University of Agronomic Sciences
and Veterinary Medicine of Bucharest
Faculty of Veterinary Medicine

SCIENTIFIC WORKS

SERIES C. VETERINARY MEDICINE

VOL. LXVIII (2)

2022
BUCHAREST

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VOLUME LXVIII (2), 2022

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PUBLISHERS:

University of Agronomic Sciences and Veterinary Medicine of Bucharest, Romania - Faculty of Veterinary Medicine
Address: 105 Splaiul Independentei, District 5, Zip code 050097, Bucharest, Romania
Phone: + 40 213 182 564, Fax: +40 213 182 888
E-mail: veterinarymedicinejournal@usamv.ro, Webpage: http://veterinarymedicinejournal.usamv.ro

CERES Publishing House
Address: 29 Oastei Street, District l, Bucharest, Romania
Phone: + 40 21 317 90 23, E-mail: edituraceres@yahoo.com, Webpage: www.editura-ceres.ro

To be cited: Scientific Works. Series C. Veterinary Medicine, Vol. LXVIII (2), 2022

ISSN 2065-1295, ISSN 2343-9394 (CD-ROM), ISSN 2067-3663 (Online), ISSN-L 2065-1295

International Database Indexing:
Index Copernicus; CABI; Google Scholar; Scipio; OCLC; PNB (Polish Scholarly Bibliography); Cite Factor; Research Bible; Universal Impact Factor

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SUMMARY

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Scientific Works. Series C. Veterinary Medicine. Vol. LXVIII (2), 2022
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

COMPARATIVE STUDY REGARDING THE PROTEIN, ENERGY AND MINERAL PROFILES IN THE DIFFERENT CATEGORIES OF INTENSIVE AND HOUSEHOLD BRED CATTLE

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Abstract

In micro-farms or households where there are no veterinary health problems, it is recommended that the metabolic tests should be performed during periods when the metabolic strain is more intense, as they represent a method of monitoring the animals' health, especially during critical periods. A total of 52 cattle were divided into 5 groups according to their physiological condition, age category and health status. Total serum protein showed significantly higher values ($p<0.05$) in adult animals compared to the other age groups. Calves with digestive disorders had moderate hypoproteinemia, as a result of consecutive protein depletion, malabsorption and maldigestion syndrome from gastroenteritis, especially in the case of chronic ones, accompanied by an intake and improper use of ration proteins, respectively by mass elimination following intestinal protein loss. The mean blood glucose values did not differ significantly ($p>0.05$), the unitary response of the studied cattle categories being remarked. In the case of the mineral profile, the variations that appeared are mainly due to the different nutritional support, specific to the two breeding systems, but also to the different pathological conditions detected.

Key words*: cattle, metabolic profile, breeding system.*

INTRODUCTION

In cattle breeding, metabolic disorders initiate or favour the onset of distinct or intricate morbid entities that reduce the growth and exploitation efficiency. Due to this fact, the problem of metabolic disorders must be considered with special attention by practitioners.

The main purpose of this paper is to provide a comprehensive picture of the metabolic profile in cattle, which should contribute to the proper assessment of the main biochemical constituents' variations (LeBlanc, 2010).

In this context, the metabolic profile evaluation allows the study of the nutritional-metabolic integrity of the herds, thus establishing the laboratory diagnosis of the conditions with clinical or subclinical inapparent evolution, even in the incipient stage (Botezatu et al., 2014).

The detection of nutritional disorders must be done in the early stages, the metabolic profile introduction into the arsenal of diagnosis means, is an excellent tool for "group prognosis" (Patra et al., 2006; Whitaker et al. 1999). Therefore, the metabolic profile allows the evaluation of the nutritional-metabolic integrity of the livestock (Codreanu et al., 2012).

In addition to assessing and remedying nutritional-metabolic imbalances, metabolic tests also allow anticipating and even avoiding their occurrence, by applying appropriate measures.

MATERIALS AND METHODS

Metabolic tests were performed on cattle belonging to different age groups and physiological conditions, healthy and with different diseases, both from the intensive and household breeding systems. The aim was to ensure that the studied groups are homogeneous in terms of weight, clinical condition and performance of the production indices, in order to conduct a proper comparative study.

The selection criteria used were age categories (calves, youth and adults) and physiological status (pregnant, non-pregnant and lactating). The young cattle category was structured in

two groups: calves 1-3 months (n = 10) and youth 6-12 months (n = 11).
The adult cattle category was subdivided in three groups: pregnant in months VIII-IX - during the period of maximum metabolic load (n = 10), lactating (n = 10) - months I-II (maximum lactation period), adult cattle (n = 11) - not pregnant and not lactating.
In the appreciation of the metabolic profile for the 5 categories mentioned, the following biochemical investigations were performed: the main constituents of the energy profile: glycemia, lipids, cholesterol; the main constituents of the protein profile: total protein, albumin, globulins; the main constituents of the mineral profile: Ca, P, Ca/P ratio.

RESULTS AND DISCUSSIONS

Given the fact that blood, through its cellular and biochemical constituents, can be considered the mirror of an individual's health, in this study we opted mainly for extensive and thorough blood tests, aimed at dosing the constituents of the energy, protein and mineral profiles, in cattle raised and exploited in an intensive and household system, respectively.

The protein profile

The results regarding the protein profile for the 5 studied groups of cattle are presented in Table 1.
The investigation of the protein profile was performed by quantitative assessments at the plasma level. The plasma level of proteins and of the various protein constituents is influenced by the nutritional intake and conditioned by liver synthesis. Therefore, the quantitative changes of these proteins mean levels, are usually due to nutritional deficiency and insufficient hepatocyte synthesis (Calamari et al., 2007). It is also worth mentioning that in cattle, high proteinemia is not always correlated with high protein rations (Yuherman et al., 2017). For the evaluation of the protein profile in cattle, we considered it appropriate to dose the main constituents, namely the total protein, albumins and globulins. Laboratory determinations of the protein profile were performed, considering, first, the growth system and then each age group, physiological / pathophysiological condition (Knowlton et al., 2002).
Both total proteins and protein fractions have values that vary depending on the physiological status of the animals and the growth system. Thus, following the biochemical examinations performed, it can be mentioned that the cattle bred in the household system had higher average values of total proteins, compared to the ones bred in intensive growth system, with significant variations ($p<0.05$) in some age groups (calves 1-3 months, and non-pregnant adults). These higher values can also be attributed to the differences in the proper supply of these nutrients through feed. It is worth mentioning that the total serum protein showed significantly higher values ($p<0.05$) in adult individuals compared to other age groups. The three calves with digestive disorders (1 from the intensive breeding system and 2 from the household system) had moderate hypoproteinemia, because of consequent protein loss, the syndrome of malabsorption and maldigestion from gastroenteritis. These conditions are accompanied by an inadequate intake and use of protein in the ration and by mass elimination due to intestinal protein loss.

Table 1. Average values of the protein profile parameters in the studied groups of cattle, bred in intensive and household system

PARAMETER/ GROUP	PROTEINS (g/dL)		ALBUMINS (g/dL)		GLOBULINS (g/dL)	
	BREEDING SYSTEM		BREEDING SYSTEM		BREEDING SYSTEM	
	Intensive	Household	Intensive	Household	Intensive	Household
Calves 1-3 months	5.89 ± 1.2	6.16 ± 1.2**	2.77 ± 0.9	3.06 ± 1.1	3.12 ± 1.1	3.10 ± 1.3
Youth 6-12 months	6.28 ± 1.3	6.58 ± 1.4	2.94 ± 0.9	3.12 ± 1.2	3.34 ± 1.2	3.31 ± 1.4
Adults not-pregnant/not-lactating	6.66 ± 1.3	8.20 ± 2.4**	3.21 ± 1.2	3.85 ± 1.4	3.45 ± 1.2	4.35 ± 1.6
Pregnant	7.58 ± 2.1	7.83 ± 2.0	3.25 ± 1.4	3.61 ± 1.3	4.33 ± 1.8	4.22 ± 1.6
Lactating	7.25 ± 2.0	7.92 ± 2.1	3.53 ± 1.5	3.81 ± 1.3	3.72 ± 1.4	4.11 ± 1.5

***$p<0.05$ - significant differences*

The energy profile

The results regarding the energy profile for the 5 studied groups of cattle are presented in Table 2.

To carry out an in-depth study in terms of energy profile we considered it appropriate, to perform the dosage of blood glucose, cholesterol and total serum lipids. These metabolic investigations aim at highlighting and appreciating the possible changes in the different cattle categories, as well as their comparative assessment in terms of age and breeding system criteria (Codreanu et al., 2013).

Regarding the glucose levels, there were no significant differences identified between the mean blood glucose values obtained in adult cattle bred in the intensive system and those bred in the household system ($p > 0.05$).

Cholesterolemia increased slightly in adult cattle (both in intensive and household breeding system).

Regarding the serum values of total lipids, it is observed that in lactating cows and adults, both in intensive and household system, there was recorded a moderate increase compared to the values recorded in the other two categories.

The mineral profile

The results regarding the mineral profile for the 5 studied groups of cattle are presented in the Figure 1 synthetic graph.

Regarding the calcium levels, the category of lactating cows recognizes values obviously lower than those recorded in all the other studied categories. The mean values were 9.8 ± 2.0 mg/dL for the lactating cows bred in intensive system, and 9.3 ± 2.1 mg/dL in lactating cows bred in household system. The differences between the two systems are statistically insignificant ($p > 0.05$), but both of the results are significantly higher ($p<0.05$) when compared to the values recorded in the other categories of adults. This moderate "physiological hypocalcemia" is caused by the intense metabolic stress during this period.

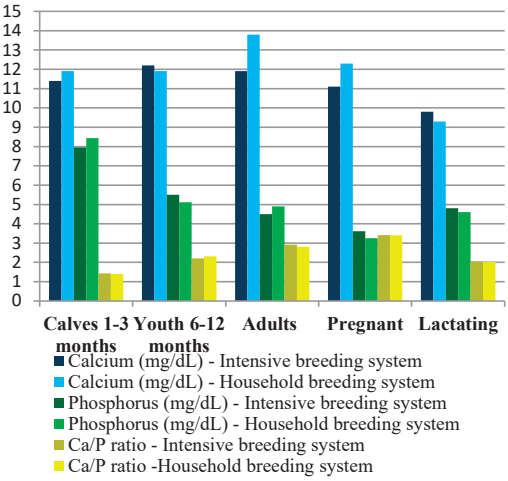

Figure 1. Graphic representation of the mineral profile average values comparative dynamics for the studied categories of cattle

Phosphoremia shows a significant decrease ($p< 0.05$) and obvious fluctuations in pregnant cows, the changes being more obvious in those exploited in the household breeding system, compared to the other categories.

Compared to calves and youth, the Ca/P ratio is higher in all 3 adult groups, physiologically exceeding the value of 2-2.5:1.

CONCLUSIONS

Regardless of the cattle breeding system, metabolic nutritional disorders initiate or favour the onset of distinct or intricate morbid entities that reduce the efficiency of cattle

Table 2. Average values of the energy profile parameters in the studied groups of cattle, bred in intensive and household system

PARAMETER/ GROUP	GLUCOSE (mg/dL)		CHOLESTEROL (mg/dL)		LIPIDS (mg/dL)	
	BREEDING SYSTEM		BREEDING SYSTEM		BREEDING SYSTEM	
	Intensive	Household	Intensive	Household	Intensive	Household
Calves 1-3 months	81.8 ±7.1	87.6 ± 7.2	131.6 ± 8.2	140.8 ± 8.4	288.8 ± 9.1	262.4 ± 9.0
Youth 6-12 months	74.6 ± 6.5	81.8 ± 7.7	142.8 ± 8.4	143.6 ± 8.4	242.8 ± 9.0	217.3 ± 8.8
Adults not-pregnant/not-lactating	69.4 ± 5.2	73.1 ± 6.5	92.9 ± 7.4	108.4 ± 7.6	238.3 ± 9.0	325.5 ± 9.2**
Pregnant	59.8 ± 4.9	58.1 ± 4.7	97.5 ± 7.5	86.8 ± 7.1	222.7 ± 8.9	279.4 ± 9.1
Lactating	72.2 ±6.4	77.4 ±6.4	93.4 ±7.4	103.6 ±7.6**	316.8 ±9.5	309.7 ±9.2

***p<0.05 - significant differences*

breeding and exploitation. The imperfections or deficiencies that may appear in the breeding technology can be detected in real time and combated by correcting the imbalanced factors. Also, the accuracy of the metabolic tests results allows the approach of an appropriate perspective in the case of the specific conditions, especially in case of entities that define the framework of "group pathology".

The components of the studied metabolic profiles (protein, energy and mineral) showed values that varied depending on the physiological state of the animals but also with the breeding system. In general, there were no significant differences ($p>0.05$) recorded between the mean values of the biochemical parameters, obtained in cattle belonging to the different categories, bred in the intensive and household system, an unitary response of the different age groups being observed regardless the breeding system.

Variations with statistical significance were recorded in pregnant and lactating cows, caused by the intense metabolic stress recorded during these periods.

In the other age categories, the variations appeared mainly due to the different nutritional intake (quantitative and qualitative), specific to the two growth systems, but also to the different pathological conditions detected with the investigations.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXVIII (2), 2022
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

LINK BETWEEN DAIRY COWS DIET AND MILK LIPID PROFILE VARIABILITY

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Abstract

Milk and dairy products are important sources of biologically active lipid substances. Interest in the nutritional qualities of dairy fats is not recent. It is now well established that milk and dairy products contain many fatty acids, some of which, with specific and sought-after physiological effects, not found in any other food. Milk, however, is not a standard product. Its fatty acid composition is very variable. At the farm level, it can be modulated either through genetics or husbandry practices. Among them, diet is the quickest and most effective way and its effects are quickly reversible. The objective of this article is to present the results of a study on the link between the feeding dairy cow's diets and the milk fatty acid profile. Two winter feeding diets were compared: A diet - maize forages as the main component of the feeding diet. B diet - maize forages and grass forage. The obtained results confirm the influence of feeding dairy cows diets in obtaining ideal milk in terms of lipid profile.

Key words*: feeding dairy caw, milk fatty acids profile.*

INTRODUCTION

Cow's milk is considered by health and nutrition specialists to be a very complete food, balanced in nutrients, rich in minerals (calcium) and containing almost all vitamins (except vitamin C). Dairy fat contains a complex mixture of triacylglycerols, diacylglycerols, monoacylglycerols, phospholipids, cholesterol, glycolipids and free fatty acids (Christie, 1995). The lipid profile of milk is very variable. The triacylglycerols in the milk are made up of over 400 different fatty acids in terms of the number of carbon atoms in the chain, the number of double bonds, their position and configuration (cis, trans) and obviously by their biological properties (Amores, 2019). The main sources of milk fatty acid variability are mainly related to the impact of genetic (breed), physiological (calving's number, lactation stage) and zootechnical factors (milking, food management) (Hanus et al., 2018). Due to these sources of variability the ruminant dairy fat contains different proportions of saturated fatty acids and unsaturated fatty acids (Figure 1). Even though saturated fatty acids (SFAs) are generally considered risk factors for cardiovascular health (Michas, 2014), milk fats have proven beneficial effects, determined by the presence in the composition of certain monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) that are involved in various physiological processes: oleic acid (C18:1 *cis-9*), linoleic acid (C18:2 *cis-9, cis-12*), rumenic acid (C18:2 *cis-9, trans-11*), α-linolenic acid (C18:3 *cis-9, cis-12, cis-15*), rumelenic acid (C18:2 *cis-9, trans-11, cis-15*), eicosapentaenoic acid (C20:5 *cis-5, cis-8, cis-11, cis-14, cis-17*), docosapentaenoic acid (C22:5 *cis-7, cis-10, cis-13, cis-16, cis-19*) (Glasser et al., 2008).

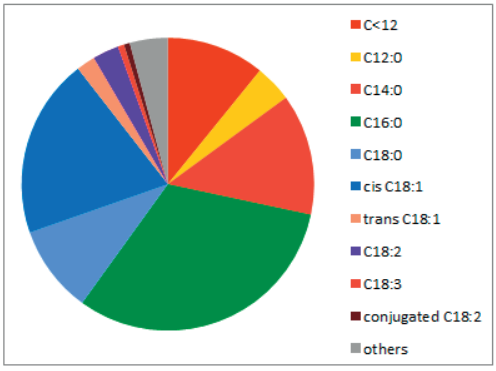

Figure 1. Average fatty acid profile of cow's milk (Glasser et al., 2008)

The physiological effects of all fatty acids are not yet fully established, but the main criteria for so-called ideal milk are known: lower concentrations of saturated fatty acid, greater concentrations of polyunsaturated omega-3 and omega-6 fatty acids (essential fatty acids), an ω-6 fatty acids/ω-3 fatty acids ratio of less than 5, a reduced content of *trans* fatty acids and an appreciable content of rumenic acid. This fatty acid is specific to ruminants (is also known as bovinic acid) but it has important beneficial effects: immune-stimulating, anti-carcinogenic, anti-inflammatory (Benjamin, 2009).

It's obviously the milk obtained in dairy farms has a fatty acid profile that cannot meet all the previously mentioned criteria. That is why real ways are being sought to obtain a milk lipid profile suitable for the requirements.

Genetic selection is one of the main ways to obtain ideal milk from the point of view of the lipid profile. But this option involves a relatively large amount of time.

A variant that can provide immediate results regarding the improvement of the lipid profile of milk is monitoring the dairy cows feed.

In ruminant diets typically consisting of forages and concentrates, fatty acids represent less than 3% of the dry matter. The majority of the dietary fatty acids are 18-carbon unsaturated fatty acids: oleic acid, linoleic acid (polyunsaturated omega-6 fatty acid) and α-linolenic acid (polyunsaturated omega-3 fatty acid). If in the concentrates the fatty acids are generally present in the form of triglycerides, in the forages most fatty acids are in the form of galactolipids (lipids with a galactose attached) what makes them more bioavailable than free fatty acids (Glasser et al., 2013).

But not all fatty acids present in dairy cows feed are found in milk, because their transfer into milk is influenced by digestive and metabolic particularities specific to ruminants (Glasser et al., 2008). Following lipolysis under the action of specific enzymes from the rumen flora, dietary unsaturated fatty acids are converted to saturated fatty acids by a process called ruminal biohydrogenation. This conversion involves PUFAs isomerization into conjugated derivatives and desaturation into C18:1-*trans* isomers, then into stearic acid (C18:0). All the intermediate fatty acids formed during ruminal metabolism are absorbed, passed into the blood and then into the mammary tissue. There, under the action of a desaturase, the C18:1-*trans* fatty acids are transformed into rumenic acid (C18:2 *cis-9, trans-11*). This transformation explains the preponderance of rumenic acid in milk fat (Jenkins et al., 2006).

Given this link between dietary fatty acids profile and milk fatty acids profile, the monitoring certain nutritional factors (e.g. type of forage and forage-concentrate ratio or use of lipid supplements) remains the most effective method to control the lipid profile. It is now known that the rapeseed it is a good source of oleic acid, the soybeans or the sunflower are rich in linoleic acid (ω-6 fatty acid) and grass is a good source of α-linolenic acid (ω-3 fatty acid) (Akbaridoust et al., 2014). Numerous experimental studies have highlighted the ability of oilseed lipid supplements to significantly reduce the proportion of saturated fatty acids in milk and to increase the proportion of unsaturated fatty acids (Glaser et al., 2008; Faulkner et al., 2018).

In this context, the paper presents the results of a study on the link between the diet of dairy cows and the fatty acid profile of milk.

MATERIALS AND METHODS

This study was conducted out in two intensive dairy farms, located in Braila County, during October 2020 - March 2021. The dairy farms raising Holsteins and Romanian Bălțata and have the same feeding strategy - winter feeding based on forages feed, supplemented with hay and concentrates - but apply different proportions of the ingredients. The first farm (A diet) has maize silage as the main component of the feeding diet. At the second farm (B diet) replaced part of the maize silage with grass silage. Both feeding diets are supplemented with other fodder crops and straw from cereals or cereal-leguminous straw (Table 1). The morning milk samples were collected from each farm, in the middle of each month. Raw milk was sampled directly from each farm milk tank during the morning milking, in standard containers (250 mL), stored at 5°C and transported to the laboratory. The milk samples were frozen until analysis.

Table 1. Ingredients (% of DM) of the two feeding diets

Ingredient	A diet	B diet
Maize silage	40	25
Grass silage	-	20
Grass hay	23	18
Concentrates	20	24
Other fodder corps	10	5
Co-products[1]	7	8

[1]Co-products including straw, bran, beet pulps, salts, mineral-vitamin premix

Fatty acids content from raw milk was analysed by gas chromatography-mass spectrometry (GC-MS) as described by Coppa et al. (2015). Chromatograms and spectra corresponding to the lipid profile of interest were analysed and interpreted by the peak area percentage method (Chilliard et al., 2009; Reis, 2011). Based on the obtained results, the concentrations of the fatty acids identified were calculated for the samples of raw milk taken into study and subsequently the fatty acid unsaturation indexes were determined.

RESULTS AND DISCUSSIONS

The lipid profile of milk corresponding to the two diets types was analysed, identifying the main fatty acids. From a quantitative point of view, the main saturated fatty acids quantified in the 12 raw milk samples analysed were long-chain fatty acids consisting of 13-21 carbon atoms: myristic acid (C14:0), pentadecylic acid (C15:0), palmitic acid (C16:0), margaric acid (C17:0) and stearic acid (C18:0). The main mono- and polyunsaturated fatty acids identified were fatty acids with an even number of carbon atoms, most of them with *cis* configuration: oleic acid (C18:1 *cis*-9), linoleic acid (C18:2 *cis*-9, *cis*-12), linolenic acid (C18:3 *cis*-9, *cis*-12, *cis*-15) and rumenic acid (C18:2 *cis*-9, *trans*-11).

The fatty acids concentrations were calculated as the ratio of each individual fatty acid to sum of all fatty acids identified in the respective milk sample. Subsequently, for each milk type, the fatty acid unsaturation indexes were calculated as the ratio of the concentration unsaturated fatty acid to the result of summing concentrations unsaturated fatty acid and corresponding saturated fatty acid, multiplied by 100 (Schennink et al, 2008). We calculated C14 index, C18 index and total index.

The variability of the milk lipid profile depending on the diet of dairy cows is presented in Table 2. From the data obtained, it can be seen how the type and concentration of fatty acids in the analysed milk samples was significantly influenced by the diet of dairy cows.

