrease worker longevity and cause considerable winter colony losses, whilst *Nosema ceranae*, introduced into European honey bees from its Asian congener (*Apis cerana*) within the last few decades is associated with colony depopulation and collapse in some areas of Europe (Higes et al., 2008a).

In Romanian bee colonies both species *N. apis* and *N. ceranae* exists in *Apis mellifera* colonies (Chioveanu et al., 2009).

Nosema spp. invades the epithelial cells of the intestinal midgut at its insertion with the Malpighian tubules, giving rise to large numbers of spores within a short period of time. The parasite is ubiquitous, in temperate conditions, nosemosis being considered to have a serious negative effect on the production capacity of honey bee colonies and the survival capacity of the affected colonies during the winter (Mitrea, 2011). Due to its subclinical evolution, usually, beekeepers do not always can estimate exactly the damages and losses due to *Nosema* infection (Popovich et al., 2012).

One of the goals of modern beekeeping is to obtain residue-free bee products, as well as a revival of researches related to using natural products for treatment in different pathological conditions (Bojor et al., 1984; Mitrea, 2002).
In phyto aromatherapy, etheric oils were awarded the outstanding properties over the time. They are used much in herbal medicine, but also in apiculture, mainly due to their emollient, calming, carminative, antispasmodic and antiseptic main properties (Chioveanu et al., 2004; Ion et al., 2008).
Given that the use of antibiotics to honey bees is prohibited for prevention and control of diseases, etheric oils, especially administered in their food, can be an alternative to assure, in vivo, at critical times, additional to energy or protein stimulators, a suppressive effect on infectious agents (Panizzi and Pinzauti, 1988; Roussenova, 2011).
Therefore, in this study we aimed to evaluate the efficacy, in vitro, of a natural product based on essential oils derived from herbs in treatment of nosemosis in honey bee colonies.

MATERIALS AND METHODS

In order to evaluate the efficacy of a product based on essential oils derived from melliferous medicinal herbs, usable in control of nosemosis in honey bees, it was determined the minimum dose of therapeutic efficacy (without side effects under laboratory conditions) and the lethal dose, through clinical evaluation, pathological and laboratory examinations.
The tested product (named ***Supresor 1***) is a mixture of essential oils derived from melliferous medicinal herbs (mint - *Mentha pepper*, melissa - *Melissa officinalis*, coriandrum-*Coriander sativum*, thyme - *Satureja hortensis*) extracted in 96° ethyl alcohol. The product is presented as a clear greenish-yellow liquid, with fragrant characteristic. It is composed by carvacarol, thymol, menthol, linalool, citronellol. The product contains about 200 mg volatile oil per 1ml hydroalcoholic solution.
The biological material used for testing consisted of honey bees from an apiary (GV-PH), Southern Romania. Before the experiment it was established the level of *Nosema* infection at both, colony and apiary, by determining the number of spores / bee (Table 1) (Ionita and Mitrea, 2013). In the experiment, there were used bees from a colony (ID=781) to which it was found an infection with *Nosema* spp. at 5 spores / unit which is equivalent to 1 250 000 spores / bee (Chioveanu et al., 2009).

Table 1. Honey-bee colonies examined for inclusion in the study

Explanations	Number of colonies
Examined honeybee colonies	20
Negative colonies	15
Positive colonies 1 spor/ unit (no 3; no 791)	2
Positive colonies 2 spores/ unit (no 774)	1
Positive colonies 5 spores/ unit (no 781; no 721)	2

Six experimental modules of honeybees with natural infection *Nosema spp.* were organized, of which to five (experimental modules) were administered the product in different concentration, and one module was the positive control - infected non-treated. Additionally, of the colonies where *Nosema* spores were not detected, two negative control groups (C = uninfected, non-treated; D = uninfected, treated) were selected to asses potential side effects of the product. Each module consisting in two groups (lots), with at least 100 bees/group (106 - 187 bees).
The groups were organized in wooden cages (190/150/50mm in size) equipped with glass, mesh ventilation and nutritious system; at least 100 bees (between 108-187) per each group were allocated (Table 3). Dead bees were collected from each cage daily, and counted. After the experiment, samples from dead bees were examined to determine the level of infection with *Nosema* spores.
The experiments were conducted in laboratory conditions, with the room temperature of 29 ± 0.5°C and humidity of about 28% (Fig. 1) (Williams et al., 2013).
The product ***Supresor 1*** (the etheric oil dissolved in alcoholic solution) was administered in sugar syrup 1/1 (*w/v*) in different concentrations in each module, as follows: 1 ml (module 1), 2 ml (module 2), 5 ml (module 3), 10 ml (module 4), and 50 ml (module 5) per liter of syrup.

The pH of the syrup was adjusted to 3.5-4 with vinegar 9%. Syrup was administrated daily, ad libitum (Fig. 1B). During of the experiments, daily observations on the general status of honey bees, mortality, and food consumption were performed; also, microclimate parameters (temperature, humidity) were monitorized (Figure 2).

Fig. 1. Organizing the experiment: A. monitoring temperature and humidity in experimental conditions; B. administration of the test product (syrup)

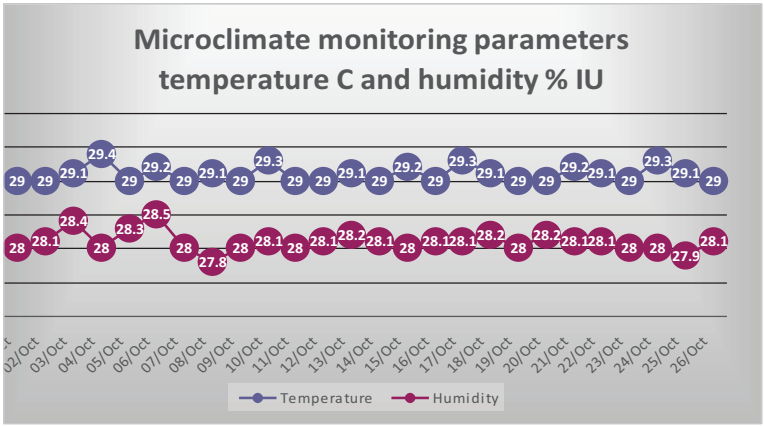

Figure 2. Microclimate monitoring parameters during the study period

RESULTS AND DISCUSSIONS

Results of the study on establishing a therapeutic dose of the tested product (*Supresor 1)* are presented in Table 2. The dose is the amount of administered tested substance. The dose is expressed as the weight of tested substance per unit. In this experiment, the dose was administered related to the quantity of honeybees' food (mg / liter). It was aimed to achieve a correlation between therapeutic dose (minimum concentration), the efficacy and possible lethal effects on honeybees, with significant clinical expression, comparative with the control groups.

The survival rate of the control groups was similar to the experimental modules 1 - 4 and higher than the 5th experimental module. The tested product mixture did not show any toxic effects on the honey bees from the experimental modules (1-4; negative control D). Referring to the toxicity scale according to HODGE and STERNER, the product can be placed in the Group 5 of toxicity, with oral administration, with limits between 5000-15000 ml / liter food for bees, practically nontoxic (OECD).

Mortality of the four experimental modules was not significant, compared with the control one; losses occurred after 4 days (96 hours) (Table 3). Considering the lots with the highest concentration (10,000 mg etheric oil / liter), after 24 hours, the losses were low (of 12.36%). Clinically, there were not registered paralyses, seizures or other signs indicating imminent or predictable death of honeybees in the experimental modules (Figure 4).

Table 2. Daily losses (number) of honeybees in experimental testing efficacy of the product *Supresor 1,* against *Nosema* infection

	Experimental modules										Positive Control		Negative Control			
	Module 1		Module 2		Module 3		Module 4		Module 5		Infected non treated		C - uninfected non treated		D - uninfected treated	
Initial number of bees	lot A	lot B	lot A	lot B	lot A	lot B	lot A	lot B	lot A	lot B	lot A	lot B	lot A	lot B	lot A	lot B
	120	**132**	**106**	**123**	**131**	**167**	**187**	**155**	**124**	**135**	**145**	**133**	**161**	**146**	**144**	**178**
Day																
1									11	21						
2									32	48						
3									48	52						
4									33	14						
5	4													2		4
6	8		7					6				8	4	2	3	5
7	8	6	10	6			12	14			10	12	7	6	7	5
8	18	10	12	14		7	5	7			7	10	10	5	8	9
9	20	17	10	16	7	6	9	12			8	11	6	7	10	8
10	10	32	12	12	6	7	7	6			9	6	8	7	6	7
11	8	15	18	18	6	11	11	8			18	13	11	9	6	11
12	5	18	9	12	9	7	9	10			14	10	12	11	10	9
13	8	22	11	17	7	9	12	12			18	12	6	8	8	11
14	9	12	17	10	7	8	11	9			22	20	12	7	9	8
15	22			18	6	12	14	11			19	16	8	11	9	10
16					10	9	11	8			16	15	9	10	10	11
17					8	9	8	11			4		11	8	9	8
18					10	11	10	7					10	10	10	9
19					9	8	12	7					10	12	7	12
20					6	12	11	8					8	9	9	10
21					8	10	12	12					6	12	11	9
22					9	9	15	7					7	10	12	9
23					8	12	18						16			8
24					4	8										15
25					5	7										
26					6	5										
LR	13s/u	12s/u	11s/u	9s/u	2s/u	3s/u	3s/u	4s/u	3s/u	5s/u	8s/u	11s/u				