Table 2. Variation in fatty acids content in milk according to dairy cows diet

Fatty acid (g/100 g total fatty acids)	A diet	B diet	*p*-value
SFA	68.14	66.09	**0.002**
< C14:0	13.08	14.17	**0.046**
Myristic acid (C14:0)	11,32	10.51	**0.048**
C15:0 - C17:0	33.68	32.34	0.114
Stearic acid (C18:0)	9.75	8.70	**0.037**
> C18:0	0.31	0.37	0.176
MUFA	26.93	28.51	**0.016**
Myristoleic acid (C14:1 *cis*-9)	0.92	1.05	0.274
Oleic acid (C18:1 *cis*-9)	18.43	19.39	**0.048**
Other MUFA	7.58	8.07	**0.004**
PUFA	3.92	4.39	**0.032**
Linoleic acid (C18:2 *cis*-9, cis-12)	1.63	1.98	**0.045**
Rumenic acid (C18:2 *cis*-9, *trans*-11)	0.31	0.41	**0.001**
Linolenic acid (C18:3 *cis*-9, *cis*-12, *cis*-15)	0.28	0.46	**0.049**
Other PUFA	1.70	1.54	**0.004**
C14 index[1]	7.52	9.08	0.583
C18 index[2]	65.40	69.03	**0.044**
Total index[3]	47.87	51.55	**0.022**

[1]C14 index = C14:1 *cis*-9/(C14:1 *cis*-9 + C14:0) x 100

[2]C18 index = C18:1 *cis*-9/(C18:1 *cis*-9 + C18:0) x 100

[3]Total index = [(C14:1 *cis*-9 + C18:1 cis-9)/(C14:1 cis-9 + C14:0 + C18:1 *cis*-9 + C18:0)] x 100

The content of saturated fatty acids is about 2% lower for milk from the B diet which replaced part of the maize silage in the cow diet with grass silage ($P<0.05$). At the same time compared to milk in A diet, the milk from B diet has reduced levels of 1.06% for short and medium chain saturated fatty acids ($P<0.05$) and reduced by approx. 1% for long chain saturated fatty acids ($P<0.05$).

Parallel to the reduction in the content of saturated fatty acids in the milk of diet B is a significantly higher content of MUFA (monounsaturated fatty acids) and PUFA (polyunsaturated fatty acids) ($P<0.05$).

These results show that milk produced with rations based on grass silage are richer in polyunsaturated fatty acids (rumenic acid linolenic acid, linolenic acid) ($P<0.05$) and lower in stearic acid ($P<0.05$) compared to rations based on maize silage.

The influence of the grass silage presence in the feeding diet is also highlighted by the values of the unsaturation indices. Thus, if the values of the C14 index (unsaturation indices of medium-chain fatty acids) are not significantly different (7.52 in A diet vs 9.08 in B diet) the C18 index (unsaturation indices of long-chain fatty acids) and the total index have much higher values for milk from diet B ($P < 0.05$). These much higher values of the milk fatty acid unsaturation index of diet B are due to the balance between the proportion of maize silage and grass silage that provides both polyunsaturated fatty acids and fibre that favours pH values close to pH optimum of biohydrogenation enzymes, simultaneously stimulating the cellulolytic microflora (Chilliard et al., 2009). Higher content of polyunsaturated fatty acids, including rumenic acid can be determined by impact of grass silage on the rumen activities (Michas et al., 2014). A high proportion of grass hay (A diet) increases the content of saturated fatty acids (SFA), while the proportion of linolenic acid decreases significantly ($P < 0.05$).

The proportion of linoleic acid in milk fatty acids is generally between 2 and 3%. The relatively low content of linoleic acid in both types of milk indicates the hydrogenation of this acid in the rumen, which severely limits its incorporation into the fatty acids of milk (Faulkner et al., 2018).

One of the requirements for milk with good nutritional qualities is a high C18:1/C18:0 ratio. The C18:1/C18:0 ratio was 1.89 in milk from diet A compared to 2.23 in milk from diet B, suggesting according to Jenkins et al., (2006) an increase in delta-9 desaturase activity that converts stearic acid to oleic acid.

CONCLUSIONS

The fatty acid profile of milk can by strongly influenced by the feeding diet type. A feeding diet incorporating grass silage alongside maize silage can provide milk with lower saturated fatty acid content (possible risk factors) and a good proportion of mono and polyunsaturated fatty acids (known for beneficial effects). The type and proportion of ingredients in the fodder offered to dairy cows is an effective way of modulating the nutritional quality of milk fat.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXVIII (2), 2022
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

STUDY CONCERNING THE THERMAL STRESS IMPACT ON HEMATOLOGICAL AND BIOCHEMICAL PARAMETERS IN DOGS

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Abstract

The environmental temperature exceeding either the inferior or the superior limit of the thermo-neutrality zone may lead to thermal stress, due to the cold or the heat. This study was conducted in order to determine the changes in hematological and biochemical parameters of dogs exposed to thermal stress. A total of 20 dogs were randomly divided into two groups (10 per group). Group 1 (control group) was exposed to an environmental temperature of 15±1ºC, while the second group to 40±2ºC, to induce thermal stress caused by the heat. Blood samples were collected and subjected to investigations. This study showed that the blood sugar level significantly increased in group 2. However, PCV, Hb, and also cholesterol, triglycerides, total protein, and albumin levels were significantly decreased in the experimental group. At the same time, MCH, MCHC, WBC and the globulin levels did not show any significant changes from the control group. It was concluded that thermal stress in dogs could negatively affect the mechanism of thermal regulation in dissipating excess body temperature, so their general condition may change considerably.

Key words*: biochemical parameters, dog, heat, hematological parameters, thermal stress.*

INTRODUCTION

During the last 50-100 years there was a tendency for climate extremes in many parts of the world (Heim, 2015). Global climate change represents one of the most significant challenge that is threatening public health; both antropogenic (industrial activities) and natural factors (volcanoes, solar variations) are increasing the temperature and the amount of precipitations (Easterling et al., 2016). It was reported that the world is experiencing fewer cold days and nights. This trend is due to the increasing number of warm days and nights (Heim, 2015).

Animals have a range of functional systems controlling body temperature, nutritional state, social interaction which allow the individuals to control its interactions with its environment and hence to keep each aspect of its state within a tolerable range (Broom, 1996). Maintaining the internal temperature within certain boundaries, even if the surrounding temperature is very different, is a process called thermoregulation (Codreanu, 2018). Heat balance occurs through the actions of heat gain and dissipation mechanism (Mazzaferro, 2009). Besides the growth, milk production, pregnancy and activity components, the exchange of heat between animals and the environment is also an important component of metabolic heat production (Berman, 2003). The environmental temperature exceeding either the inferior or superior limit of the therm-neutrality zone may lead to thermal stress, due to the cold or the heat (Cotor et al., 2014). Each species has optimal temperature limits, allowing the physiological processes to run properly; otherwise, it appears the stressful state (Barton, 2002; Cocan et al., 2018). The temperature can affect the growth rate, feed efficiency, animal behaviour and also reproductive efficiency (Bogdan, 1999; Vasile et al., 2012). The sensitive and psychic stress agents can hurt the animals and degrade their welfare. In this situation, the physiological, metabolic, and hematologic parameters are modified and the deterioration of the animal welfare degree might be valued (Paraschivescu & Paraschivescu, 2012).

Dogs are part of the category of domestic animals used by humans for thousands of years in their lives for many purposes, such as hunting, detecting drugs, searching for missing people, guarding, or guiding blind people (Culea et al., 1998). Even if the average temperature of dogs is 37.9-38.9ºC and they regulate it by the nervous system, the thermal

stress is very common in dogs (Cunningham, 1992). Multivariable analysis identified significant risk factors including geriatric age, sex, breed (brachycephalic anatomy), obesity, an active playful character (Labrador and Golden retrievers), utility (military and police working dogs) (Bruchim et al., 2017; Hall et al., 2020; Niedermeyer et al., 2020; Moon et al., 2021). Results from studies conducted in other species revealed that the lack of hair and its density caused a predisposition to thermal stress (Araúz, 2017; Pena et al., 2020). Also, heat dissipation can be affected by the use of certain drugs (diuretics, phenothiazines, negative inotropic drugs) (Romanucci & Della Salda, 2013). This study was realized to determine the changes in hematological and biochemical parameters of dogs exposed to thermal stress.

MATERIALS AND METHODS

This study was conducted in August 2020, when it was a prolonged hot period. A total of 20 clinically healthy dogs (females and males), raging between 1-3 years old and with approximately equal body weights were used. The dogs were randomly divided into two groups, with 10 dogs per group. They were placed into a room with AC installation. The dogs from group 1 (control group) were exposed to an environmental temperature of 15±1ºC, while the group 2 to a higher temperature, to induce thermal stress caused by the heat. For this to be done, the dogs from the second group were exposed to an environmental temperature of 40±2ºC for 3 h/day, 7 days.

Blood samples were collected by puncturing the cephalic vein puncture and subjected for investigations. The samples were transferred into 2 ml EDTA vacutainers (for hematological tests) and into 3 ml Clot activator vacutainers (for the biochemical profile) and transported under refrigeration conditions (+2ºC) to the laboratory.

The hematological parameters that were taken under consideration were: red blood cells, hemoglobin, packed cell volume, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, white blood cells. The following biochemical parameters were investigated: blood sugar, total protein, albumin, globulin, cholesterol and triglycerides. Hematological parameters were determined by using IDEXX VetAutoreadTM Hematology Analyzer. Serum biochemistry parameters' analysis was performed with IDEXX VetTest 8008. The obtained results were compared with the reference values and for the statistical interpretation of the obtained data was performed Student's t-test. The data are expressed as means ± SD. Differences were compared for statistical significance at the p-level less than 0.05 ($P<0.05$). Tables and charts were designed in Word and Excel, Microsoft Office 2010.

RESULTS AND DISCUSSIONS

The hematological analyses presented in Table 1 showed significant decreases ($p<0.05$) of packed cell volume (PCV) and hemoglobin (Hb) in group 2, in comparison to group 1. However, the red blood cell count (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and white blood cells (WBC) did not show significant changes ($p>0.05$) in group 2 from the control group (Figure 1).

Table 1. Hematological data of dogs from group 1 and group 2

Hematological parameters	Group 1	Group 2
RBC (1×10^6/mm^3)	6.32±0.28	5.25±0.23*
Hb (g/dl)	14.87±0.27	10.13±0.27**↓
PCV (%)	43.75±0.33	32.97±0.33**↓
MCV (μ^3)	69.31±2.69	62.41±2.83*
MCH (pg)	23.52±0.80	19.27±0.48*
MCHC (%)	34.04±0.63	30.69±0.73*
WBC (1×10^3/mm^3)	9.68±0.16	10.13±0.15*

(Mean ± Standard Deviation)
*p>0.05 - statistically non-significant differences;
**p<0.05 - statistically significant differences

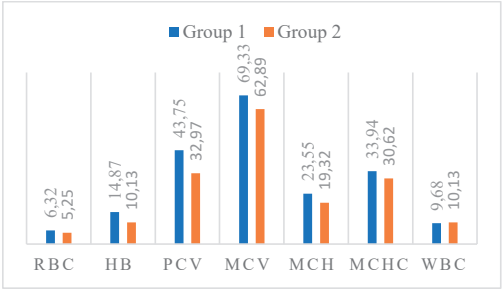

Figure 1. Variation of hematological parameters of dogs in group 1 and group 2

The biochemical profile and the effect of different environmental temperatures on it are presented in Table 2, Figure 2a, and Figure 2b. The blood sugar level increased significantly ($p<0.05$) in group 2 from the first group. On the other side, the levels of total protein, albumin, cholesterol and triglycerides showed significant decreases ($p<0.05$) in group 2 from the control group. The globulin level of group 2 shows a statistically non-significant increase ($p>0.05$) from group 1.

Table 2. Biochemical data of dogs from group 1 and group 2

Biochemical parameters	Group 1	Group 2
Total protein (g/dl)	6.12±0.23	4.92±0.35**↓
Albumin (g/dl)	3.91±0.23	1.97±0.17**↓
Globulins (g/dl)	2.21±0.14	2.95±0.21*
Blood sugar (mg/dl)	82.17±0.23	96.1±0.39**↑
Cholesterol (mg/dl)	143.25±0.49	90.38±0.37**↓
Triglycerides (mg/dl)	61.9±0.37	35.6±0.63**↓

(Mean ± Standard Deviation)
*p>0.05 - statistically non-significant differences;
**p<0.05 - statistically significant differences

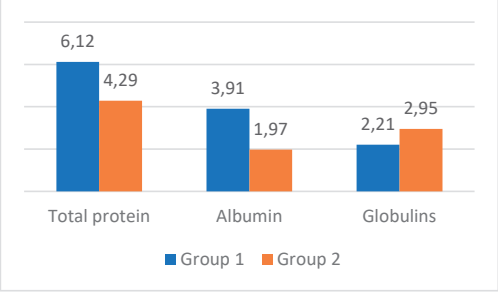

Figure 2a. Variation of proteic profile of dogs in group 1 and group 2

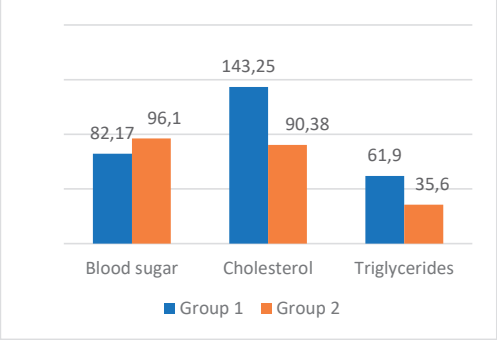

Figure 2b. Variation of energetic profile of dogs in group 1 and group 2

Blood is the most efficient stress indicator (Hattingh, 1977; Marin et al., 2015; Simide et al., 2016). The environment may have a significant impact upon the hematological parameters in mammalian and non-mammalian vertebrates (Gabriel et al., 2004).

The caloric needs of working or stressed dogs may exceed the levels of a maintenance diet, depending on the animal and the extent of work performed. Most diets designed for work or stress have increased levels of animal fats, with the other nutrients appropriately balanced to the increased energy density (The Merck Veterinary Manual). Hyperthermia leads to an increased metabolic state and oxygen consumption that raise both caloric and water requirements by approximately 7% for each 0,6ºC above accepted normal values (Miller, 2009). In addition, hyperthermia leads to suppression of the appetite center in the hypothalamus, causing a decrease in feed consumption (Miller, 2009; Wojtas et al., 2014). Studies conducted by Taha (1998) and Alkam (1999) explained that exposure of dogs (group 2) to high environmental temperature led to malabsorption of essential elements for red blood cells formation like iron and cobalt. The reduction of packed cell volume (PCV) and hemoglobin (Hb) could be considered as a result of RBC reduction (Al-Shammari et al., 2019).

Regarding the protein profile, the significant decrease in the average concentrations of total blood protein and albumin levels in group 2 can be due to the thermal injury and increased cellular metabolic demand and oxygen consumption; the thermal injury may cause widespread cellular necrosis through protein denaturation (Flournoy et al., 2003).

According to Patriche et al. (2011) and Suljević et al. (2015), a stress indicator valuable biomarker is the level of glycemia. The significant decreases in cholesterol and triglycerides levels observed in group 2 may be resulted from the lower metabolic rate, due to the lower thyroxin (T4) concentration, as Chandra et al. (2009) observed.

CONCLUSIONS

It was concluded that exposing dogs to an environmental temperature of 40±2º C leads to

an increased metabolic state and oxygen consumption.

The physiological response to the thermal stress factors is manifested by significant variations of cortisol and blood glucose.

The glycemia in dogs exposed to an environmental temperature of 40±2ºC (for 3 h/day, 7 days) was significantly increased in thermal stress conditions, explained by highly increased cortisol secretion by the adrenal gland.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXVIII (2), 2022
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

INVESTIGATION INTO THE PHOSPHORUS METABOLISM IN RED-MINI-ROCK HENS FED ON DIFFERENT AVAILABLE PHOSPHORUS DIETS

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Abstract

This paper aimed the particularities of the phosphorus (P) metabolism in 40-week-old Red-Mini-Rock hens subject of different available phosphorus (aP) supplemented diet feeding. Six diets containing 4.0, 3.5, 3.0, 2.5, 2.0 and, respectively, 1.5 g aP / kg diet of food were tested. The experimental feeding lasted five weeks. Research on the effect of different levels of aP in the diet on the metabolism of P in Red-Mini-Rock layers allowed the identification of particularities related to the ability to absorb, to store in bones and to release for egg formation of the P. The mobilization of the P from the bone is amplified as the dietary supplement decreases, increasing the loss of soluble P through manure. The total P content of the blood serum decreases with the level of aP supplementation but the variations are not linear. The total P content of the eggs increases only with high levels of aP. Food intake and laying rate are not affected by different levels of aP supplementation. In conclusion, a supplement of 2.0-2.5 g aP/kg diet of diet seems to better meet the metabolic requirements of Red-Mini-Rock hens from 40 to 45 week of age.

Key words*: hen, diet, phosphorus metabolism.*

INTRODUCTION

Phosphorus is an essential element for the animal organism being involved in many physiological processes. 80% of phosphorus is stored in bones in the form of hydroxyapatite, along with calcium, whose bone deposits amount to 90% of the total calcium in the body (Proszkowiec-Weglarz & Angel, 2013). The growth rate of the chicken and laying rate of the laying hens has increased in recent decades. These gains in production efficiency are coupled with metabolic disorders and increased mortality. Such rapid growth requires adequate nutritional supply and, in the case of rapid bone growth, adequate calcium and phosphorus supply. The deficiency or excess of one of these elements interferes with their homeostasis metabolism (Kebreab et al., 2005).

Birds' feed diets are supplemented with mineral phosphorus in the form of monocalcium phosphate and dicalcium phosphate to compensate for the loss of phosphorus in the diet in the form of indigestible phytate. On the other hand, the aim of the work of many researchers is focused on reducing phosphorus supplementation in the diet. Many investi-gations have been conducted to determine the exact phosphorus requirements of laying hens (Keshavarz & Nakajima, 1993; Van der Klis et al., 1997; Punna & Roland, 1999; Bar et al., 2002; Sohail & Roland, 2002; Snow et al., 2004; Ahmadi & Rodehutscord, 2012). According to Lambert et al. (2014) hens raised in alternative systems, especially domestic ones, can better tolerate lower levels of phosphorus in the diet, thus reducing expenses on the one hand and reducing digestive losses of phosphorus added to diets on the other. The reasoning of Lambert et al. (2014) is based on the observation that hens reared in alternative systems have better bone development and appear to utilize dietary phosphorus more efficiently. A reduction in dietary phosphorus in these birds would not have negative consequences on egg quality. Proszkowiec-Weglarz & Angel (2013) studied

the metabolism of phosphorus and calcium in broiler breeders (Proszkowiec-Weglarz & Angel, 2013).

Phosphorus supplementation levels in laying hens' diets are still under discussion; one of the reasons being the insufficient knowledge of the metabolic pathways of this element in the bird organism. This paper aims to investigate the ability to absorb, retain in the body and eliminate phosphorus from various sources in 40-week-old Red-Mini-Rock laying hens.

MATERIALS AND METHODS

Experimental design. Six hen groups of 40-week-old Red-Mini-Rock each one were constituted and noted from P1 to P6. Each group had a number of 60 hens and was housed in 4.2/5.4 m cages. The six layers groups were fed on diets (Table 1) containing different quantities of available phosphorus as follows (in g/kg diet): P1 = 4.0, P2 = 3.5, P3 = 3, P4 = 2.5, P5 = 2.0 and P6 = 1.5. No artificial phytase was added in any diet. The birds were fed *ad libitum* and had free access to water. The light schedule was 16.5 hours a day, from 5:00 A.M. to 10:30 P.M. The housing rooms were provided with wooden slatted floors to allow the collection of manure. During the five-week experimental period, egg production and feed consumption were monitored and manure was collected to determine the phosphorus content removed by manure. At the end of the five-week monitoring period, blood was sampled from the axillary vein to perform biochemical determinations. Excreta were collected per cage in the last three days of the experimental period. Serum was immediately removed from the blood samples as appropriate. Serum samples were stored at −20°C until biochemical processing. Five birds from each group were also slaughtered for bone and ileal content sampling. For this, the laying hens were euthanized by an intracardial injection with an

Table 1. Structure and composition of the diets used in the experiment (calculated values)

Ingredient (g/kg)	P1	P2	P3	P4	P5	P6
Wheat	515	515	514	515	520	525
Maize	85	85	87	65	65	57
Rapeseed meal	70	70	70	69	50	70
Soybean meal	175	177	178	200	218	202
Lucerne meal	20	20	20	20	20	20
Rapesead oil	27	25	25	25	25	25
Dicalcium phosphate	17,5	11,5	4,5	-	-	-
Monocalcium phosphate	-	-	-	15	10	5
Sodium chloride	2	2	2	2	2	2
Limestone[1]	81	87	92	82	83	87
L-lysine	1	1	1	1	1	1
DL-metionine	1.5	1.5	1.5	1	1	1
Vitamin - mineral premix	5	5	5	5	5	5
Nutrient contents						
Dry matter	876.4	886.5	889.5	885.5	872.4	886.5
AME_N (MJ/kg)	10.2	9.6	11.03	11.3	12.4	10.5
Crude protein	160.6	162.20	168.3	165.7	169.4	165.4
Calcium	35.0	33.6	35.9	34.9	35.4	35.4
Total phosphorus	7.6	6.7	6.4	5.3	4.6	3.0
Available phosphorus	4.0	3.5	3.0	2.5	2.0	1.5

Note: Diets contained 65% fine limestone and 35% coarse limestone

euthanasia solution (T61). In order to determine the phosphorus content eliminated by the eggs, 60 eggs were sampled from each of the six experimental layer groups.

Analysis. The eggshell was separated, weighed and used to determine the total phosphorus in the shell. The shell, bone, ileal content and manure samples were calcined at 550°C ± 5°C and then used to determine the phosphorus in them. A spectrophotometric method described by Manta et al. (1974) was used to determine the total phosphorus content from mineralized samples of eggshell, bone, ileal content and manure. A standard curve was drawn using a standard solution of 1 mg phosphorus/mL. For this 4.387 g of potassium dihydrogen phosphate p.a. were dissolved in 1000 mL of water. The curve will be linear for concentrations between 0 and 40 µg/ml. The optical density was measured at 430 nm using a blank solution obtained by adding 10 ml of molybdovanadate reagent to 10 ml of water. The amount of phosphorus in the test sample was determined using the calibration curve. The results were expressed as a percentage of the sample. A spectrophotometric method described by Kuttner & Cohen (1927) was also used to determine the phosphorus content of serum samples, using a standard phosphate solution (0.4394 g of dried monopotassium phosphate in 1 liter of distilled water) (normal values range from 2.8 to 4.5 mg/dL of serum in adult hens). The same method was addapted to determine the soluble phosphorus in manure.

Statistics. Data are expressed as the mean and SEM calculated using the GLM procedure in the SAS statistical package (version 9.4; SAS Institute Inc., Cary, NC). One-way ANOVA was used to compare mean between groups. Tukey *post hoc* test was performed to determine which experimental groups differed significantly from the control group. The Kruskal–Wallis non parametric test was used to analyze the effects of experimental diets on phosphorus contents in bone, eggshell, and blood plasma. Differences were considered significant at $P<0.05$. Correlations of phosphorus supplement levels (independent variable) with the investigated dependent variables were determined using Pearson's r values.

RESULTS AND DISCUSSIONS

The analysis of the data presented in Table 2 shows that food intake was not linked with the levels of available phosphorus supplementation (r = +0.12) and the same values of daily consumption were not significantly changed during the experimental period (P>0.05, which is accord with Lambert et al. (2014) and Lordelo et al. (2017). However, the total weight of the egg followed a downward trend, directly correlated with the dietary level of the available phosphorus supplement (r = -0.49). The egg weight loss was statistically significant (P< 0.05) and amounted to a total of 3.3%, which in absolute terms was 1.9 g/egg/period. A decrease in egg weight in chickens fed on diets supplemented with available phosphorus reported Lambert et al. (2014) in Dekalb White laying hens. According to these authors, at weeks 36-45, Dekalb White hens fed on 3.2 g rP/kg (rP = retainable phosphorus) of diet had a lower egg weight when compared to hens fed 2.8 g rP/kg of diet, which is not in agreement with our results. But at 55-65 weeks of age, egg weight of hens fed 2.8 g rP/kg was significantly higher than hens fed 2.6 g rP/kg, which is in agreement with our results.

Table 2. Feed intake and laying performances of Red-Mini-Rock hens fed on different supplement levels of phosphorus in diets (data are presented as means of a minimum 5 samples)

Ingredient	P1	P2	P3	P4	P5	P6	SEM	r	P
Food intake (g/cap./day)	92.2	94.3	101.5	88.5	97.0	98.8	12.3	+0.12	0.843
Laying performance (%)	85.6	89.4	87.5	86.0	88.4	82.2	22.3	+0.05	0.808
Feed convertion ratio (g/g)	2.44	2.43	2.50	2.54	2.49	2.61	0.32	+0.22	0.546
Egg weight (g)	59.4	58.9	58.8	58.7	57.3	57.5	7.55	-0.49	0.044
Mortality (%/month)	0.56	0.86	0.88	0.34	0.89	0.66	0.03	-0.05	1.433

Means with the same exponent indicate significant differences (*: < 0.05; **: P < 0.01). All means were compared with the control group.

The analysis of the data presented in Table 3 shows that the different levels of available phosphorus in the diets significantly changed the quantities of absorbed phosphorus, the accumulation in the bone, and the quantities eliminated in the egg and manure, as it follows. The phosphorus content in the feces decreased as the dietary phosphours intake decreased, finding a very close correlation between the level of fecal and dietary phosphorus (r = +0.94). Such a decrease is confirmed by other authors (Snow et al., 2004; Rama Rao et al., 2006; Lambert et al., 2014) but for some ages of the birds only. The exact reason why fecal phosphorus content is not altered by diets supplemented in rP at other ages is not known. There are also large differences between total fecal phosphorus and total ileal phosphorus (insoluble and soluble). Difference in total phosphorus between ileal and faecal digesta is on average 2.02 g/kg. An explanation on the physiologic mechanism of these high difference was done by Lambert et al. (2014) is this difference is due to the large amount of soluble P in faecal digesta. The soluble P fraction is related to the resorption of medullary bone and is excreted by the uric acid fraction in the faeces. There is increased demand for calcium during the period of egg shell formation in the shell gland. Because this usually occurs during the night when supply of calcium from the digestive system is low, a high proportion of shell calcium comes from resorbed medullary bone (Whitehead, 2004). Calcium is stored in bones as calcium phosphate, so both calcium and phosphorus are resorbed at the same time from the medullary bone. Calcium is used for egg shell formation and phosphorus must be excreted by uric acid (Lambert et al., 2014). The rate of absorption of phosphorus and its introduction into the intermediate metabolism depends on the functional capabilities of the digestive tract, knowing that phosphorus absorption occurs throughout the digestive tract, but especially the duodenum and jejunum, and is controlled by transmembrane transporters (Olukosi, 2011).

The level of phosphorus in the bones was highly influenced by the level of phosphorus in the diet (r = +0.55). Phosphorus supplementation in food tends to increase bone deposits. On the other hand, phosphorus in the bones is mobilized daily together with calcium in order to form eggshells in laying hens. Thus, the level of phosphorus and calcium in the bones is the algebric sum of the intervention of two diametrically opposed processes (Li et al., 2016). In our experiments we found a downward trend in phosphorus in the tarsal bones of Red-Mini-Rock hens but the curve of decreasing phosphorus in the bone is not linear. Lei et al. (2011) and Snow et al. (2004) reported an accumulation of dietary retainable phosphorus levels in bone and carcass of Dekalb White and LSL Classic laying hens and this agrees with our results. On the other hand,

Table 3. Phosphorus contents of bone, blood serum, eggshell, and manure of Red-Mini-Rock hens fed on different supplement levels of phosphorus in diets (data are presented as mean of a minimum 15 samples)

	P1	P2	P3	P4	P5	P6	Mean	SEM	r	P
Ileal P (g/kg DM)	$9.4^{\#;*;\times}$	9.6	8.8	6.8×	6.6*	$4.4^{\#}$	7.6	2.12	+0.99	0.002
Ileal soluble P (g/kg DM)	0.57	0.55	0.58	0.52	0.54	0.54	0.55	0.64	+0.33	0.544
Fecal P (g/kg DM)	$10.3^{e;f}$	10.2	9.3	7.3	6.9^{f}	4.6^{e}	8.1	1.11	+0.88	0.032
Fecal soluble P (g/kg DM)	$2.44^{a;b;c}$	2.44	2.34	2.50^{c}	2.68^{b}	2.78^{a}	2.53	0.54	-0.76	0.000
Bone (g/kg DM)	177.7	178.5	165.5	134.4	133.3	143.1	155.4	65.0	+0.55	0.021
P in blood serum (mg/dL)	4.3	5.2	4.6	4.6	3.0	2.9	4.1	0.54	+0.70	0.029
Egg shell P (g/kg DM)	1.22^{a}	0.95^{a}	1.04	1.00	1.09	0.98	1.04	1.09	+0.21	0.554
Egg albumen P (g/kg DM)	0.77^{a}	0.58^{a}	0.55	0.57	0.62	0.70	0.63	0.65	+0.33	0.433
Egg yolk P	11.11^{a}	10.25^{a}	11.4	9.56	9.99	10.54	10.47	2.22	+0.31	1.320

Values with a common superscript within the same row differ significantly ($P \leq 0.05$)

Punna & Roland (1999) and Sohail & Roland (2002) reported a decrease of the bone mineral density in hens fed on low levels of retainable phosphorus.

Total blood phosphorus ranged from 2.3 to 4.3 mg/dL of serum, following a non-linear downward curve, with a Pearson correlation coefficient of +0.70, which reveals a high degree of synchronization with the level of phosphorus supplementation in the chicken diet (Fig. 1). Klingensmith & Hester (1983) did not find singnificant differences in plasma inorganic phosphorus levels of 90-week-old Leghorn hens fed on different dietary phosphorus supplementation levels (0.2, 0.4 and 0.4% respectively, which is in agreement with our results on 40-week-old Mini-Rock hens, but reported significant differences in high incidence soft-shelled and shell-less layers (6.1 mg/dL) vs. low incidence soft-shelled and shell-less layers (5.2 mg/dL).

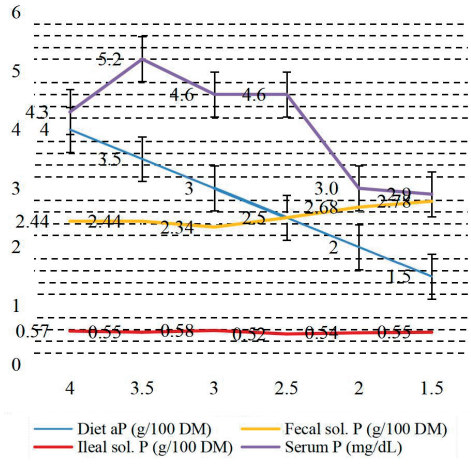

Fig. 1. Evolution of the fecal ileal and serum soluble phosphorus according to diet available phosphours supplementation. Abscissa: g suppl. aP/100 g DM diet; ordinate: P

The phosphorus content of eggs was not influenced by the level of phosphorus in the diet, except for chickens fed on a supplement of 4.0 g/kg diet, whose phosphorus values in shell, yolk and egg white were significantly higher ($P < 0.05$) than of the next experimental fed groups. Lower phosphorus values were found in groups receiving supplements of 2.0 and 1.5 g/kg diet, respectively.

The particularities of breed, age, diet, etc. of the phosphorus metabolism make the recommendations regarding the level of phosphorus supplementation in the diets still far from being in consensus. According to Lambert et al. (2014) a rP level of 2.4 to 2.6 g/kg diet could be sufficient to support the maximal egg number, egg weight, egg mass and feed conversion ratio from 36 to 90 weeks of age. According to Skřivan et al. (2010) the requirements of laying hens would be completely met with 0.27% available phosphorus in wheat-based diet and 0.30% available phosphorus in maize-based diet without added phytase. Regular use of phytase in common recipes in practice remains a method of improving the phosphorus metabolism and reducing the available phosphorus content of the diets, thus reducing mineral supplements, which are more difficult to assimilate metabolically.

CONCLUSIONS

Research on the effect of different levels of phosphorus in the diet on the metabolism of phosphorus in laying hens allowed the identification of particularities related to the ability to absorb, store in bones and release for egg formation. The mobilization of bone phosphorus is amplified as the dietary supplement decreases, increasing the loss of soluble phosphorus through manure. The total phosphorus content of the blood serum decreases with the level of phosphorus supplementation but the variations are not linear. The total phosphorus content of the eggs increases only with high levels of supplementation. Food intake and laying rate are not affected by different levels of supplementation.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXVIII (2), 2022
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

CONTRIBUTIONS TO THE MORPHOLOGY OF THE THORACIC AUTOPODIAL NERVES IN HORSE

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Abstract

As it is known, taxonomy is one of the most dynamic aspects of biology. Ever since Antiquity, throughout the linnaen and post-linnaen period and until the new cladistics era, all branches of the phylogenetic tree were subject to plenty of modifications. The Equus genus was no exception, one of the most important evolutionary transformations being the reduction of the number of fingers in this genus, criterion considered crucial for correctly placing a species. Numerical reduction is also accompanied by a reorganisation of vascular and nervous trajectories. Based on autopodial parts of 10 individuals, this study aims to identify details and potentially individual variants which may complete existing literature data and which will represent additional arguments to the taxonomic classification of the species. The main objectives of this study were represented by the description of individual variants and clarifying of data regarding anatomical terminology, hoping that once achieved the results will aid veterinary research and practice.

Key words*: horse, autopodium, nerves, forelimb.*

INTRODUCTION

Out of all domesticated mammals, only in carnivores are the nerves of the limbs almost completely developed.

Seeing as in the evolutionary process the locomotor way has changed, from the plantigrade stance to the digitigrade and finally the unguligrade one, there has been a concomitant numerical reduction of fingers, a remodelling of both the active and the passive locomotor apparatus as well as an associated reorganisation of the vasculo-nervous formations (Barone & Simoens, 2010).

However, in spite of the strict specialisation of the free extremity of the limbs, as is the case for example in equines, the organisation of the trajectories of vascular and nervous formations still maintains some similar elements to the ones observed in penta-dactyl limbs (Robu et al., 2018; Schummer et al., 2013).

The nerves of the autopodium originate in the zeugopodial segment, through fascicles disposed on the cranial and caudal side of the latter. These nerves' participation in the horse is special due to their necessity to adapt to the phylogenetic differentiation of the passive and active locomotor apparatus, an example being the almost total lack of dorsal autopodial nerves both in the case of the thoracic and the pelvic segment.

In time, anatomists have paid great attention to the study of the autopodial segment in equines and the interest for this area is known: any problem, no matter how apparently insignificant, can remove an animal (oftentimes a valuable one) from the competitive or economic cycle (Ganță & Pentea, 2006; Spătaru, 2013).

Morphological research of great detail appears as far back as two thousand years ago, studies of more recent artistic anatomy as well as studies for the simulation of models which respect the biomechanics of equines prove how wide the interest sphere really is (Coțofan, 1969; Gheție et al., 1955; Gudea et al., 2009).

Even though at first sight it may appear an exhausted subject, we consider that there are always individual variations waiting to be discovered and interpreted especially in order to understand the evolutionary process mentioned at the beginning of this work.

MATERIALS AND METHODS

The study material was represented by limbs originating from ten horse bodies. After skinning, the thoracic limbs were detached by sectioning the connecting muscles between the scapula and the trunk.
The dissection was performed by classical methods, on successive planes, carefully following the topography of the nervous formations in the autopodial region.
The most suggestive images on the studied material were photographed, and then edited in the Adobe Photoshop C3 program. The identification, description and homologation of the formations was done in correlation with the Nomina Anatomica Veterinaria - 2017

RESULTS AND DISCUSSIONS

The nerves of the autopodium are characterised through the reduction of those on the dorsal side, which is largely served by nerves on the palmar side that are very well represented in contrast. On the dorsal side, dorsal digital nerves are absent. They are replaced in the dorso-medial half of the carpus and metacarpus with the last ramifications of the medial cutaneous nerve of the forearm (from the musculo-cutaneous nerve), and in the dorso-lateral half of the same regions with the caudal cutaneous nerve of the forearm (from the ulnar nerve) and inconstantly (in 40% of cases) with the lateral cutaneous nerve of the forearm (from the radial nerve). Out of the dorsal metacarpal nerves, the only present one, albeit very thin, is the lateral one which originates in the dorsal branch of the ulnar nerve. This branch has a descendent trajectory on the lateral side of the carpus and obliquely crosses the proximal extremity of the lateral metacarpal bone. It continues as a dorsal metacarpal nerve, descending near this bone until the metacarpo-phalangeal joint to which it provides fibres. The palmar branch of the ulnar nerve contributes to the formation of the nerves of the palmar side through two fascicles. On the medio-palmar margin of the pisiform bone, it joins the lateral branch of the median nerve (which basically constitutes the lateral palmar nerve). At the level of the second row of carpal bones its fibres distribute into two terminal branches: the superficial branch remain definitively interwoven with the lateral palmar nerve; the others form the profound branch (known in the past as "profound palmar branch") which curves between the origin of the III interosseous muscle and the accessory ligament (the carpal bridle) of the tendon of the profound flexor muscle of the fingers in order to provide the palmar metacarpal nerves after sending a branch to the III interosseous muscle (Fig. 1). The palmar metacarpal nerves, one medial and one lateral, each descend between this bone and the corresponding accessory metacarpus. It sends fibres to the II and IV interosseous muscles and the lumbrical muscles, as well as to the periosteum of the metacarpus. A bunch of final fibres reach the palmar side of the metacarpo-phalangeal joint.
The common digital palmar nerves are strong.

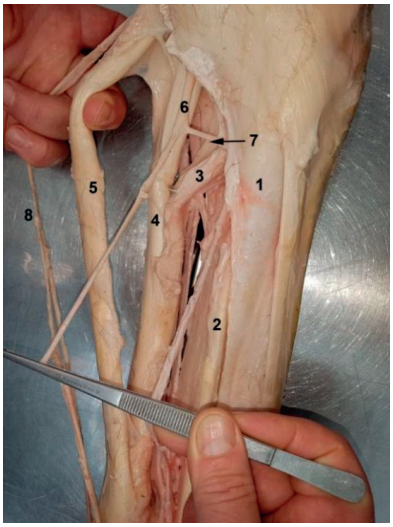

Fig. 1. Topography of the profound palmar nerve in the horse, right thoracic limb - lateral view (original): 1 - lateral rudimentary metacarpal IV; 2 - the median interosseous muscle; 3 - carpal bridle for the tendon of the deep digital flexor muscle; 4 - deep digital flexor muscle tendon; 5 - tendon of the superficial digital flexor muscle; 6 - lateral palmar nerve; 7 - deep palmar nerve (for the median interosseous muscle); 8 - medial palmar nerve

The medial palmar nerve is the medial branch through which the median nerve ends. It accompanies the palmar margin of the median artery on its trajectory, then the common digital artery II, descending on the medial side of the tendon of the deep flexor of the finger muscle, in

the palmar sheath of the carpus (the greater postcarpal sheath), then under the palmar metacarpal fascia (where it can be anaesthetised). Above the metacarpo-phalangeal joint it ends in the proper digital nerve which it continues, the latter giving a dorsal branch on its origin. On its trajectory it emits multiple fibres to the skin and the tendons of the digital flexor muscles. A remarkable communicant branch detaches in a very sharp angle from the palmar side, in the middle third of the metacarpus, crossing the tendons of the flexors superficially in order to unite with the lateral palmar nerve.

The lateral palmar nerve is formed through the joining of the lateral branch of the median nerve with the superficial palmar branch of the ulnar nerve. It joins the palmar margin of the III common digital palmar vein, thus descending on the lateral margin of the tendon of the profound digital flexor, relatively similar to its medial homologous. Halfway to the metacarpus it receives a communicant branch from the medial palmar nerve. It ends at the same level as the medial palmar nerve through the lateral proper digital nerve.

The two proper digital nerves, lateral and medial, have exactly the same distribution. Each proper digital nerve is located on the palmar side and supplements the absence of the dorsal digital nerves by emitting a strong dorsal branch which constitutes the first collateral branch (Fig. 2). This occurs above the metacarpo-phalangeal joint, the detachment place of the branch representing the limit between the palmar nerves and the proper digital nerves.

Alongside the proper digital artery and vein, the nerve will form a vasculo-nervous fascicle located under the thick skin of the metacarpo-phalangeal region, and then the pastern region. The nerve occupies the palmar margin of this fascicle, the digital vein is located dorsally and the artery intermediary (Fig. 2). Fine nervous threads originating from the dorsal branch accompany the vessels; one is located on the dorsal side of the vein and another between the artery and vein. The aforementioned vasculo-nervous fascicle obliquely crosses the ergot ligament which orients from the III finger to the ergot, crossing the profound side of the vein and then the surface of the artery and nerve.

At the level of the proximal margin of the ungular cartilage the fascicle dissociates and only the artery and the proper digital nerve pass on its profound side to distribute to deep portions of the ungular region.

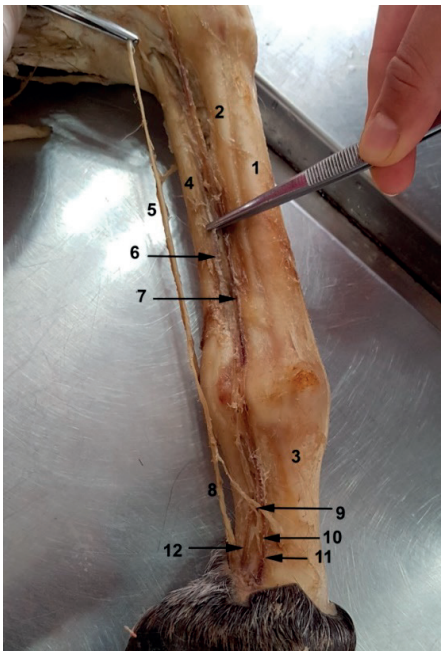

Fig. 2. Topography of vascular-nervous formations in the thoracic autopodium region in horses, left limb - medial view (original):
1 - main metacarpal bone III; 2 - rudimentary metacarpal bone II; 3 - phalanx I; 4 - flexor tendons; 5 - medial palmar nerve; 6 - palmar artery; 7 - common digital vein II; 8 - posterior digital nerve; 9 - anterior digital nerve (dorsal branch); 10 - middle digital nerve; 11 - proper digital medial vein; 12 - proper digital medial artery

The dorsal branch crosses the surface of the proper digital artery and vein, dorsal to which it ramifies dorso-distally. These nervous fibres distribute to the skin on the dorsal side of the finger until the dermis of limbus of the burelet, as well as in the sub-ungular dermis of the lateral regions of the hoof capsule. A remarkable and constant branch is the intermediary one, disposed between the artery and vein. Almost as thick as the dorsal branch, it detaches usually from its initial portion, but it can directly originate from the proper digital nerve, with which it exchanges dermis of the palmar portion of the hoof capsule.

Other branches of the proper digital nerve distribute as such: 1) near the proximal sesamoids are branches for the tendons of the digital flexors and a small nerve for the ergot (metacarpal torus); 2) the branch for the digital torus which accompanies the homonymous artery to distribute to the digital cushion; 3) deep branches for the tendons of the flexors; 4) coronary branches which emerge under the ungual cartilage to distribute together with branches of the dorsal artery of the middle phalanx to the interphalangeal joints and to the deep side of the ungual cartilage; 5) opposite to the latter, a thread is emitted which crosses the palmar process of the distal phalanx and traverses the ungual cartilage to participate in innervating the lamellar dermis, alongside branches of the coronary artery; 6) branches destined to the velutous tissue of the digital torus; 7) a thread which crosses the notch of the palmar process of the distal phalanx to accompany the dorsal artery of this phalanx to participate in the innervation of the lamellar dermis and the bone; 8) terminal branches are the ones which interweave with the arterial branches in the solar canal before distributing threads to the distal sesamoid and the adjacent recesses of the distal interphalangeal joint.

It is worth mentioning that the dorsal branch also innervates, aside from the skin of the pastern and the coronal regions, most of the lamellar dermis and velutous tissue. The proper digital nerve innervates mostly the deep organs of the hoof. At this level sensitivity is heightened, maintaining the functional activity of the keratogenic tissue, especially the lamellar dermis which ensures the fixation of the hoof wall to the distal phalanx.