LR- Laboratory results: quantitative determination of spore number of *Nosema* spp.

s/u- Spores of *Nosema spp*. Per/unit (/bee)

Table 3. Toxicity results, in terms of registered mortality rate in honeybees from the study

Supresor 1 (ml/mg ethereal oil) / l syrup)	Experimental modules Product administered/: number of ml / l syrup (mg etheral oil)					Positive Control	Negative Control	
	Module 1	Module 2	Module 3	Module 4	Module 5	Infected non treated	C-uninfected non treated	D-uninfected treated
	1 ml (200 mg)	2 ml (400mg)	5 ml (1000mg)	10 ml (2000mg)	50 ml (10000mg)	-	-	5 ml (1000mg)
Bee losses in 24/h	-	-	-	-	12.35%	-		
Bee losses in 48/h	-	-	-	-	30.88%	-		
Bee losses in 72/h	-	-	-	-	38.62%	-		
Bee losses in 96/h	-	-	-	-	18.15%	-		
Bee losses in 8 days	21.43	21.40%	2.35%	12.86%	-	14.08%	11.73%	12.73%
Bee losses in 16 days	78.57	78.60%	42.62%	46.79%	-	81.59%	46.25%	44.41%
Bee losses in 26 days	-	-	55.03%	40.35%	-	4.33%	42.02%	42.86%

Based on the recorded results on the efficacy of the administered product in different concentrations in food, the best results in terms of survival rate of the honeybees and the rate of *Nosema* infection (number of spore/unit), were obtained for the module 3 to which a dose of 5 ml/l was administered.

On this module, the mortality started 2 -3 days later than in the positive control and the rest of the experimental modules; also, the number of nosema spores was reduced (2-3s/u compared to 8-13 s/u). This dose represented by 5 ml product Supresor 1 per litre sugar syrup was considered as optimal dose for using it. The study on establishing a therapeutic dose had a prospective nature.

The product ***Supresor 1*** was not proved to be dangerous for the viability and vitality of bees, compared with the control groups, both positive and negative, respectively. It was also considered that for the dose of 10 ml (2000 mg etheric oil) / l syrup (module 4), although there were obtained good results without toxic effects for bees, however due to the strong flavour felt, it can not be used in the hives, because it could affects "the smell of the hive".

Moreover, providing of bio-stimulators of energy or protein with suppressive effect on bees, in critical times is more necessary in this respect. The present study will be extended for further observations on the apiary level.

The ultimate objective was to investigate the efficacy of medicated feed with *Supresor 1* against the development of *Nosema* spp. sporozoan in field conditions.

There are few scientific studies regarding the use of essential oils in the fight against *Nosema*. One of these experiments (Maistrello et al., 2008) shows that thymol and resveratrol undoubtedly have potential in the development of alternative strategies for the control of *Nosema* infection. In the same time a concentration of 0.12 mg/l and 0,60 mg/ l thyme esential oil obtained from Thymol (Sigma) minimum 95,5% causes decreased levels of infection with Nosema.

High concentrations of thyme essential oil – 2.5 mg/g (from *Thymol minimum* 99.5%, Sigma) can have toxic effects on honeybees (in accordance with its classification as a moderately toxic product) and may not be palatable.

Other experiments (Gherman et al., 2012) showed that propolis which contains 10% essential and aromatic oils has no effect on the spores of *Nosema* spp.

But, the majority of experiments on the use of different types of essential oils, such as: peppermint, eucalyptus, orange, lemon, etc., are performed by beekeepers, and results there were obtained in treatment of *Nosema* there are not scientifically substantiated.

CONCLUSIONS

Suppressor 1 product, a mixture of medicinal herbs and etheric oils obtained from melliferous plants, dissolved in ethylic alcohol, added to food in quantities of 1 to 10 ml per / l had good results on the survival rate of the honeybees and for control of *Nosema* infection; the product did not determined toxicological effects to honeybees. The dose of 5 ml product / l syrup was considered a therapeutic dose, whose effectiveness will be tested on the apiary level.

In conditions of an organic beekeeping, natural products based on plant extracts can be an alternative to control of honeybees' diseases; by using of these products can be also avoided risks of drug residues in apiculture products.

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****Toxicity Scale according to Hodge and Sterner https://ro.wikipedia.org/wiki/Doză_letală*