CONCLUSIONS

The dorsal side of the autopodium in horses lacks nerves with the exception of a metacarpal nerve, represented in older terminology by the so- called dorsal nerve of the hand, a superficial branch of the ulnar nerve.

There are individual variations regarding the innervation of the dorso-lateral side of the carpus and metacarpus in 40% of cases the lateral cutaneous nerve of the forearm overlaps its territory with the caudal cutaneous nerve of the forearm.

The lateral and medial palmar nerves are terminal branches of the median nerve. In this sense the indications of the Nomina Anatomica Veterinaria must be respected, namely the participation of the ulnar nerve with superficial terminal threads in the consolidation of the lateral palmar nerve.

Deep terminal branches of the ulnar nerve will form a fascicle, known previously under the improper term of deep palmar nerve, which will serve the median interosseous muscle and will represent the origin of the palmar metacarpal nerves.

The two palmar nerves, lateral and medial, are symmetrical both as topography and as distribution of the proper digital nerves and their terminal branches.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXVIII (2), 2022
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

RESEARCH REGARDING THE MORPHOLOGY OF THORACIC LIMB BONES IN THE CARPATHIAN LYNX (*Lynx lynx* ssp. *carpaticus* - Linnaeus, 1758)

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Abstract

According to the actual taxonomic classification, Lynx lynx is one of the four species of the Lynx genus. The bones studied belong to individuals originating from the area of the Carpathian Mountains, belonging to the "carpathicus" subspecies. The lynx is a top predator in a food chain, its most important role being to control the populations of small vertebrates in the ecosystem and to maintain the populations of roe deer (Capreolus capreolus) and red deer (Cervus elaphus) under control. Specialty literature includes works of various authors regarding the morphology and morphometry of the locomotor apparatus of the Lynx lynx species and other species of the Lynx genus. However, upon a detailed study of the material, some interesting anatomical aspects could be identified. Worth mentioning are details regarding the contour of the scapula, the morphology of the spina scapularis and the coracoid process, the aspect of the distal extremity of the humerus, the aspect of the radio-ulnar interosseous space, as well as of the distal extremity of the radius and olecranon. These elements may complete existing literature data and may prove useful to clinicians in various interventions (radiological exams, MRI scans or surgical interventions).

Key words: *lynx, scapula, coracoid process, humerus, olecranon.*

INTRODUCTION

The lynx *(Lynx lynx* ssp. *carpathicus)* (Linnaeus, 1758) is classified in the *Carnivora* Order, which includes carnivorous mammals belonging to the *Felidae* Family, *Lynx* Genus. It is a species which inhabits the territory of Romania in the mountainous areas. Their population is relatively low, and they are a protected species (Cotta & Bodea, 1969).

Out of all felines encountered in zoos or reservations, studies were done on the tiger (*Panthera tigris*), the cheetah (*Acinonyx jubatus*), the lion (*Panthera leo*) and the jaguar (*Panthera onca*), regarding the morphology and morphometry of different skeletal components (skull, vertebral column, limb bones) (Belu et al., 2012; Kirberger et al., 2002; Nzalak et al., 2010; Roșu et al., 2016).

Specialty literature includes studies regarding the morphology and morphometry of the locomotor apparatus of the *Lynx lynx* species as well as other species in the *Lynx* genus (Karan et al., 2016; Mandal & Talukder, 1975). In spite of this, following a more detailed study some interesting anatomical aspects were identified.

The study focused on the bones of the appendicular skeleton of the thoracic limb in two lynx (*Lynx lynx*) individuals, and it aimed to present some particularities on the basis of which it can be differentiated from other carnivorous species.

MATERIALS AND METHODS

The study material was represented by the bones of two adult lynx (*Lynx lynx* ssp. *carpathicus*), both males that died of natural causes, one originating from the Bucuresti-Baneasa Zoo and the other from the Grigore Antipa Museum of Natural Sciences.

Maceration was done in containers maintained at a constant temperature for a long time (approximately 50 days) under constant supervision. ***Washing*** was first performed under a continuous stream of water for 24-48 hours. ***Post-maceration cleaning*** was performed with the tip of a scalpel to remove all organic remnants. ***Degreasing*** was done using usual detergents diluted in the wash water. The next step was ***washing using slightly acidic water*** to remove all traces of organic material. ***Drying*** was then performed under constant supervision for 48-56 hours at an average temperature of 18-22^{0}C in order to avoid the fissuring of osseous structures and compromising their integrity.

The most interesting aspects were described and photographed. Describing, identifying and naming of the formations were done according to Nomina Anatomica Veterinaria (N.A.V.) 2017.

RESULTS AND DISCUSSIONS

In lynx *(Lynx lynx* ssp. *carpathicus)* the scapula presents, on the lateral surface, a rectilinear spina scapularis, slightly bent over the infraspinous fossa. On the distal extremity the spina scapularis ends in an acromion, flanked by a well-developed, relatively trapezoid shaped para-acromion, with the lesser side disposed proximally. The ratio between the supraspinous and infraspinous fossae is of 1:1 (Figure 1).

The caudal angle is thickened and the cranial angle is rounded. The suprascapular cartilage is absent, replaced by a thick epiphyseal lip. The caudal border is flattened medio-laterally, and the distal extremity presents a rough articular surface for muscular insertion. The cranial border is rounded. The scapular notch is reduced. The neck of the scapula is very short. On the distal extremity of the supraspinous fossa there is a first order vascular hole.

The subscapular fossa has a groove which corresponds to the detaching place of the spina scapularis on the lateral side - aside from this it is crossed by numerous lines of muscular insertion (Figure 2).

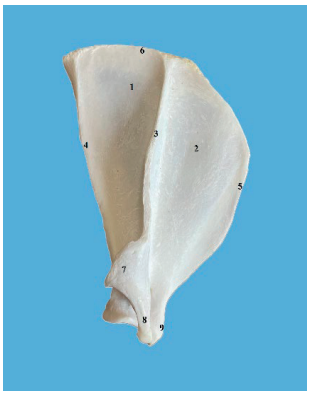

Figure 1. Lateral surface of scapula in lynx (*Lynx lynx* ssp. *carpathicus*): 1. Supraspinous fossa; 2. Infraspinous fossa; 3. Scapular spine; 4. Caudal border; 5. Cranial border; 6. Rough epiphyseal lip; 7. Paraacromion; 8. Acromion; 9. Supraglenoidal tuberosity

On the medial surface of the scapula in the distal extremity, nearing the neck of the scapula, there is a first order vascular hole.

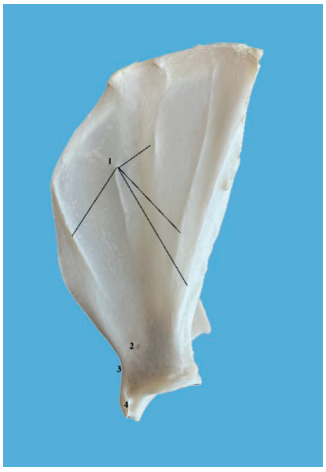

Figure 2. The medial surface of the scapula in lynx (*Lynx lynx* ssp. *carpathicus*): 1. Muscular insertion lines; 2. First order vascular hole; 3. Scapular notch; 4. Coracoid process

The glenoid cavity is circular in shape, and from the glenoid cavity detaches the supraglenoidal tuberosity, disposed cranially. From the supraglenoidal tuberosity the coracoid process detaches, very well developed and oriented cranio-medio-distally (Figure 3).

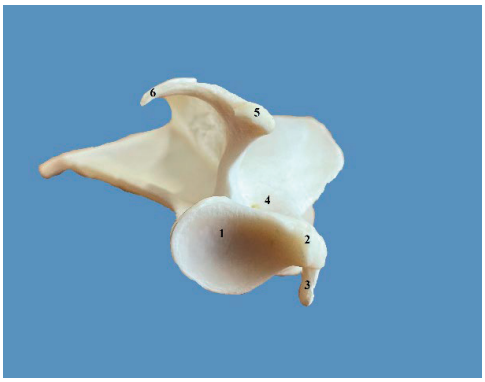

Figure 3. Distal extremity of scapula in lynx *(Lynx lynx* ssp. *carpathicus*): 1. Glenoid cavity; 2. Supraglenoidal tuberosity; 3. Coracoid process; 4. First order vascular hole; 5. Acromion; 6. Paraacromion

The humerus has the articular head oriented caudally, with the articular surface elongated cranio-caudally. The greater tubercle, undivided, slightly overtakes the articular surface of the humeral head. Immediately under this tubercle the relatively circular *facies infraspinata* can be evidenced, disposed laterally. On the proximal extremity of the humerus, on the lateral side there is a reduced tubercle for the *teres minor* muscle.
The lesser tubercle is reduced, with a rough elongated surface. The intertubercular groove is wide and shallow (Figure 4).

Figure 4. Medial side of the humerus in *lynx* (*Lynx lynx* ssp. *carpathicus*): 1. Greater tubercle; 2. Lesser tubercle; 3. Intertubercular groove; 4. Articular head; 5. Tubercle for the teres major muscle; 6. Supracondylar foramen; 7. Medial lip of the trochlea

In the proximal and middle third, on the cranial side, a rough crest can be observed.
On the medial side, in the superior third of the humerus, there is a reduced tubercle for the *teres major* muscle.

Figure 5. Cranial side of the humerus in lynx (*Lynx lynx* ssp. *carpathicus*): 1. Greater tubercle; 2. Lesser tubercle; 3. Rough crest; 4. Supracondylar foramen; 5. Coronoid fossa; 6. Radial fossa; 7. Condyle; 8. Trochlea

On the lateral side of the body, on the proximal extremity, there is an obvious anconeal crest, which continues distally with a sharp deltoid crest. The body of the humerus is slightly curved, relatively S-shaped, similar to the one in canides.
The distal extremity of the humerus presents, on the caudal side, a wide and deep olecranon fossa, and on the cranial side a superficial radial fossa, disposed above the humeral trochlea and a coronoid fossa which is more reduced, disposed above the condyle (Figure 5)
The articular surface is represented by a reduced trochlea, oblique, with unequal lips, the medial one being taller and sharper. The lateral lip of the trochlea is laterally flanked by a reduced condyle, visible on the cranial side. Above the medial lip of the trochlea there is an elongated supracondylar foramen.
The distal articular surface is flanked by the two epicondyles, lateral and medial. The crest of the lateral epicondyle is tall, ending in the inferior third of the caudal side of the humerus (Figure 6).

Figure 6. Lateral side of the humerus in the lynx (*Lynx lynx* ssp. *carpathicus*): 1. Greater tubercle; 2. Anconeal crest; 3. Deltoid crest; 4. Tubercle for the *teres minor* muscle; 5. Supracondylar foramen; 6. Crest of the lateral epicondyle; 7. Condyle; 8. Trochlea; 9. Coronoid fossa; 10. Radial fossa

The radius and ulna articulate only at the level of their extremities, delimiting a large interosseous space.

The radius has a relatively rectilinear body.

On the proximal extremity it presents an elliptic glenoid cavity.

The medial tubercle is well evidenced in the proximal extremity of the medial margin, under the articular cavity (Figure 7). Latero-caudally, on this extremity, there is an obvious oval-shaped tubercle. Distally, the cranial surface of the body presents three obvious tendinous grooves, two of them disposed longitudinally and one oblique medio-distally.

The distal articular surface has the aspect of an elongated cavity. On the distal extremity of the lateral margin there is a relatively oval articular surface for the ulna. Distally, on the medial margin of the radius there is an obvious notch.

The ulna presents an obvious olecranon, with an olecranal tuber that is divided cranially by a median groove, resulting in two tubercles, lateral and medial, with the medial one being more prominent. The beak of the olecranon is pulled cranially, the large semilunar notch is semicircle shaped and the radial notch is pulled medially (Figure 8).

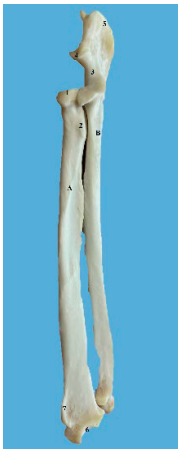

Figure 7. The medial surface of the radius and ulna in the lynx (*Lynx lynx* ssp. *carpathicus*): A. Radius; B. Ulna: 1. Glenoid cavity; 2. The medial tubercle; 3. The large semilunar notch; 4. The beak of the olecranon; 5. Olecranal tuber; 6. Distal articular surface; 7. Lateral crest

The body of the ulna is flattened latero-medially, with the caudal margin widened in the superior and middle thirds while in the inferior third it is thin and sharp. On the lateral side in the middle third there is an obvious groove. The ulnar styloid process is also evident, with an articular surface for the carpal bones.

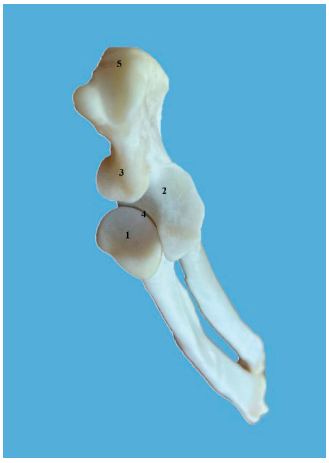

Figure 8. The proximal extremity of the radius and ulna in the lynx (*Lynx lynx* ssp. *carpathicus*): 1. Glenoidal cavity; 2. Large semilunar notch; 3. The olecranal tuber; 4. Radial notch; 5. Olecranon tuberosity

The carpal bones are seven in number, with the most voluminous one being represented by the radial carpal bone (Figure 9).
The radial carpal is the biggest carpal bone, presenting, proximally, a convex articular surface for the radius. Medio-palmar there is an obvious tubercle. Distally, it presents an articular surface for the bones in the second row, dorso-medially to the second carpal bone and latero-palmar for the third and second carpal bones.
The accessory carpal bone, located in the proximal row of carpal bones, presents on the free extremity an elongated tuberosity and two articular surfaces: a proximal one for the ulnar bone and a distal one for the ulna. Also in the proximal row is the ulnar bone which presents articular surfaces for the ulna, accessory carpal and fourth carpal bones.

Figure 9. Carpal bones in the lynx (*Lynx lynx* ssp. *carpathicus*): 1. Accesory bone; 2. Ulnar carpal bone; 3. Intermediate carpal bone; 4. Fourth carpal bone; 5. Third carpal bone; 6. Second carpal bone; 7. First carpal bone

In the distal row, the following bones are disposed latero-medially: fourth carpal bone, which articulates with the proximal extremity of the IV and V metacarpals, the third carpal bone articulates distally with the proximal extremity of the III metacarpus, the second carpal boned articulates with the II metacarpus and the first carpal bone articulates with the I metacarpus.
There are 5 metacarpal bones, of which metacarpus I is the shortest (Figure 10). At the level of the proximal extremity there are articular surfaces for the carpal bones on one side and on the other surfaces for articulating with each other. The distal extremity of the metacarpals presents articular surfaces for the phalanges and sesamoid bones.

Figure 10. Thoracic autopodium in lynx (*Lynx lynx* ssp. *carpathicus*): 1. Carpal bones; I - Metacarpus I; II - Metacarpus II; III - Metacarpus III; IV - Metacarpus IV; V - Metacarpus V; 2. Claw processes

The phalanx of the I digit is the shortest, the others with lengths directly proportional to the corresponding metacarpals. The middle phalanges act similarly to the proximal ones, corresponding to the metacarpals II, III and IV. The most developed middle phalanges are represented by digits III and IV.
On the distal phalanges well developed, recurved claw processes can be observed. The claw process of the first finger is the most well developed.

CONCLUSIONS

The spina scapularis ends with an acromion flanked by a well-developed para-acromion.
In the distal extremity of the supraspinous fossa there is a first order vascular hole.
The medial surface is crossed by numerous lines of muscular insertion.
The coracoid process is well developed and oriented cranio-medio-distally.
In the proximal and middle third of the humerus, on the cranial surface, there is a rough crest.
The anconeal crest and the deltoid crest are well evidenced.

The crest of the lateral epicondyle is tall, ending in the distal third of the caudal side of the humerus.

On the medial margin of the distal extremity of the radius there is an obvious ridge.

The thoracic autopodium has no particular characteristics compared to other felines.

All of these are characteristics which allow establishing the species and reduce the risk of confusion upon examination.

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CLINICAL SCIENCES

Scientific Works. Series C. Veterinary Medicine. Vol. LXVIII (2), 2022
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

SARS-CoV-2 DETECTION IN THREE CATS (*Felis catus*) BY REAL-TIME REVERSE-TRANSCRIPTASE POLYMERASE-CHAIN-REACTION IN BUCHAREST, ROMANIA

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Abstract

Since the COVID-19 pandemic began, there have been more and more studies and case-reports regarding the transmission of SARS-CoV-2 to several animal species. In cats, SARS-CoV-2 susceptibility was proved through experimental infection and natural settings (direct contact with infected humans). In this paper, the real-time reverse-transcriptase polymerase-chain-reaction (rRT-PCR) results obtained from four cats living in SARS-CoV-2-infected households in Bucharest (Romania) were described. All cases were indoor cats living in close contact with infected owners (in one household, owners were asymptomatic and, in the other households, owners had mild clinical signs). All the cats were lethargic and had a moderate loss of appetite. One cat was slightly dyspnoeic. The genomic material was extracted from deep oropharyngeal swabs using the QIAamp cador Pathogen Mini Kit. The rRT-PCR analysis used the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel and primers designed to detect the SARS-CoV-2 nucleocapsid (N) protein gene, supplied by Integrated DNA Technologies (IDT, USA). The sample from the cat living with an asymptomatic family was negative, while cats of owners with clinical signs provided positive results, as follows: $Ct_{(Cat\#1)}$=27.04, $Ct_{(Cat\#2)}$=24.13, and $Ct_{(Cat\#4)}$=35.0. Results revealed the risk of indoor cats' infection with SARS-CoV-2 in households where owners have COVID-19, especially if they show clinical signs.

Key words*: SARS-CoV-2, COVID-19, Coronavirus, feline diseases.*

INTRODUCTION

In December 2019, a new pandemical situation was declared, starting from Wuhan, China. The first official communications incriminated bats as an infectious reservoir, and the new virus was suspected of having the ability to cross the species barrier (Wang et al., 2020; Hosie et al., 2021a). SARS-CoV-2 is a novel Coronavirus responsible for the COVID-19 global spread. It is related with SARS-CoV-1 [the etiological agent of Severe Acute Respiratory Syndrome (SARS), first identified at the end of February 2003) and MERS-CoV [the etiological agent of Middle East Respiratory Syndrome (MERS), first reported in June 2012], but unlike these, SARS-CoV-2 has caused a pandemic still evolving, with still emerging strains and still rising questions (Likhacheva, 2006; de Groot et al., 2013; Turcu et al., 2021).

Coronaviruses are enveloped single-stranded, positive-sense RNA viruses. At least 39 distinct coronavirus species have been described and classified in 27 subgenera. They belong to genera *Alphaletovirus*, *Alphacoronavirus*, *Betacoronavirus*, *Deltacoronavirus*, and *Gammacoronavirus*, in subfamilies *Letovirinae* and *Orthocoronavirinae* of the family *Coronaviridae*, suborder *Cornidovirineae,* and order *Nidovirales*. SARS-CoV-1, SARS-CoV-2, and MERS-CoV belongs to the genus *Betacoronavirus* (Baraitareanu, 2020; Gaudreaulta, 2020).

Since the COVID-19 pandemic years began there are more and more studies and case reports regarding the transmission of SARS-CoV-2 to several species. First, the bat was recorded as a natural reservoir, but along with new researches, new animals proved to be receptive to this virus. In the early days of the pandemic, studies like the one conducted by Xiao et al. (2020) showed the presence in pangolins of a virus strongly related (until 99%) with SARS-CoV-2. The most studied species are cats, dogs, ferrets, and minks. In addition, experiments have shown that birds

such as ducks and chickens, as well as pigs, are not susceptible to the virus (Hosie et al., 2021a).
The most frequently studied and proven natural interspecies SARS-CoV-2 infections are human-cat and cat-to-cats. There is more evidence of natural infections of animals resulting from close coexistence with infected people, but also infections between animals of the same species demonstrated by experimental infections (Braun et al., 2021).
Hong Kong reported the first SARS-CoV-2 infections in a cat: a domestic short-haired cat that lived with the owner confirmed with COVID-19. Subsequently, numerous scientific reports have reported this in Belgium, the USA, France, Spain, Germany, the UK, Italy, Switzerland, Russia, Denmark, Sweden, Chile, Japan, Brazil, and Argentina (Barrs et al., 2020; Garigliany et al., 2020; Michelitsch et al., 2020; Musso et al., 2020; Newman et al., 2020; Sailleau et al., 2020; Segalés et al., 2020; Hosie et al., 2021a, 2021b; Klaus et al., 2021; Ruiz-Arrondo et al., 2021).
Recent analytical evaluation of real-time reverse-transcriptase polymerase-chain-reaction (rRT-PCR) for SARS-CoV-2 supports the gold standard value of this test method in COVD-19 diagnostic (Rahbari et al., 2021). Moreover, the studies of Rahbari et al. (2021) and Lee et al. (2021) provided information about sources of error (e.g., sampling, storage, processing, RNA extraction, cDNA synthesis, amplification, interpretation, and analysis and test reporting) and findings of the CDC investigation conducted to identify the causes of the N1 and N3 false-positive reactivity (Lee et al., 2021; Rahbari et al., 2021). This paper describes the results obtained by rRT-PCR for SARS-CoV-2 in indoor cats (*Felis catus*) that were in close contact with infected owners.

MATERIALS AND METHODS

Samples, animals, and housing
Deep oropharyngeal swabs from four cats (#1, #2, #3, and #4) that were living in the same apartment with SARS-CoV-2 infected owners were collected by their owners during quarantine. The swabs were safely collected by the owners who had medical training (veterinarians for cats #1, #2, #3, and a human anaesthetist for cat #4).
All samples were refrigerated (2-8°C) until the owners left the isolation period and brought the samples to the laboratory themselves (swabs were not older than 3 days after sampling).
Cat #1 is Siamese two years old female, Cat #2 is British Shorthair two years and 8 months old female, Cat #3 is British Shorthair 1 year and 2 months old female, and Cat #4 is European shorthair two years old female.
All cats were lethargic, with moderate loss of appetite. Cat #1 was slightly dyspnoeic, but with no radiologic findings (Figure 1). For cat #1 basic biochemical and haematological assays were performed, but there no changes were found.
The cats lived indoors, in apartments, with no outside access. The owners of cat #4 were two doses-vaccinated, asymptomatic, and PCR-positive. The owners of the cats #1, #2, and #3 were two doses-vaccinated, with a mild flu-like syndrome, and PCR-positive. No owner needed hospitalization.

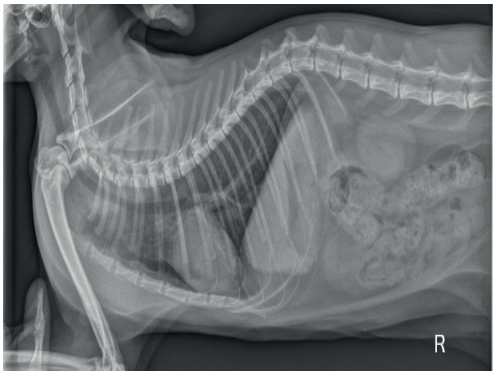

Figure 1. No pulmonary X-ray abnormalities (left, latero-lateral incidence) in a SARS-CoV-2 positive cat with mild respiratory syndrome. Cat #1 (Siamese breed, 2 years old, female).

rRT-PCR
For this analysis, it was used the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic-Panel (https://www.fda.gov/media/134922/download) Briefly, this test method use oligo-nucleotide primers (2019-nCoV_N1-F; 2019-nCoV_N1-R; 2019-nCoV_N2-F; and 2019-nCoV_N2-R;) and probes (2019-nCoV_N1-P label FAM, BHQ-1; and2019-nCoV_N2-P label FAM, BHQ-1) which detect the SARS-CoV-2 nucleocapsid (N) protein gene

(www.fda.gov/media/134922/download). The primers and probes were supplied by Integrated DNA Technologies (IDT, USA). The extraction of the ARN was made manually using QIAamp cador Pathogen Mini Kit (Qiagen, USA), following the manufacturer's extraction protocol. The amplification of the DNA was accomplished using qScript XLT One-Step RT-qPCR Tough Mix (Quantabio, USA) and a Light Cycler 2.0 analyser.

RESULTS AND DISCUSSIONS

The nucleotide-chain sequence for SARS-CoV-2 was identified and amplified in oropharyngeal swabs from three cats (#1, #2, and #3) and the Cat #4 sample was negative.
The Ct values of Cat #1 and Cat #2 were 27.04 (Figure 2) and 24.13 (Figure 3), which represents a medium-low amount of viral genome in the analysed samples.

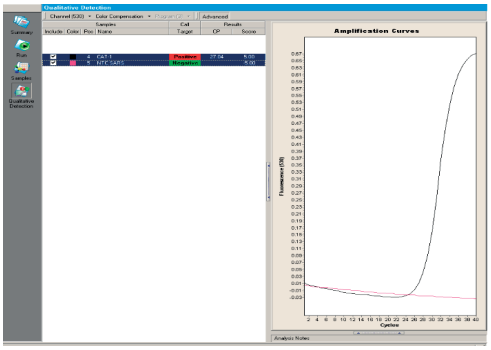

Figure 2. Amplification curve of rRT-PCR: oropharyngeal swab from Cat #1 (Siamese breed, 2 years old, female): Ct=27.07

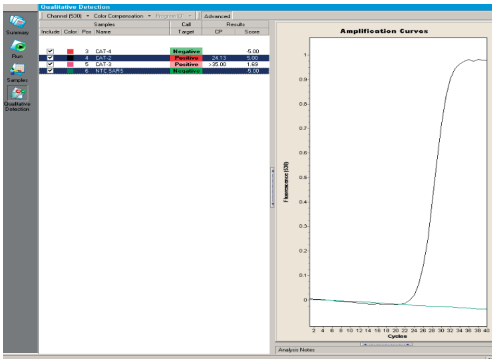

Figure 3. Amplification curve of rRT-PCR: oropharyngeal swab from cat #2 (British Shorthair breed, 2 years and 8 months old, female): Ct=24.13

A Ct=35.0 (Figure 4) value was found in the sample from cat #3 that can be related to a very small amount of virus in the extracted swab.
For the cat #4 no SARS-CoV-2 genome was detected in the received sample, meaning that the qPCR result was negative (Figure 5).

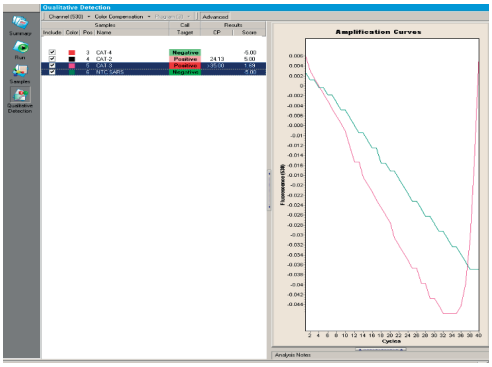

Figure 4. Amplification curve of rRT-PCR: oropharyngeal swab from cat #3 (British Shorthair breed, 1 year and 2 months old, female): Ct=35.0

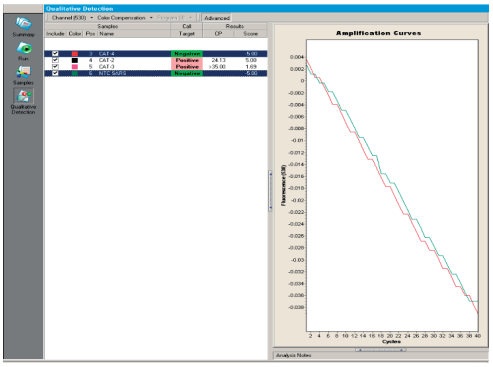

Figure 5. Amplification curve of rRT-PCR: oropharyngeal swab from cat #4 (European shorthair breed, 2 years old, female): Ct>40

The rRT-PCR used in our study proved to be a very useful, quick, and accurate diagnostic tool for an infection with the new Coronavirus, which, at least for the animals included in our study, has no characteristic symptoms. Also, the amount of sample required by this assay is very small and a properly sampled swab is sufficient to extract the required RNA.
Our results support the previous reports of cats and dogs' natural infections with SARS-CoV-2 in households with confirmed COVID-19 human subjects (Musso et al., 2020; Segalés et al., 2020; Hamer et al., 2021; Hosie et al.,

2021a; Klaus et al., 2021; Tewari et al., 2021) and demonstrate that cohabitation of a SARS-CoV-2-positive humans with a cat can lead to transmission of the virus to the cat. However, the role that cats play in COVID-19 epidemiology should be determined in further research studies because the relationship between humans, animals, and the environment is complex and dependent (Hernández et al., 2020).

The possible susceptibility of the cat to SARS-CoV-2 was considered because Martina et al. (2003) demonstrated the ability of SARS-CoV to infect ferrets and cats (Martina et al., 2003) and by first reports of Chen et al. (2020), Halfmann et al. (2020), and Shi et al. (2020) made early in the COVID-19 pandemic.

Moreover, Zhang et al. (2020) study provided serological evidence for SARS-CoV-2 infection in pets with the main recommendation to investigate the route of transmission of SARS-CoV-2 from humans to cats (Zhang et al., 2020).

In our study, only the cats living with the symptomatic owners provided positive results, possibly due to a higher amount of virus spread by owners with clinical signs, which increased the risk of contamination of these cats (statement also supported by the fact that the negative cat belongs to the infected owners without clinical signs).

Our data support the recommendation of Zhang et al. (2020) to implement preventive measures to maintain a suitable distance between owners with COVID-19 and companion cats (Zhang et al., 2020).

Spada et al. (2021) surveyed stray colony and shelter cats from the Lombardy region in pre- and during pandemic SARS-CoV-2 infection and obtained negative molecular results and very low seroprevalence. These results indicate that the likelihood of cats that do not live in close contact with humans (free-ranging stray or shelter cats) becoming infected is very low, as well as their role of them in the transmission of SARS-CoV-2 during the pandemic (Spada et al., 2021). However, cats living in households where people are infected with SARS-CoV-2 may act as vectors and close contact with these cats should be avoided, especially if owners showed signs of COVID-19 (Hosie et al., 2021b). Therefore, veterinarians should pay special attention to contact with cats and other pets that require medical care during the quarantine period of owners with COVID-19.

CONCLUSIONS

The risk of indoor cats' infection with SARS-CoV-2 in households where owners have COVID-19 is high, especially if they show clinical signs. In infection with SARS-CoV-2, cats can become lethargic, lose their appetite and be slightly dyspnoeic. In our study the cats recovered without treatment. Further research on the epidemiology of COVID-19 to establish the role that companion animals play in SARS-CoV-2 spreading should be carried out.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXVIII (2), 2022
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

CAN IT MAY BE A MIXED NEOPLASIA WITH THE COMPONENT OF BOTH CARCINOMATOUS MASTITIS AND T-CELL LYMPHOMA WITH SKINL LOCATION - COMPARATIVE STUDY

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Abstract

Carcinomatous mastitis or the inflammatory mammary carcinoma is a malignant entity described in human, dog and cat clinical oncology; it is characterized by a sudden onset, edema, erythema, peau d'orange, hardening and increased local temperature of the mammary gland, with or without the presence of mammary nodules. Blockage of superficial lymphatics with neoplastic cells is the cause of severe edema in the region. This type of cancer is characterized by an extremely fast rate of growth, development and invasiveness. Tumor cells break the basement membrane and invade regional lymphatic vessels and satellites lymph nodes. Cutaneous T-cell lymphoma in the dog is a rare neoplastic condition with unknown etiology. The reaction at dermis level is characterized by infiltration of neoplastic T lymphocytes with a specific tropism for the epidermis and the ancillary structures. The abnormal division of lymphocytes present plaques or other lesions within the skin. It often involves enlarged satellite lymphnodes, edema, erythema and hardening of the malignant nodules. The link between the immune role of the T-cells and the fulminant inflammatory reaction of the carcinomatous mastitis, frameable in the category of autoimmune diseases, is just one of the puzzles researchers are trying to solve in order to prove the connections between the two neoplasias. One of the pieces is the role of cytokines, having value both in canine inflammatory mammary cancer and T-cell lymphoma. Their purpose of these small proteins that act as cell-to-cell messengers and play an important role within the immune system by stimulating or inhibiting cells in response to a range of stresses has been evaluated due to it's valuable contribution as diagnostic biomarker, biologic predictive marker and the therapeutic significance. The purpose of this study was to characterize the two malignant pathologies clinically, histopathologically and regarding response to treatment in order to identify common ground. Following the information obtained from the studies considered for this article, supplemented with personal case studies, the presence of a mixed neoplasm in the form of an undifferentiated carcinoma interspersed with T lymphoblasts is suspected.

Key words: *inflammatory carcinoma, carcinomatous mastitis, cutaneous T-cell lymphoma.*

INTRODUCTION

Developed on a biological substrate and with a similar endocrine metabolism, the diseases of the mammary gland in humans, dogs and cats have many similarities in clinical evolution and in therapy.

Carcinomatous mastitis is a neoplasm composed of undifferentiated mammary neoplastic cells possibly epithelial and mesenchymal stem. It is characterized clinically by evolutionary bursts (EB).

These EBs are the expression of acute development between the degree of neoplastic aggression and metabolic and immunological antitumor resistance of the affected body. Stage EB1 represents the initial moment, the equivalent of T1, EB2 is in the case of localized forms (T2) and EB3 in the case of diffuse, extended (T3-T4), it has a local pseudoinflammatory appearance manifested by edema with erythema of the entire breast, local hyperthermic, with the characteristic appearance of peau d'orange. It usually represents the clinical stage T4, being characterized by the lymphatic dissemination, in the intradermal structure of the epithelium of the gland, of the highly anaplasic undifferentiated malignant cells from an invasive ductal carcinoma. Clinically in inflammatory mammary carcinoma or the acute EB undifferentiated form, the affected mammary gland is severely edematous, with infiltrated skin, intense congestive blush

coloration, with characteristic appearance of "flush" caused by stenosis of dermal lymph capillaries, hypodermic and glandular parenchymal structure with microscopic tumor thrombi but with embolizing effect. (Crînganu, 2009)

Following selected studies, we present essential information that demonstrates the presence of similarities that indicate the possibility of a mixed form of cancer.

This data is essential for updating diagnostic and treatment protocols, thus leading to their efficiency. This comparative essay is essential for the development of ideas related to the similarity of neoplastic pathologies at this level, which cannot be differentiated from a clinical point of view.

Given the aggressiveness of this type of cancer, the poor response to treatment and the prognosis, it is necessary to continue the studies we have so far.

A COMPARATIVE REVIEW OF THE EXISTING SPECIALTY LITERATURE

There are few reports concerning histological aspects of IMC[1]. Infiltrating ductal carcinomas, other carcinomas and unspecified malignant tumors have been described as involved with IMC. Mammary involvement be can be localized to one or both glands with or without regional lymph node involvement known as PBL[2]. It can be a part of disseminated disease considered as secondary involvement of the breast. Lymphoepithelial lesions in ducts, lobules and vascular involvement have been seen in breast lymphomas. In both primary and secondary breast lymphomas, diffuse large B cell lymphoma (DLBCL) is the most common type. Diagnosis is not often delayed due to the similarity of signs with inflammatory mammary carcinoma.

According to Vianello most cases of LBL[3] have T-cell phenotype, the majority of breast NHLs[4] are B-cell phenotype. Cases with this atypical presentation of lymphoma mimicking an inflammatory mammary carcinoma are rare, but raise questions about clinical diagnosys of malignant formations.

This is because, extensive observation of clinical cases have identified that the most common signs and symptoms of breast lymphoma include a painless enlarged palpable mass. Local inflammatory signs such as skin retraction, erythema, and peau d'orange are usually associated with high-grade lymphoma or diffuse parenchymal involvement. Which means, a clinical differentiation between mammary lymphoma and inflammatory mammary carcinom is impossible (Pena, 2003).

Cytokines released in the tumor microenvironment play a major role in cancer pathogenesis. In human cancers and corresponding animal models, cytokine expression contributes to tumor growth and progression, as well as regulation of the host anti-tumor response. The elucidation of the function and importance of cytokines in canine cancers is still in an early stage, although relevant data have been obtained in classical examples of comparative models of human cancers, such as osteosarcoma, melanoma, mammary tumor and lymphoma.

Cytokines are another piece of the puzzle that links the inflammatory mammary carcinoma and mammary/skin lymphoma together.

Cytokines are small proteins that act as cell-to-cell messengers and play an important role within the immune system by stimulating or inhibiting cells in response to a range of stresses. They also control the differentiation, activation and growth of different cell types. The effects of individual cytokines on immunity depend on the local cytokine concentration, the expression of specific receptors and the activation of multiple signalling pathways (Irac, 2019).

Canine lymphoma represents the most well-studied cancer in terms of cytokine or blood biomarker involvement. T cell lymphoma associated with increased levels of IL-6, whilst IL-10 and De Andres et al. (2013) have demonstrated increased serum levels and tissue expression of IL-6 and IL-10 in dogs with inflammatory mammary carcinoma compared with the non-inflammatory malignant mammary tumors.

Thus similar effects of the cytokines are found in both types of cancers.

[1] inflammatory mammary carcinoma
[2] Primary Breast Lymphoma
[3] Lymphoblastic leukemia/lymphoma
[4] Non-Hodgkin Lymphomas

Cancer often originates from a site of persistent inflammation, and the mechanisms turning chronic inflammation into a driving force of carcinogenesis are intensely investigated. Cyclooxygenase-2 (COX-2) is an inducible key modulator of inflammation that carries out the rate-limiting step in prostaglandin synthesis. Aberrant COX-2 expression and prostaglandin E2 (PGE2) production have been implicated in tumor genesis.

Malignant T cells isolated from skin specimens of patients with MF[5] showed COX-2 expression, whereas non-malignant T cells did not. Moreover, lymphocytes with a malignant phenotype also showed COX-2 expression in situ, indicating that malignant T cells express COX-2 in vivo in a large fraction of patients with advanced MF.

Furthermore expression of COX-2 has been uncovered in inflammatory mammary carcinoma biopsy specimens which mirrors the results obtained from the MF patients.

Anaplastic lymphoma kinase (ALK) gene has been found to be altered in several solid and hematologic tumors. Immunohistochemical analysis showed ALK is overexpressed in a substantial proportion of inflammatory mammary carcinoma and possibly plays a significant role in the aggressive behavior of this cancer. In order to confirm the common ground for these markers in inflammatory mammary carcinoma and/or mammary/skin lymphoma we have selected a canine patient, Mops, 9-year-old female, previously treated for a histopathological diagnosis of undifferentiated carcinoma (Figure 1).

The formation is recurrent after radical mastectomy, with an unfavorable evolution due to the incomplete therapuetic protocol.

Clinically, the dog presents massive thoraco-abdominal edema extended to the hind limbs.

Complete blood-work, thermography (Figure 2) and histopathology have lead to the diagnosis.

The treatment consisted of a complex approach to the patient. Chemotherapy: multi-agent second-line cytostatic drug consisting of the combination of Ifosfamide at a dose of 200 mg/m^2 i.v., every 14 days alternating with Epirubicin, (anthracyclinic pivot) at a dose of 20 mg/m^2 iv, every 21 days.

Recommended approach for this case is a complete mastectomy with axillary and inguinal lymph node excision after first the preoperative protocol described above.

Postoperatively, we recommend cytostatic chemotherapy to prevent lung and bone metastases (Gemcitabine 200 mg/m^2 iv at 14 days and Carboplatin 30 mg/m^2 at 21 days). In this case postoperative cytostatic therapy for IMC was supplemented with Leukeran 2 mg/m^2, every 14 days, per os, as a specific alkylating agent for lymphoma and Massivet 10mg./m^2 per os, daily, for 30 days as a tyrosine-kinase inhibitor to prevent the onset of cytostatic resistance by suppressing MDR genes[6]. Additional therapy with Cefort 25 mg /kg iv, Furosemide 0.2 ml sc, Meloxicam 0.35 ml sc. Adjuvant therapy included liver protection (Samilyn, Hepatiale, Ornitil), cardiac protection (Cardiostrength), renal protection (Renalvet), immunotherapy (Impromune, Corpet) and paraneoplastic syndrome therapy (antiemetics, transfusion, painkillers).

RESULTS AND DISCUSSIONS

The values of the presented markers fall both in the category of mammary carcinoma and in the category of a skin lymphoma.

Their prognostic value is confirmed by the patient's response to treatment.

Results of the patiens evaluation have been consistent with the specific characteristics determined by these markers.

Biochemical:

Elevated ALP: 520 (0-212) U/L

Hematologic:

Increased neutrophils: 13.71 billion/liter (3-12 billion/liter)

Low percentage lymphocytes: 6.8% (12-30%)

Monocytes percentage increase: 7.6% (2-4%)

Low percentage eosinophils: 0.7% (1-8%)

MCV (average volume of citrate) increased: 78% (60-77)

MCHC (mean hemoglobin concentration) low: 29.4 - g/dL (31-34 g/dL)

[5] Mycosis fungoides - cutaneous T-cell lymphoma

[6] Multidrug resistance gene

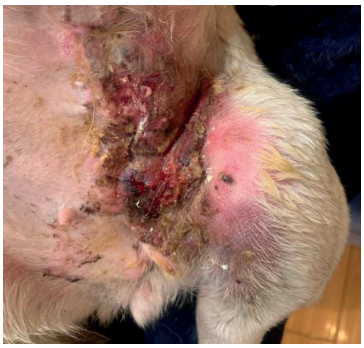

Figure 1. Mops, 9-year-old female, diagnosis of undifferentiated carcinoma (original)

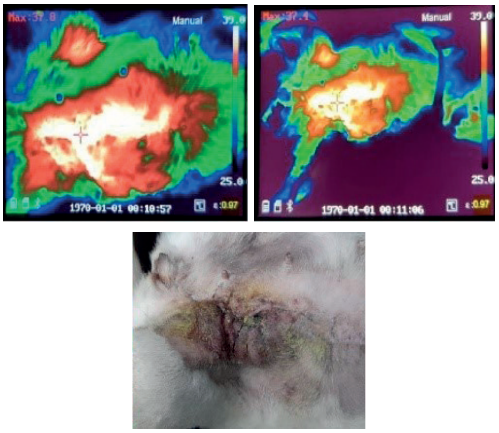

Figure 2. Thermography revealed important changes in the physiological perimeters that demonstrate the presence of inflammation (original)

Two hypotheses exist regarding the origin of PBL. The more accepted concept suggests lymphoma arises from existing intramammary masses. The less accepted notion theorizes that PBL has a connective tissue origin from the intralobular periacinar regions of lymphocytes, plasmocytes, and other cell types from undifferentiated mesenchymal cells. Our theory of a potential mixed tumor is based on the first concept.

Differential diagnosys is mostly done through cytodiagnosis of the primary breast lymphoma and the inflammatory mammary carcinoma. PBL presents lymphoepithelial lesion characterized by infiltration of lymphoma cells into the sparsely present duct epithelium of the breast . The major problem is obtaining adequate material due to thick skin, edematous stroma, and lack of a discrete mass so diagnosis of PBL by fine-needle aspiration cytology (FNAC) is reported infrequently. The same issue rises in inflammatory mammary carcinom (Militaru 2010).

Histopathologically, there is a weakly differentiated ductal carcinoma with lympho-plasma cell infiltrates that includes the wall of galactophore ducts, invasion of vascular spaces, lymphatic embolization and dermal infiltration, the tumor comprising both the skin and the subcutaneous and glandular connective tissue and with possible T lymphocyte entanglement that virtually determines an associated skin lymphoma with mammary involvement although they are different histo-structurally and ontogenetically .

The mark of histological confirmation is the invasion of dermal lymphatic vessels by undifferentiated malignant neoplastic cell embolisms associated with possible T cell lymphocyte infiltrate which must be confirmed by Parr test for lymphocyte phenotyping.

The aberrant COX-2 expression involved in the regulation of proliferation of malignant MF T-cells is mirrored in inflammatory mammary carcinoma although inflammatory cell infiltrates are no a common histologic finding and do not differentiate IMC from other forms of locally aggressive breast cancer, despite the clinical signs of inflammation. The presence of inflammatory cytokines is negligible (de Souza, 2009; Kopp, 2010).

Anaplastic lymphoma kinase (ALK), a tyrosine kinase receptor residing on chromosome 2p23 was first described in a subset of anaplastic large cell lymphoma (ALCL) patients. ALK alterations such as increased ALK copy number, gene amplification and translocation have been shown to be present in 80 % of inflammatory breast cancer and 25 % of triple-negative breast cancers (TNBC), which are considered to be the most aggressive subtypes of breast cancers. ALK positive anaplastic large cell lymphoma (ALCL) of the breast masquerades as a inflammatory mammary carcinoma with an increased incidence following a repetitive and/or frequent trauma (in humans the use of breast implants is incriminated) (Sathyanarayanan, 2010).

Based on the importance of these common factors, we evaluated a case of undifferentiated inflammatory breast carcinoma.

CONCLUSIONS

Clinical cases confirm that these common markers predict response to treatment and have prognostic value. Therapy result are inconsistent and palliative.

Histological study of the skin in our selected studies demonstrated embolization of lymphatic dermal vessels.

The exam showed various histological patterns of neoplastic dermal invasion: one of a tubular/papillary pattern with well-differentiated structures, one very anaplastic with independent highly malignant cells resembling a sar-coma (sarcomatous-like type). Adding serum levels of selected cytokines to diagnostic options in canine cancer patients would allow a better prognostic evaluation and would assist therapeutic decisions. Moreover, cytokines may give reliable information on the efficacy of therapy and very early response for cases that involve IMC, PBL, NHL or even mixed cancers.

COX-2 is expressed in MF, and a dependent growth factor for malignant T cells and has a strong presents in IMC thus being able to improve novel therapeutic targets in MF, IMC or mixed tumors with cyclooxygenase-2 expression. The recommended therapy took into account the markers presented in this paper, predictors of response to treatment and prognosis, and cases treated with a complete protocol had a favorable respons. The case presented emphasizes the importance of following all the steps.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXVIII (2), 2022
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

CASE REPORT: AORTIC THROMBOEMBOLISM RELATED TO POLYCYSTIC KIDNEY DISEASE IN A CAT

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Abstract

A cross-breed female cat was presented with clinical signs of abdominal pain, paralysis of both hind limbs, tachycardia, tachypnea and bilateral absence of the femoral pulse. Necropsy confirmed an aortic thrombus at 2 cm above the adrenal glands, the heart with left atrial dilation and hypertrophied left ventricle, multiple bilateral kidney cysts and ectopic thyroid tissue. Histopathological examination reveals the kidney with multiple cysts, diffuse loss of renal structural details without inflammatory cells, hypertrophic cardiomyopathy, and no ectopic thyroid tissue changes. This communication describes a case of aortic thromboembolism associated with hypertrophic cardiomyopathy due to autosomal dominant polycystic kidney disease and ectopic thyroid tissue.

Key words*: aortic thromboembolism, hypertrophic cardiomyopathy, cardiorenal syndrome, polycystic kidney.*

INTRODUCTION

In cats, arterial thromboembolism (ATE) is a common clinical syndrome. It is caused by the unexpected migration of a thrombus or a thrombus fragment from the left atrium (LA) (Vezzosi et al., 2020).

This syndrome was reported for the first time by Collet in 1930 (Smith & Tobias, 2004; Tomaiuolo, Litvinov, Weisel, & Stalker, 2020).

It is, most often, a consequence of the heart diseases, such as hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), restrictive cardiomyopathy (CMR), unclassified ischemic cardiomyopathy (Molina, Estrada G, Salas, & González, 2012; Schoeman, 1999), chronic myocardial infarction (Tsujino et al., 2005).

In 50% of cats with HCM, ATE has been reported (Michaud, Herbert, Elkins, & Gozalo, 2017). Generally, LA is the source of emboli.

This statement is supported by the fact that 21% of cats with HCM examined postmortem have left atrial thrombi.

Moreover, ultrasound examination in cats with heart diseases showed that intracardiac thrombi are quite commonly identified (Hogan, 2017; Smith & Tobias, 2004).

In cats without heart damage, ATE had been, sometimes, associated with neoplasms or hyperthyroidism. Neoplasia, especially lung carcinoma, is a risk factor for ATE, although in this situation there is a tumour embolism and less thromboembolism. In cats with thyroid disease and thyrotoxic cardiomyopathy ATE has been reported as being associated (Silva et al., 2016; Smith & Tobias, 2004). Dilated LA and intraatrial blood stasis are the main risk factors for thrombus development. In cats, the increasing of LA volume occurs most often in HCM, being concurrent with concentric hypertrophy of the left ventricle. Although it is known that in humans the improper functioning of the affected left ventricle may be a predictor of the onset of ATE than the increase in the volume of AS, in cats the systolic function has not been evaluated as a risk factor for ATE (Acierno et al., 2020; Baty et al., 2001). Male are more affected than female, with a male:female ratio of 2.5: 1, due to their predisposition to develop HCM (O'Dwyer, 2015; Tomaiuolo et al., 2020).

In the present case report we describe the clinical, pathological and histological features in a cat with aortic thromboembolism associated with hypertrophic cardiomyopathy

due to autosomal dominant polycystic kidney disease (PKD) and ectopic thyroid tissue. No other cases of cats that present these types of associated injuries were found in studied databases.

MATERIALS AND METHODS

A mixed-breed long-haired cat, female, aged seven years, weighing 3.4 kg, was presented with paraplegia, tachypnea, cyanotic mucous membranes, hypersalivation, vomiting, vocalizations. The cat was under medical observation for hypertrophic cardiomyopathy with left atrial dilation, severe mitral regurgitation and pleural effusion. Before recommending the therapeutic protocol, a cytopathological examination of the thoracic fluid was performed after previuos centrifugation for 5 minutes at 1500 rpm. Almost all of the surnatant was discarded and the obtained cell pellet was resuspended with the small amount of surnatant left. Cytological impression smears of the resuspended centrifuged pellet cell sample were performed, air-dried and stained with May Grunwald Giemsa (MGG) for light microscopic evaluation. Due to the rapid worsening of the clinical condition, the cat was euthanized. Post-mortem examination was performed and samples were collected for histopathological examination, from all modified organs. The selected specimens were fixed in 10% neutral buffered formalin, processed routinely, embedded in paraffin wax, cut into 3 µm sections, and stained with hematoxylin and eosin (HE).

RESULTS AND DISCUSSIONS

At the clinical examination were observed abdominal pain, paralysis of both hind limbs, tachycardia, and tachypnea. Lack of superficial and deep sensitivity, absence of femoral pulse, hypothermia, and cyanosis of the footpads was observed at the level of the hind limbs. The body temperature was 38°C. At the neurological evaluation, depression was present without neural reflexes. The thoracic fluid revealed grossly a pinkish, clear, watery appearance and the microscopical analysis showed inflammatory cells in approximately equal proportions: neutrophils, active macrophages, lymphocytes, plasma cells and eosinophils. A suspicion of modified transudate with chronic inflammatory process was suspected. The diagnosis of lumboaortic thromboembolism was established by the clinical investigation. Supportive therapy was instituted with anticoagulant, diuretic, steroidal anti-inflammatory drugs, antihypertensive drugs and fluids. Antibiotic was given to prevent secondary infections. The post-mortem examination confirmed the bilateral hydrothorax, and approximately 150 ml of pinkish fluid was evacuated. The lungs presented, bilaterally, diffuse oedema and congestion, a suggestive aspect of cardiogenic oedema. At the level of the dorsal, anterior mediastinum, an ectopic thyroid tissue of approximately 3 mm in diameter was found (Figure 1).

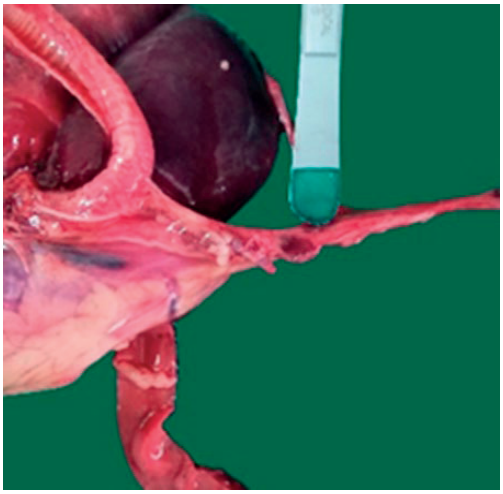

Figure 1. Ectopic thyroid tissue at the anterior mediastinum of the cat: the ectopic tissue is 3 mm diameter, dark red

0.8 ml of pinkish fluid were extracted from the pericardial sac, and that fits within the average values of 0.25 ± 0.15 ml/kg (Davies & Forrester, 1996; Holt, 1970). The left atrial dilation (Figures 2 and 3.), the thickening of the free left myocardial wall and the interventricular septum (Figure 4) were confirmed. The free left ventricular wall measured 9.82 mm, and the interventricular septum was 7.75 mm. Those dimensions are correspondent for HCM. Pathological

hypertrophy is defined by thicknesses of the left free ventricular wall and the interventricular septum ≥6 mm. At least one of these two myocardial segments must exceed the value mentioned above to establish the HCM diagnosis (Baty et al., 2001; Gouni et al., 2008) The values obtained following the ratio g heart/kg body was 0.88%, the cat heart having 0.46% ("Appendix 1: Normal Organ Weights - Percentage Body Weight", 2017).

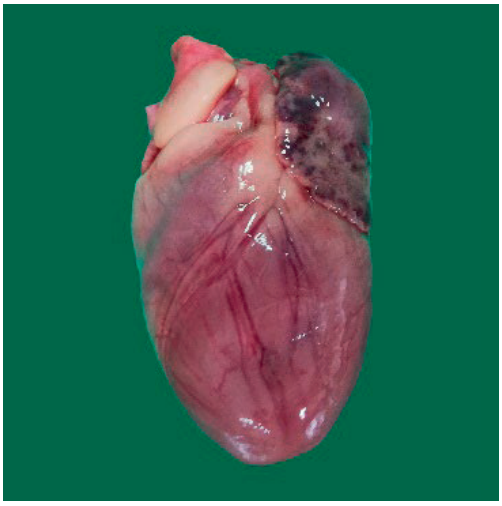

Figure 2. Gross features of the heart with HCM: LA dilation and diffusely enlargement heart volume

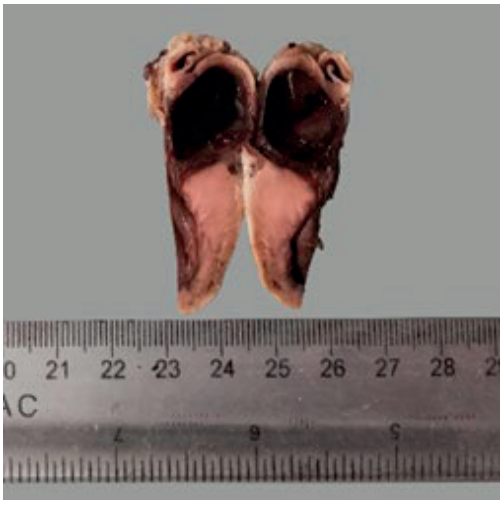

Figure 3. Gross left atrial chamber section view of the heart with HCM: dilation of left atrium

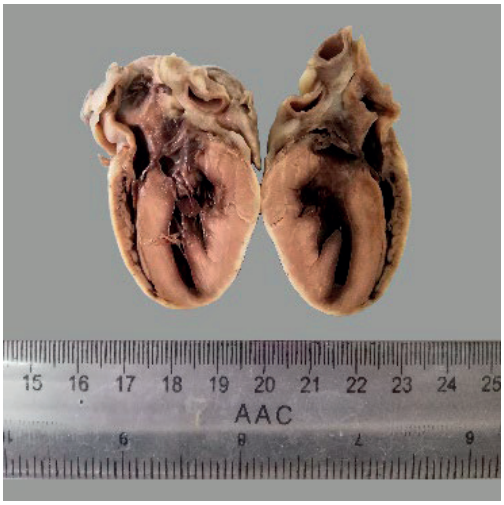

Figure 4. Four-chamber cross-section view of the heart with HCM in cat: disproportion between ventricular septum and free left ventricular wall hypertrophy with associated reduction in left ventricular cavity size

The liver has the appearance of passive congestion and areas of multifocal, subcapsular haemorrhage. The kidneys bilaterally showed numerous cysts and areas of fibrosis. Those modifications replaced more than half of the renal parenchyma (Figure 5). Multifocal necrosis of the renal papillae was also noted. The renal cortico-medullary area showed congestion, suggestive of prerenal ischemia. Yellow, transparent liquid leaked when the cysts were sectioned.

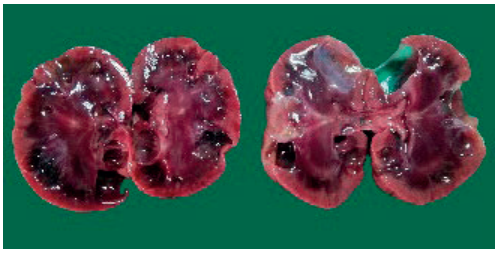

Figure 5. Longitudinal section of polycystic dysplastic kidneys: irregular external perimeter; the parenchyma was replaced by multiple cysts filled with fluid, varying in size; corticomedullary congestion suggestive of hypoperfusion

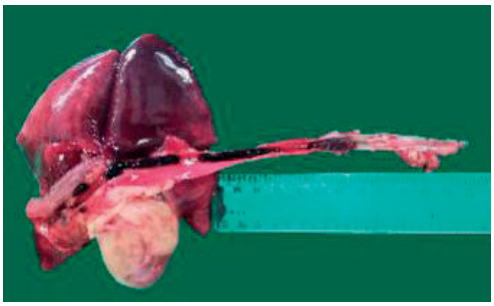

Figure 6. Longitudinal section of the aorta with thrombus, post-mortem prethrombotic haemoglobin imbibition, suggestive of obliterating thrombosis

The abdominal aorta showed a dilated reddish colour shape, at 2 cm above the adrenal glands (Figure 6). After dissection, a 2 cm long dark red, dry, friable, granular, obliterating, adherent to endothelium thrombus. The tail of the thrombus is extended downstream from the area of vascular attachment, in this case showed the cardiac origin. The cause of the complete obstruction of the lumen was found, confirming the clinical diagnosis of aortic thromboembolism (Figure 7).

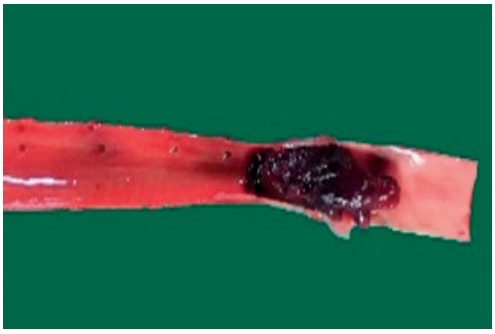

Figure 7. Longitudinal section of the aorta with thrombus obliteration: solid dark red mass, rough, matte, adherent to wall

Histopathological examination of the obliterated aortic segment revealed a massive accumulation of layers of fibrin, erythrocytes and organized cell debris, also an altered endothelium with partially denuded basement membrane (Figure 8).

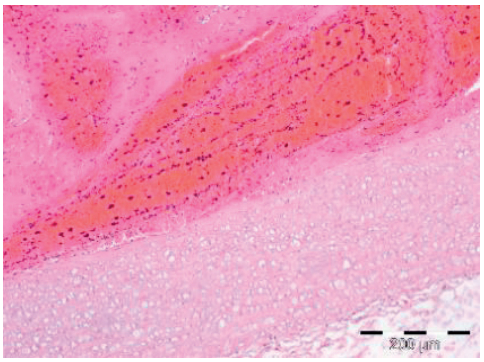

Figure 8. Thrombus details: layers of fibrin with erythrocytes; continuous adhesion to the intima of the aorta, ob 100, HE

Renal parenchyma presented multiple bilateral cysts with homogeneous liquid, with diffuse loss of renal structural details. The cysts were lined by a single layer of epithelial cells. (Figure 9).

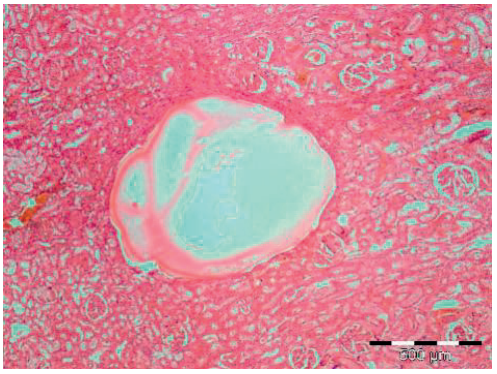

Figure 9. Renal cortex: large simple cyst lined by flattened cells, that contain granular eosinophilic material (probably protein). The glomeruli, are compressed, ob 100, HE

Disorganisation and hypertrophy of cardiomyocytes, as well as interstitial fibrosis, were revealed in the heart (Figure 10). Ectopic thyroid tissue did not show any changes in histopathology (Figure 11). Spherical and oval follicles filled with eosinophilic colloid could be observed in different functional stages, bordered by simple cuboidal epithelial tissue. Restricted areas of clustered cells with vesicular nuclei, small nucleoli and eosinophilic cytoplasm can be observed. No parathyroid tissue was observed.

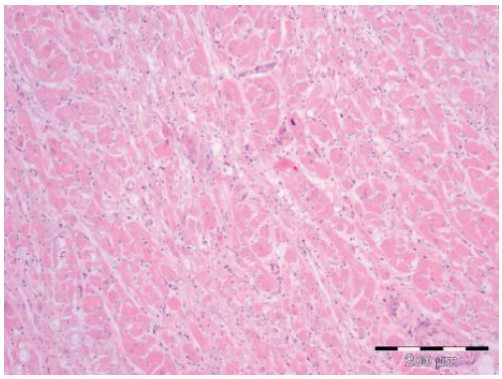

Figure 10. Ventricular septum myocardium with HCM: myocyte hypertrophy and disarray characterized by the interweaving of myofibers, associated with proliferation of the interstitial fibrous connective tissue (fibrosis), ob 100, HE

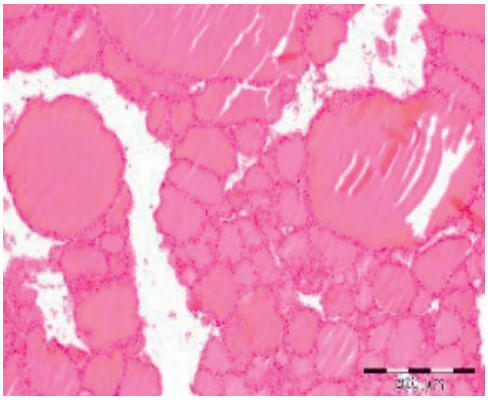

Figure 11. Ectopic thyroid tissue: follicles in different functional stages, bordered by simple cuboidal epithelial tissue, filled with eosinophilic colloid, ob 100, HE

The diagnosis of aortic embolism/aortic thromboembolism or natural aortic occlusion was made based on clinical signs shown by cat (Carter, amp, & Van Heerden, 1994; Silva et al., 2016). These clinical signs associated with ATE were due to acute ischemia of the tissue served by the occluded artery. The vocalisations of the cat were attributed to pain, the cats responding to pain through self-mutilation and vocalisations (Smith & Tobias, 2004). It is a well-known fact that cats with heart disease have high-risk condition for thrombus formation. Risk factors include changes in blood flow, endothelial cell lesions and hypercoagulopathy. Those modifications are known as Virchow's triad (Molina et al., 2012; Schoeman, 1999). Knowing that the cat was antemortem diagnosed with HCM and LA dilation, it may be considered that formation of the thrombus that led to the aortic embolism took place at the level of LA (Vezzosi et al., 2020). It is known that maximum speed of the blood in the left atrial auricle is lower in cats with cardiomyopathy (0.31 m/sec) compared to healthy cats (0.46 m/sec). In cats with concomitant left atrial thrombus or ATE, the maximum known velocity of blood flow is 0.14 m/sec, suggesting that stasis may indeed contribute to left atrial thrombus formation. These events are classified as cardiogenic because the source of the thrombotic material comes from a cardiac chamber, usually from LA (BUTLER, 1971; Hogan, 2017; Silva et al., 2016). As no endothelial lesions were observed at the AS level, and the cat was undergoing anti-platelet therapy, we can consider that the thrombus was formed following blood stasis at this level. The emboli are dislocated, reaching the aorta or one of its major branches. The blockage will occur when size exceeds the diameter of the vessel, thus obliterating the blood circulation. Subsequent consequence involves the absence of blood supply of one or more limbs. This cat had only aortic thromboembolism. It is known that thromboembolism can rarely affect the cerebral, renal and mesenteric arteries (Smith & Tobias, 2004; Tomaiuolo et al., 2020),(Hogan, 2017). The infarction of the area served by the blocked blood vessel is not exclusively due to the loss of blood flow. Experimental models have shown that when the activation of the coagulation system occurs, similar to the situation when an embolus obliterates the terminal aorta, the collateral circulatory network builds up in vasoconstriction, probably due to the release of vasoactive substances. Experimental studies have shown that complete ligation of the terminal abdominal aorta does not impede arterial flow to the pelvic limbs, nor result in clinical signs, as there is a collateral circulatory network in the vertebral and hepatic systems that maintain arterial flow around the ligature. Aortic ligation does not affect locomotion either, paralysis depending on the presence of blood thrombus in the aorta (BUTLER, 1971; Hogan, 2017; Săvulescu-Fiedler, Gherghiceanu, Militaru, Brăslașu, & Bruckner, 2014; Silva et al., 2016).

Corroborating the anamnesis with the result of clinical and necropsy investigations of this case was concluded that ATE was the consequence of HCM. It is known that certain breeds have a high risk to develop HCM. In Maine Coon and Ragdoll breeds, HCM is an inherited autosomal dominant condition. It is caused by a mutation of the gene, which is encoding cardiac myosin-binding protein C (MYBPC3). In these breeds, genetic testing for reproductive acceptance is recommended (Juliana Mariotti Guerra et al., 2020; Luis Fuentes et al., 2020).

In common breed cats cases of HCM have been reported, but that has not previously been diagnosed with an infectious or metabolic disease leading to this disease. It has not been shown that the disease was genetic. (Baty et al., 2001) The cat from the presented case does not belong to the breeds prone to developing HCM, but it has not been possible to determine if being a cross-breed of one of them. HCM, the most common cardiomyopathic phenotype in cats, is characterised by thickening of the interventricular septum and/or left ventricular wall, diffuse or asymmetric, without dilation of the right ventricular chamber. HCM is a condition with a high risk of morbidity and mortality, featured by lesions corresponding to congestive heart failure and consecutive ATE, being the most common causes of clinical signs of heart disease and sudden death in this species (Juliana Mariotti Guerra et al., 2020; Luis Fuentes et al., 2020).

The association between PKD and HCM, also observed in this case, has rarely been described in cats. In humans, 89% of patients with autosomal dominant polycystic kidney disease (ADPKD) who died of the cardiac disease had, as in the present case, left ventricular wall hypertrophy (LVH). In humans, 89% of patients with ADPKD who died of the cardiac disease had LVH, as in the presented case. Expansion of renal cysts and local hypoperfusion activates the intrarenal renin-angiotensin system, causing hypertension, while increased intracardiac pressure stimulates myocardial remodelling (Juliana Mariotti Guerra et al., 2020). The increase in blood pressure is also mediated by the increase in cardiac output. The result is compensatory left ventricular hypertrophy (Luis Fuentes et al., 2020)·(Sim Lam et al., 2020; Spencer, Wheeler-Jones, & Elliott, 2020), injury reported in this case as well. The interrelationship between heart and kidney diseases is quite common in cats; the association between the two diseases having a poor prognosis (Bongartz et al., 2012). In the presented case, it could be observed that the necrotic pressure caused by renal cysts of different sizes caused the denaturation of over 60% of the normal renal parenchyma. Loss of renal architecture has been associated with impaired renal function. Polycystic kidney disease (PKD) is one of the most common inherited disorders of the cat. It is common in the Persian breed or related breeds, and inconsistent in their mixed breed. An inherited dominant autosomal mutation caused PKD, and the animals may be asymptomatic for years. The cause of PKD is a mutation in the PKD1 gene. It has been identified as heterozygous in 48 different breeds of cats including Persians, Siamese and short-haired cats. Grossly, PKD is characterised by the presence of one or more fluid-filled cysts, of various sizes, in the cortex, medulla, or both renal areas. They can occur in a single kidney or bilaterally. Renal cysts tend to multiply in number and increase in size over time as the animal ages. This leads to progressive deterioration and necrosis of kidney tissue due to pressure, causing chronic renal failure and eventually cat death. The disease is usually subclinical until middle age or older. The diagnosis of PKD can also be established by genetic tests for the mutation of the PKD1 gene (Bilgen et al., 2020; Guerra, Cardoso, Daniel, Onuchic, & Cogliati, 2020; Sim Lam et al., 2020; Yu et al., 2019).

Knowing that PKD is a genetic condition with the potential to cause HCM, it was concluded that, in this case, HCM is secondary to chronic kidney disease induced by PKD. This lesion is known as being responsible for cardio-renal/reno-cardiac syndrome. Disorders of the heart and kidneys, manifested by acute or chronic dysfunction of one organ, could induce acute or chronic dysfunction in the other organ. The presented case is included in type 4 of this syndrome being known as five types in total. Type four represents chronic renocardiac syndrome consisting of chronic kidney disease, which causes injury, illness and/or heart dysfunction (Damman, Voors, Navis, van Veldhuisen, & Hillege, 2011; Orvalho &

Cowgill, 2017; Pouchelon et al., 2015). In the presented case, chronic kidney disease led to HCM.

It was concluded, at the histopathological diagnosis, there are no changes in the ectopic thyroid tissue, so it was possible to exclude the hyperthyroidism given by hypersecretion of thyroid hormones at this level (Peterson, 2012). Unfortunately, no blood tests were performed antemortem to confirm the hyperthyroidism. Corroborating the histological result with the body fur and muscles well-maintained appearance, it has been chosen to exclude this condition. It is known that hyperthyroidism leads to HCM in cats (Luis Fuentes et al., 2020).

It is known that ectopic thyroid tissue rarely undergoes pathological transformations, such as hyperplasia, adenomas or adenocarcinomas. Accessory thyroid tissue can occur anywhere from the larynx to the diaphragm. In cats, calcitonin-containing C cells appear at 38 days of gestation as single scattered cells and migrate into interfollicular spaces on the 50th day of gestation. The examined tissue showed the presence of C cells so we could conclude that ectopia could have appeared after the 38th day of gestation (Knowles, Uhl, Blas-Machado, & Butler, 2010; Patnaik, Peterson, & Hidgon, 2000).

CONCLUSIONS

The case showed that PKD leads to chronic kidney disease, renal-cardiac syndrome and hypertension. In addition, the onset of left atrial dilation is a consequence of hypertension, producing subsequent disturbances of blood circulation and blood flow deceleration. Thus, predisposing factors for ATE are created, being considered a syndrome with a severe prognosis.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXVIII (2), 2022
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

PARACLINICAL DIAGNOSIS AND THERAPEUTIC APPROACH IN ETHYLEN GLICOL POISONING IN DOGS

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Abstract

One of the most affected species by various toxic substances is, undoubtedly, the canides. Among them, ethylene glycol is one of the most possible causes, its use as an antifreeze solution making it a frequently encountered substance. Because of its sweet taste, it is eaten with pleasure by dogs and not only. The aim of the study was to optimize the diagnostic and treatment methods in ethylene glycol poisoning in dogs. In this study were included 8 clinical cases of dogs (2 males and 6 females), aging from 4 months to 13 years, that were isostenuric after 3 hours after ingestion. Also, calcium oxalate crystalluria becomes detectable after 3-6 hours after consumption. After the onset of renal impairment, the value of blood urea nitrogen and creatinine increased, which resulted in glomerular filtration damage. The present study highlights the effectiveness of the treatment in the first 8 hours after ingestion, being very important to slow down the oxidation metabolism of ethylene glycol by alcohol dehydrogenase. The administration of ethyl alcohol or fomepizole (4-methylpyrazole) to dogs presented to the veterinary clinic following exposure to ethylene glycol increase their chances of survival.

Key words*: diagnosis, treatment, ethylene glycol, dogs.*

INTRODUCTION

One of the most affected species by various toxic substances is undoubtedly the canine species. Along with other glycols, ethylene glycol is one of the most possible causes of dog poisoning, its use as an antifreeze solution making it an almost domestic agent (Davis et al., 1997; Dobre, 2019).

Because of its sweet taste, it is eaten with pleasure by dogs and other species. After hepatic metabolism by alcohol dehydrogenase, the metabolites formed produce CNS depression and nephrotoxicity, which can lead to acute renal failure (Gupta, 2018).

The mortality rate in dogs, due to this type of intoxication, varies between 50% and 70%, most cases being accidental (Barton and Oehme, 1981; Rowland, 1987).

According to data provided by some poison control centers, antifreeze poisoning is the second most common cause of fatal animal poisoning (Hornfeldt and Murphy, 1998). In addition, ethylene glycol is not toxic by itself, but by toxic products resulting from hepatic metabolism under the action of alcohol dehydrogenase (ADH) - glycolic, glycoxalic and oxalic acids.

Interestingly, this intoxication is somewhat seasonal, with the highest incidence occurring in late autumn and early spring, with the change in antifreeze solution.

The aim of the study was to optimize the diagnostic and treatment methods in ethylene glycol poisoning in dogs.

MATERIALS AND METHODS

In this study were included 8 clinical cases of dogs that were registered between September 1, 2020 - March 31, 2021 at a veterinary medical clinic near Bucharest.

Data about the animals included in the study are presented in Table 1.

Table 1. Data about dogs examined following ethylene glycol exposure

Dog ID	Breed	Sex	Age
D1	Belgian shepherd	Male	1 year
D2	German shepherd	Female	4 months
D3	Half breed	Female	2 years
D4	Bichon	Female	13 years
D5	Half breed	Male	1 year
D6	Half breed	Female	6 years
D7	Half breed	Female	10 years
D8	Half breed	Female	10 years

All animals were clinically examined, the anamnesis was collected. Thereafter, blood samples were taken for paraclinical examination (hematological and biochemical) and treatment was instituted.
Blood samples were analyzed using a 5-Diff haematological analyzer and a biochemistry analyzer, respectively.
After evaluating the anamnesis and the hematological and biochemical results, antidote treatment and rehydration treatment were administered to the patients.
Taking into account that we didn't have Fomepizole (4-methylpyrazole) in our clinic, we used 40% ethyl alcohol, which was brought to a concentration of 20% by 1:1 dilution with saline, at a dose of 5.5 ml/kg, IV (Mathews, 2006).
For rehydration a therapy with saline and glucose solutions was administered, and for the correction of acidosis 8.4% sodium hydrogen carbonate was used.

RESULTS AND DISCUSSIONS

The anamnesis tried to establish the time elapsed from ingestion to the time of presentation to the clinic. Hematological examination quantified the values of lymphocytes, granulocytes, hemoglobin and hematocrit in all cases studied (Table 2). Broadly, lymphocytes and granulocytes were above the maximum values of the reference values, possibly due to the stressful situation experienced by the patients and, respectively, the overlapping infections over this situation. Hematocrit was also increased due to dehydration of the examined animals.

Table 2. The results of the hematological parameters determined in the 8 dogs exposed to ethylene glycol

Parameter	D1	D2	D3	D4	D5	D6	D7	D8
WBC x10^9/L	23 ↑	40.2↑	21↑	22.6↑	20.7↑	21.5↑	32.2↑	22.1↑
GRAN x10^9/L	19.8↑	36.7↑	10.8	12.4	16.9↑	19.8↑	25.4↑	14.8↑
HGB g/dL	19.8↑	18.9	21.3↑	20.6↑	18.5	17.8	18.6	19
HCT %	58.2↑	37.9	65↑	42.4	50.1	47.2	43.5	55.4

The biochemical examination showed an increase in azotemia values in 7 of the 8 cases studied, while the creatinine value showed a significant and constant increase in all cases. Increased levels of azotemia and creatinine indicate impaired renal function in animals in this condition. Also, in 50% of cases the serum glucose showed increases that can be interpreted by the stress conditions that the subjects had to face (Table 3).

Table 3. The values of the biochemical parameters determined in the 8 dogs exposed to ethylene glycol

Parameter	D1	D2	D3	D4	D5	D6	D7	D8
GLU mg/dl	107	**448**	112	**158**	127	**237**	116	**156**
BUN mg/dl	21.2	**94.7**	**42.2**	**143**	**105.8**	**82**	**53**	**159**
CRE mg/dl	**1.84**	**7.63**	**2.89**	**6.87**	**11.82**	**7.9**	**5.3**	**16.4**

The therapeutic protocol used included antidote and supportive treatment. We used 20% ethanol to competitively inhibit alcohol dehydrogenase and prevent the metabolic conversion of ethylene glycol. The dose used was 5.5 ml / kg every 8 hours on the first day (3 times a day) and every 12 hours on the next two days (twice a day), so that the animal was kept for 72 hours in a drunken state (Table 4). We used 40% ethyl alcohol which was diluted with saline in a ratio of 1: 1, the amount being administered slowly intravenously. When determining the administration interval, we also took into account the disadvantages of ethyl alcohol, such as the ability to depress the CNS, to form acetaldehyde, which in turn affects carbohydrate metabolism and the fact that it is irritating to the brain (Money et al., 1989).
In the body's supportive therapy, we aimed to antagonize the acidity with sodium bicarbonate infusion solution 8.4% (1000 mEq / L) at a dose of 6.2 mEq / kg every 8 hours and to combat dehydration with 5% glucose infusion solution. 10% calcium gluconate was used to control hypocalcemic attacks (0.25 ml / kg / day). We used supportive therapy for 7 days to support the regeneration of the renal tubules and the resumption of renal function in optimal conditions.

Table 4. Treatment schedule administered to the dogs exposed to ethylene glycol (drugs and doses are provided)

Patient ID	Weight (kg)	Etanol 20%/ admin	NaHCO3/ admin	Gluconat de Ca/admin
D1	18	99 ml	112 mEq	4.5 ml
D2	10	55 ml	62 mEq	2.5 ml
D3	13	71.5 ml	81 mEq	3.25 ml
D4	3	16.5 ml	18.6 mEq	0.75 ml
D5	9	49.5 ml	56 mEq	2.25 ml
D6	12	66 ml	74 mEq	3 ml
D7	20	110 ml	124 mEq	5 ml
D8	13	71.5 ml	81 mEq	3.25 ml

During the course of the therapy, we noticed the reluctance of the owners towards the duration of such a treatment in the conditions of the reserved vital prognosis. The unpromising clinical course of some of them eventually led them to request euthanasia. Also, some of the owners did not return to our clinic to continue the treatment.

It is worth noting the importance of early initiation of antidote therapy with ethanol, which was confirmed by the clinical study. Remarkably, ethanol dosing recommendations vary from author to author, with some preferring continuous infusion at a constant rate (CRI). In this case, it is recommended to use a concentration of 5% at a rate of 5.5 ml / kg / h which can avoid the concentration of ethanol in the blood, which can exacerbate the clinical effects (Mathews, 2006).

The cases presented more recently after exposure have evolved towards healing, while the cases that arrived at the clinic late went to the exitus. As it can be seen in Figure 1, 25% of patients recovered, compared with 37% who succumbed. It is interesting to note that the latter showed increased values of azotemia and impaired renal function upon arrival.

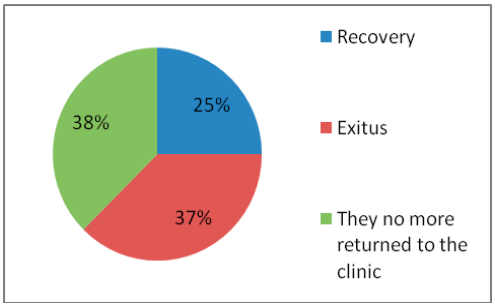

Figure 1. The evolution of the studied cases

In this way, we notice how important the therapeutic intervention is before the increase of azotemia and the disorder of the renal function.

It can be seen that in 38% of the cases, the owners did not return to the clinic, which means that either the animals in their possession died or, on the contrary, the animals recovered, so the owners considered unnecessary the return to the clinic. From this perspective, it may be useful, a centralized system for recording and monitoring poisoning, following the model practiced in other states, knowing that dogs are especially victims of poisoning.

Regarding the distribution by age groups in our study, we observed a higher frequncy in adults (63%) compared to young animals (37%), which supports the opinion that ethylene glycol tastes good for dogs in general, and that experience does not prevent adults from avoiding the harmful substance (Figure 2).

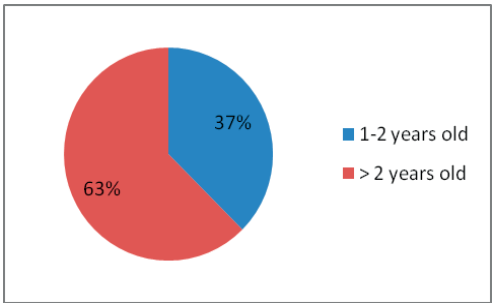

Figure 2. Distribution of age-related intoxication (youth and adults)

CONCLUSIONS

In this study, we had a large number of cases in a short period of time, thus indicating an increased incidence of ethylene glycol poisoning cases. Particularly important is the evaluation of the patient based on the hematological and biochemical examination as well as the evaluation of the time elapsed since the exposure.

20% ethanol treatment should be instituted as soon as possible in order to prevent metabolic transformation of ethylene glycol and the occurrence of renal failure.

The adverse effects of ethanol must also be taken into account in determining the antidote therapy protocol. It is very important to perform a long-term supportive therapy that allows the resumption of renal function in good conditions.

Despite the prompt application of the therapeutic protocol, the survival rate remains low, which supports the importance of preventing animals from accessing this type of substance.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXVIII (2), 2022
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

HEALTH IMPACTS AND CONTROL MEASURES IN INFECTIOUS BOVINE RHINOTRACHEITIS – A REVIEW

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Abstract

Infectious bovine rhinotracheitis is a viral, endemic, specific bovine, goat and swine (genital) disease with acute evolution, clinically characterized by hyperthermia and respiratory involvement, inflammation of the anterior respiratory tract (rhinitis, tracheitis) or genital disorders (abortions, infertility, balanopostitis), often accompanied by conjunctivitis. This entity is globally spread and is considered to be one of the most costly diseases affecting bovine livestock, and can have major economic consequences by decreasing productivity (milk production, low yield on fattening, animal culling), high morbidity, lethality, which can reach 12% for youth, as well as by restrictions in trade between countries. The review examined the impact of the infection on health status of affected bovine and possibilities to control the disease based on general and specific measures.

Key words: *bovine, IBR, clinical outcome, reproductive problems, prevention.*

INTRODUCTION

Bovines have a particular socio-economic importance in the economy as a whole and in agriculture. This results from the fact that they provide an increased and varied volume of products of primary importance for the consumption of the population, and raw materials for the processing industries. At the same time cattle breeding is an intensive agricultural production, a market for production means and industrial products, a source of income for the economy and a means of capitalizing on natural resources (Georgescu, 1995).

The main purpose of cattle breeding is to provide some of the necessary means of subsistence for humans. Thus, cattle provide 96% of the world's total milk production, 33% of the world's meat and 90% of the total weight of leathers processed in the world's tannery industry. In human food, bovine account for about 12% of their energy intake, ie 56% of all animal protein in ration (Morar et al., 2005).

Each mammal lives in its living environment alongside a multitude of microorganisms that occupy every ecological niche. To maintain animal integrity against aggressors' pathogenic effects, there is a wide range of defense mechanisms, from simple barriers that protect against insect attack and complex immune responses directed against viruses and bacteria.

When the aggressor is a virus, the immune defense mechanism protects the recipient host against the initial attack, especially when the animal has been previously specifically immunized. For the synthesis of antibodies and, implicitly, long-term protection, the presence of the micro-organism or their fragments is required. In such cases, the survival of a small viable population is beneficial, a condition called pre-immunity, a type of non-sterile immunity that is necessary to prevent reinfection.

Infectious bovine rhinotracheitis is a viral, endemic, specific bovine, goat and swine (genital) disease with acute evolution, clinically characterized by hyperthermia and respiratory involvement, inflammation of the anterior respiratory tract (rhinitis, tracheitis) or genital disorders (abortions, infertility, balanopostitis), often accompanied by conjunctivitis. This entity is globally spread and is considered to be

one of the most costly diseases affecting bovine livestock, and can have major economic consequences by decreasing productivity (milk production, low yield on fattening, animal culling), high morbidity, lethality, which can reach 12% for youth, as well as by restrictions in trade between countries.

1. ETIOLOGY

Bovine herpesvirus 1 (Bovine herpesvirus - 1; BHV-1), belonging to the family *Herpesviridae*, the subfamily *Alphaherpesvirinae*, Varicellovirus genus, is incriminated in the etiology of bovine infectious rhinotracheitis. The viral genome consists of double strained DNA, and the viral particle presents a capsid with icosahedral symmetry, surrounded by a pericapsid. Glycoproteins (gB-gE, gG-gI, gK-gM) have been identified at the level of the viral envelope having a primary role in interacting with host cells and the humoral and cellular immune system of the host. Some glycoproteins, such as especially gE, but also gC, gG and gI, are not essential and as such, deletion of the gene encoding these glycoproteins only affects viral multiplication to a small extent. The absence of a serological response to gE allows the differentiation of vaccinated animals (gE-deleted vaccines) from naturally infected animals.

Genetic research has revealed the existence of two distinct subtypes: the respiratory subtype (BHV-1.1) and the genital subtype (BHV-1.2), the latter with two variants: BHV-1.2a in Europe and BHV-1.2b in Australia. All BHV-1 strains are very similar in both genomic and antigenic terms, so a BHV-1 subtype 1 vaccine confers immune protection against both subtype 2 infection and inverse (cross-immunity).

Regarding BHV-1 virulence, hypervirulent, virulent and hypovirusemic strains are distinguished.

2. EPIDEMIOLOGY

Only cattle, especially young and adult animals in large herds (intensive system), are susceptible to the natural infection.

2.1. Susceptibility and risk factors

There are authors who consider that receptivity is not influenced by age, breed, and sex. However, various epidemiological studies indicate that clinical symptoms in young cattle include severe forms of development (oculo-respiratory syndrome, gastroenteropathy, meningoencephalitis, etc.).

Certain risk factors for the installment of BHV-1 infection also highlight the high importance of the animal exploitation system. Thus, the disease expresses itself exclusively on large farms, being virtually unknown in extensive raising, this situation being correlated with the inappropriate carrier state; the severe progression of the disease may be due either to the increase in virulence of the virus through repeated passages or to greater probability of direct transmission between susceptible individuals (Solis-Calderon et al., 2003; Boelaert et al., 2005). The size of the flock is therefore a risk factor.

2.2. Sources of infection and transmission pathways

The main infectious source is represented by the infected animals showing or not clinical signs, which eliminate the virus through respiratory, genital and ocular secretions. Nasal discharge is the major source of the virus, the BHV-1 virus being present in the nasal mucus at 24 hours post-infection, at a sometimes very high titer. The primary excretion period, corresponding to an intense dissemination of the virus in the external environment, ranges from 10 to 16 days, with a peak between the 4th and 6th day post-infection.

Due to the immune response installed immediately after the infection, the animal is able to control the infection and stop primary viral excretion, but becomes a latent, asymptomatic, virus. The virus is no longer detected in the nasal mucus, being present in neurons of the regional ganglion as a non-integrated viral genome. Under the influence of various factors: transport, parturition, glucocorticoid administration, secondary infection with other viruses, bacteria and parasites, latency can be annulled and the virus resumes the multiplication cycle in the body. Viral reactivation results in a re-expression of BHV-1, with or without symptoms of disease, with an increase in the circulating specific antibody titer, depending on the degree of immunization of the animal.

The presence of latent carriers is the essential element of the persistence of the virus in flocks.

Epidemiological research indicates that in some high-prevalence populations of BHV-1 infection, some latent carriers do not have a detectable antibody titer, being classified as false and which, being undetectable, are the source of infection for the rest of the flock. The unresponsive state of latency was observed in calves infected but benefiting of colostrum immunity or in virulent strain infection (Bradshaw and Edwards ,1996). This category of animals (seronegative and latent carriers of the virus) is a major draw-back in controlling the disease (Ackermann and Engels, 2006; Mollema et al., 2005, Anita, 2008, Anita et al., 2008).

Disease transmission is achieved either by direct, nose to nose contact or indirectly through secondary sources. The infection is possible by respiratory route, by inhalation of virulent aerosols from the nasal and ocular secretions of diseased animals. Caretakers may intervene in spreading the virus to other susceptible cattle through various objects or clothing contaminated by nasal secretions. Transmission through artificial insemination or mounting is also frequent, the virus being isolated from serum positive serum bulls. The risk of transmission of the infection by embryo transfer is very low, although BHV-1 is able to adhere to the oviduct or embryo pellucid membrane after *in vitro* infection.

2.3. Evolutionary dynamics

Infectious bovine rhino-tracheitis is of an endemic character, with high outbreak spreading, affecting all susceptible animals within the infected herd. The maximum number of sick animals is recorded 2-3 weeks after the first case occurred, and the duration of endemic is generally of 2-6 weeks, up to several months. Although the morbidity is high (20-100%), mortality does not exceed 10-12%. In fattening units, however, the disease becomes stationary, appearing 10-15 days after the introduction of a new batch of calves (Noordegraaf et al., 1998; Mollema et al., 2005).

3. IMMUNITY

Immune protection against BHV-1 involves various mechanisms, both specific and non-specific. It is also considered that bovine, either naturally infected or vaccinated, develop an immune response primarily focused on the synthesis of specific neutralizing antibodies and lymphocyte proliferation (Loehr et al., 2000; Van Drunen et al., 2006 and 1994). In the natural infection, by passing through the disease, the healed animals become resistant to the pathogenic action of the virus. However, the immunity in this disease is somewhat relative, because despite a pronounced immune response, the virus does not disappear, so the healed animal remains a latent carrier of the virus and potentially shedding. The presence of neutralizing antibodies in the serum does not exclude the possibility of virus removal and their absence does not indicate that the animal is not a carrier (Ackermann and Engels, 2006). Thus, immunity in infectious bovine rhinotracheitis is not sterilizing, in the cured animals the infection and immunity persist simultaneously.

3.1. Specific immune response (cellular and humoral)

BHV-1 infection stimulates a specific response, provided by B lymphocytes and T lymphocytes, which have receptors for this virus (Babiuk et al., 1996, Platt et al., 2006). T lymphocytes respond to BHV-1 infection by recognizing the specific antigens presented in connection with the major histocompatibility complex and B lymphocytes by producing specific neutralizing antibodies. The primary neutralizing antibodies in cattle are directed against gB and gD. These antibodies also participate in complement-dependent complement activation mechanisms or cytotoxicity, to lysis of infected cells. In the antiviral immune response, it is important to involve cytotoxic T lymphocytes, which, given the "spectrum" of their immune activity, can contribute to limiting viral spread.

Conventional anti-BHV-1 vaccines usually induce a strong humoral response but no cytotoxicity. In contrast, a marker vaccine resulting from DNA techniques was able to induce moderate antibody synthesis to gB, but the cellular response was very intense (Huang et al., 2005). The augmentation of the cellular immune response to BHV-1 was also observed in the testing of a plasmid encoding gD but without providing protection against clinical signs (Pontarollo et al., 2002).

Concerning the category of infected animals and latent carriers, serological positivity (especially to gE) is mentioned for a period of 2-3 years.
Also, Lemaire et al. (2001) have shown that negative gE calves which have benefited of passive immunity from vaccinated negative gE mother can produce antibodies to this glycoprotein after infection with a natural strain of BHV-1.

3.2. Non-specific immune response

In addition to the specific immune response, bovine herpesvirus infection 1 also stimulates a non-specific immune response characterized by the secretion of type 1 interferon (IFNα and IFNβ)(Woolums et al., 2003). This non-specific response is mediated by polymorphonuclear leukocytes (neutrophils), macrophages and natural killer lymphocytes. The moment of intervention of these immune cells coincides with the early phase of the infection because, unlike B and T lymphocytes, they do not have antigenic memory.
The onset of BHV-1 infection is also dependent on the activity of non-specific defense mechanisms that provide a first line of defense against viral extension. The more nonspecific the immune response is, the easier the role assigned to the specific immune component represented by the B and T lymphocytes is. Activation of systems involved in non-specific defense can occur under natural conditions (natural infection or body-virus contact) or may be induced artificially by administration of so-called non-specific inducers, "NSD inducers" or immunomodulators. Thus, there are authors who consider it appropriate in the case of IBR to administer an immunomodulator, eg Baypamun (Bayer), in an *in vivo* study demonstrating the following effects: reducing the gravity of the clinical picture, reducing the susceptibility of animals to the virus, significantly reducing the level of elimination viral (Castrucci et al., 2000).

3.3. Disturbing factors of immune reactivity in IBR

Vitamin - mineral deficiencies, especially vitamins B (vitamin B6, pantothenic acid, vitamin B12), vitamin C (Dubeski et al., 1996) and vitamin E (Cusack et al., 2005) have negative consequences on the immune status of calves, disturbing the immune response to vaccination and natural infection. In the Hereford x Angus crossbreeds, experimentally induced with Cu deficiency by molybdenum administration, it was found that this mineral deficiency causes in BHV-1 infected animals the alteration of acute phase protein concentrations during the acute phase protein response to viral infection and can also affect lymphocyte response to mitogen stimulation (Arthington et al., 1996).
Starting from the consideration that water and feed deprivation during commercial operations and transport may adversely affect the ruminant synthesis of vitamin B at the time of maximum susceptibility to infectious agents, the effect of parenteral vitamin B administration on infection and immune status in restricted calves. The parenteral intake of vitamins did not significantly affect viral concentration, interferon titer in nasal secretions, and blastogenic lymphocyte activity, however, the post-infective IgG titer tended to increase. In conclusion, it is argued that the immune response of stressed cows at the time of vaccination can be favorably influenced by the vitamin B level in the body.
Due to the fact that in monogastric species, stress and disease cause the increase of the need for vitamin B6, folic acid, pantothenic acid and ascorbic acid, thus the effects of food restriction, of the herpesvirus infection, as well as the effects of vitamins on the vitamin B plasma are critical to the immune response.
It appears that the levels of vitamin B6, B12, pantothenic acid and ascorbic acid influence the immune response to infection or vaccination in stressed cows.
Since there is a close relationship between stress, nutritional status and thyroid status, the effects of thyroid hormone administration on immune and metabolic response are dependent on the nutritional status of the animal (Cole et al., 1994).

4. CLINICAL PICTURE

The incubation period is 4-7 days. Clinically, depending on strain tropism and age of infected animals, evolutionary forms with respiratory, conjunctival, encephalic, cutaneous, genital and abortions may occur.

4.1. Respiratory form (infectious bovine rhinotracheitis)
This IBR form is the most common and affects bovines of all ages. Calves become sensitive at age 3-4 months when protection from colostrum antibodies disappears. The disease begins with hyperthermia, numbness, diminished appetite, sudden decrease in milk production, than the respiratory changes become obvious: nasal discharge, initially serous, then mucopurulent, while breathing becomes accelerated and superficial, hyperemia and ulcers in the nasal mucosa appear.
Sometimes, due to secondary bacterial infections, the process may expand to the posterior respiratory tract, clinical manifestation being bronchitis and pneumonia.
At the level of the nipple there is an initial erythema, followed by the formation of crusts which, by detachment, leaves ulcerated areas of red color. In the absence of bacterial complications, healing occurs after 15 days. Some very virulent strains of BHV-1 can induce a high mortality rate. During the development of this form, embryo mortality in early pregnancy or in heifers after mating, repeated heat and abortion between months 4-7 of gestation were noticed.
4.2. Conjunctival form may sometimes develop in the absence of other signs, usually in benign form, but following secondary infections with *Moraxella bovis* or other bacteria, it can lead to irreversible lesions of the eyeball, including panophthalmia.
4.3. The encephalitic form may occur in calves below the age of 5 months. It starts with hyperthermia (40ºC), nasal and mucous membrane hyperemia, lacrimation, nasal discharge and foamy saliva, but without respiratory symptoms. After 1-2 days from onset, nervous signs appear in the form of seizures, which occur at at shortening intervals. Death occurs within 6-7 days in all cases with nervous signs.
4.4. The cutaneous form is localized at the interdigital space as a round ulcer with smooth edges and healing tendency within the next 4-5 days. Frequently, by the intervention of the secondary bacterial flora, an ulcerative lesion healing very slowly is produced. Sometimes skin lesions also occur in the perineum as a decuamative dermatitis that extends to the scrotum.
4.5. The genital form (infectious pustular vulvovaginitis - IPV) develops sporadically and is sexually transmitted by artificial insemination with BHV contaminated sperm. It is the most benign form and is expressed by hyperthermia (up to 41.5 ° C), pustular vulvovaginitis and balanopostitis. Females are anxious, look stunned, frequently urinate, the vulva is swollen with a yellowish secretion present. In males, especially the gland and foreskin, initially pustules are formed, which turn to erosions. The passage areas from the foreskin to the gland are edematous and subsequent necrosis of the gland can occur. Symptoms persist for 1-2 weeks, depending on their severity and the disease ends with healing. Prolapse of the uterus can sometimes occur due to the efforts of females following pain caused by lesions, and in males, adhesions and fibrosis accompanied by phimosis or paraphimosis.
4.6. *Generalized form* is often found in newborn calves if they are not protected by colostrum or active immunity, induced by vaccination.
The disease usually occurs at the age of 3-4 days, with the following clinical picture: severe hyperthermia, severe botulism (red muzzle disease), anorexia, lacrimation, nasal discharge, coughing, laryngeal ulcers, exceeding salivation, bronchopneumonia and diarrhea, followed by death in 3-4 days.

5. PATHOLOGY

At the necropsy, besides the injuries observed at the clinical examination, exsudation with fibrin and puss, petechiae, necrosis and ulcers in the anterior respiratory tract mucosa, the presence of false membranes in the larynx, erosive-ulcerative foci in the pharynx, esophagus, abomasum and gut mucosa chosen in generalized forms, serous or mucopurulent exudates in the sinuses, edema of regional lymph nodes. In the case of bacterial complications, bronchopneumonia can be observed at various development stages.
Fetuses have subcutaneous and pulmonary edema, hemorrhagic liquid in the cavities, small hemorrhages in the epicardium, endocardium, pleura and lungs, and small, point-like necroses in the liver and other organs, destruction of the renal cortex.

The histological examination reveals congestive-hemorrhagic, necrotic, ulcerative and infiltrative processes, meningoencephalitis with lympho-monocytes and acidophilic intranuclear inclusions in the respiratory and vaginal epithelium.

6. DIAGNOSIS

For the diagnosis, epidemiological, clinical and pathological data are corroborated with the laboratory ones (histological, virological and serological examination) (OIE Manual of Diagnostic, 2004).
For virus isolation, testicular or renal cell cultures of calf, primary or secondary cells obtained from bovine pulmonary cells or bovine kidney cell lines are used. In 3 days after inoculation cytopathic effect appears (rounding of cells and their clustering, the occurrence of dropsy, intranuclear oxyphilic inclusions).
BHV-1 can be identified by SN, IF, ELISA (very sensitive) (Rosskopf et al., 1994, Perrin et al., 1996), immune peroxidase test, electron microscopy, PCR. Viral antigen research can be performed on frozen tissue sections (mucous membranes and organs) and on cell smears (nasal swabs or tracheobronchial washings) with IF. By the avidin-biotin complex method, the viral antigen appears localized intra- and perinuclear in the epithelial cells.
The indirect, retrospective diagnosis, based on the detection of specific antibodies, includes as most important, SN methods and different ELISA techniques. Of these, the blocking allows the differentiation of naturally infected animals from those vaccinated with marker vaccines.

7. DIFFERENTIAL DIAGNOSIS

The differential diagnosis of infectious bovine rhinotracheitis is related to a wide range of diseases, most notably:
• Viral diarrhea - mucosal disease in which diarrhea is usually present and development is more severe - the laboratory exam is used;
• Malignant catarrh fever, which occurs sporadically, the symptoms are more polymorphic, mortality is high;
• Rinderpest, which is enzootic-epizootic, the progress of the disease is severe, with high mortality;
• Allergic rhinitis, which occurs during grazing, no temperature increase, the animals sneeze, show inspiratory dyspnea, greenish-orange, caseous nasal discharge.
Consideration will also be given to: pasteurellosis, calf diphtheria, viral pneumonias.

8. TREATMENT

At present, there is no specific treatment against bovine infectious rhinotracheitis. The usefulness of a mixed antipasturelic and a bivalent bovine antiviral serum (anti-IBR-IPV and PI-3) in the dose of 0.5-0.6ml / kg is quoted. Also, in the event of an IBR outbreak, antibiotic therapy is recommended to minimize the risk of bacterial secondary infections, accompanied by symptomatic and dietary therapy and good hygiene. Vaccination of bovine during the outbrake, in the early phase of BHV-1 infection with a live attenuated vaccine by intranasal administration has been observed to reduce the number of clinical illnesses. However, this measure does not influence the development of clinical cases.

9. PREVENTION AND CONTROL

9.1. Prophylaxis

Prophylaxis is achieved through general and specific measures.
9.1.1. General measures: the general measures concern: the purchase of animals only from vaccinated flocks, the establishment of prophylactic quarantine, the supervision of the breeding and exploitation technologies, the avoidance of stress factors, serological surveillance, the use of the serologically negative bulls, current disinfection.
9.1.2. Specific measures (immune prophylaxis). In specific IBR prophylaxis, various inactivated or attenuated, monovalent or polyvalent vaccines have been used with definite effectiveness only in preventing clinical signs associated with bhv-1. To date, no vaccine is capable of providing complete protection against BHV-1, so it is necessary to establish a repeat vaccination protocol supplemented by

strict sanitary and hygienic measures to reduce the risk of contamination (Ackermann and Engels, 2006). An alternative to conventional vaccines are those designated as marked or marker vaccines, consisting of viral strains from which genes encoding glycoprotein gE or gC were deleted. They immunize the animals against all bhv-1 antigens, except for gE (gC), thus allowing seronegative animals to be distinguished from gE (animals vaccinated with marker vaccines) from naturally infected seropositive bovine animals. in perspective, it is hoped that recombinant subunit vaccines or those made up of plasmid DNA, will provide improved protection. The benefits of the latest generation vaccines are: superior levels of seroconversion, reduction of viral excretion, booster effect from a third administration, possibility of differentiation between diseased and vaccinated animals.

Live modified, inactivated, conventional or modern generation IBR (subunit, marker deleted) vaccines reduce only the gravity of clinical symptoms, viral replication and viral transmission but are not capable of preventing BVH-1 infection. From this point of view some authors argue that there are no differences between conventional and marker vaccines (Lemaire et al., 2000a and b, Ellis et al., 2005, Gogev et al., 2004). However, it has been observed that the transmission of the virus via the marker vaccines was minimal (Mars et al., 2000). The problem is the possibility of inducing the latency status, sometimes associated with reactivation and elimination of the virus in the herd, following the use of attenuated preparations (Castrucci et al., 2002). It has also been demonstrated, though experimental studies, the possibility of combining a deleted gE vaccine strain with a wild-type strain in the vaccinated herds (Schynts et al., 2003). However, the time interval between two successive infections has a major influence on this recombination (Meurens et al., 2004).

Although the protective effect against viral infection cannot be guaranteed and there are also other inconveniences, of which the induction of latency was quoted above, vaccination represents a choice since it was found that the presence and spread of the virus in the herd is thus significantly diminished (Trapp et al. , 2003; Bowland et al., 2000).

In IBR eradication programs, marker vaccines are recommended, given the two characteristics: they protect against the viral pathogenic effect and offer the possibility of differentiation between vaccinated animals and diseased ones (Bosch et al., 1998, 1997, 1996). Detection of infected animals is based on the assumption that all wild-type viruses express glycoprotein gE and that all these animals will produce anti-gE antibodies (Egyed et al., 2000). Different combined live vaccines (IBR-IPV; IBR-IPV, PI-3; PI3-IBR-BVD), inactivated monovalent vaccines or others, depending on the country or producer, are available on the market (Fulton et al., 1995, 2003, 2004, Kerkhofs et al., 2004, Kujik, 2002, Silva et al., 2006).

The administration protocols depend on the vaccine type. The IBR-IPV vaccine is administered at a dose of 2 ml, irrespective of age, boosted after 21 days in the previously unvaccinated breeding herds and after 7 days in the other age groups, when the first vaccination was by aerosol or nasal instillation. Immunity is maintained through annual vaccinations, i.m., at the same dose.

Bi- and trivalent vaccines are applied to calves under the same conditions as the trivalent vaccine against BVD-MD (Gogev et al., 2002, 2004, Gupta et al., 2001). Thus, the vaccine is administered by intranasal route (1 ml in each nostril) starting with the first few days of life, with repeat after 15-20 days (im, 2ml) or from 2-3 weeks (im, 2ml) and repeat after 15-20 days.

The inactivated vaccine is applied to cows and bulls on breeding farms, 5-ml, s.c. injection, repeated after 15-20 days and then every 6 months.

9.2. Control

On farms with disease outbreaks, the diseased animals are isolated and treated symptomatically and the healthy ones, depending on the epizootiological situation, are serum treated using the mixed serum (0.3-0.4 ml / kg, s.c.) or vaccinated by emergency procedure. For the genital form, reproduction is discontinued throughout the course of the disease, the artificial insemination is carried out

with the semen from non-infected bulls. The diseased animals are isolated and treated symptomatically with antiseptics or antibiotics, in the form of sprays or ointments. Since the healed animals become latent carriers of the virus, the most drastical method to eradicate the disease is by eliminating the seropositive animals (Ackermann and Engels, 2006). A very strict monitoring procedure should be in place, ie, in Romania, in the strategic disease control program includes in the case of IBR, active surveillance by serological screening (ELISA), only upon request, for bulls and breeding buffaloes (2 times a year, sem. I and II); mothers of bulls, on licensing, and candidate bulls mothers (once a year); breeders, after the age of 6 months.

10. ERADICATION

BHV-1 infection is highly contagious and causes important economic losses. In numerous European countries, there are programs to control and / or eradicate IBR. Disease free countries or those where the disease was eradicated impose restrictions on the import of bovines and their products from countries where the virus is present, since the (re)introduction of this herpesvirus in free herds leads to a rapid spread of the disease. Therefore, EU directives also include special conditions for the import of bovine, semen and embryos.

IBR / IPV eradication refers to the eradication of BHV-1 virus in bovine populations. Bovine herpes virus becomes latent due to infection, so all seropositive animals are considered to be carriers and permanent viral shedders. In order to eradicate BHV-1 from a bovine herd, it is necessary to identify and eliminate all seropositive animals.

This approach has been used in countries with low prevalence of infection, namely in the Scandinavian countries, Austria and Switzerland.

In high-prevalence countries the elimination of all HIV-positive animals is not an economically feasible solution; the only viable solution for eradicating this disease is to reduce the incidence of seropositive animals at national level and to use marker vaccines to differentiate between infected and vaccinated animals. The IBR eradication program is based on vaccination of cattle with marker vaccines, controlled bovine movement, stock monitoring and rigorous bio-security measures. In order to follow progress in the eradication of rhinotracheitis, permanent sampling of cattle belonging to several age categories is recommended, as follows: - two years after the start of vaccination, samples are taken from animals between 6-24 months of age, including bovine no longer having maternal antibodies, non-pregnant heifers as well as pregnant heifers. A negative result in this age group (6-24 months) suggests the absence of the virus from the herd.

In the subsequent years, the same age category (6-24 months) will be monitored along with the surveillance of the previously monitored groups from the herd.

ACKNOWLEDGEMENTS

This research work did not benefit of any financing.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXVIII (2), 2022
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

ISOLATION AND IDENTIFICATION OF TWO *Pasteurella* STRAINS, RESPONSIBLE FOR AN OUTBREAK OF PNEUMONIA IN SHEEP

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Abstract

The present study was carried out with the purpose of identifying the etiological agent(s) of a reemmergent outbreak of respiratory syndrome affecting a flock of sheep. The first cases of pneumonia on the farm were recorded during warm season (June - July 2021), affecting mainly lambs, with a few isolated cases among adult sheep. Symptoms included nasal discharge, coughing, dispnea, loss of appetite and severe weight loss, a morbidity of 80% among young animals and 5% mortality. Pasteurella spp. was isolated from lung tissue samples collected during necropsy. The lambs were treated with Enrofloxacin administered orally, and the clinical status improved after 6 days of therapy; however, during the fall of 2021, a recurrence of respiratory distress was reported, this time affecting both young and adult animals. Bacteriological examinations were performed on nasal and palatine tonsil swabs collected from live animals and lung and lymph node tissue collected from slaughtered animals for diagnostic purposes. Two strains of Pasteurella were isolated, Pasteurella multocida and Pasteurella spp. The isolates were characterized biochemically and antibiotic susceptibility tests were performed. Following test results, the entire flock of 2000 sheep was treated with Enrofloxacin for 6 days and complete remission of respiratory symptoms was achieved.

Key words: *Pasteurella multocida, Pasteurella spp., pneumonia, respiratory infection, sheep.*

INTRODUCTION

Small ruminant husbandry plays an important role in worldwide economy. Ovine infectious pathology is complex and diverse, comprising numerous morbid entities, such as anthrax, mycoplasmosis, anaerobiosis and viral diseases, which have to be considered when the prophylactic regime is established for a certain herd (Turcu et al., 2010; Enache et al., 2017; Negru et al., 2021). Diseases caused by bacteria belonging to the *Pasteurella* genus among sheep populations can be a cause of major economic loss, especially due to manifestations such as pneumonia and sepsis in lambs. The main species responsible for ovine pasteurellosis is *Pasteurella* (*Mannheimia*) *haemolytica*; very rarely, *Pasteurella multocida* is the causative agent of pneumonia in sheep. Prevention of the disease is difficult to accomplish, due to the large number of circulating strains and low immunogenicity of the bacteria (Manzat, 2001). *P. multocida* represents a heterogenous group of microorganisms, characterized by antigenic variation, diverse host predilection and pathogenesis. Some of the strains included in the group are primary pathogens, determining severe outbreaks of respiratory infections in various species, and others are ordinary commensals of the respiratory tract, able to multiply and invade tissues, causing respiratory disease, as comorbidity, in immuno-suppressed individuals (Weisser et al., 2003). The purpose of the current study was to identify the etiological agent(s) of an reemergent outbreak of respiratory syndrome affecting a flock of sheep, and to establish a correct course of treatment, in accordance with the antibiotic susceptibility tests.

MATERIALS AND METHODS

The outbreak of pneumonia described in the current study affected a flock counting 2000

sheep, located in the south-eastern region of Romania, in the Danube Meadow. The animals were breeded in the traditional husbandry system, both in enclosed sheds, during cold season, and on pastures during the warm season. Besides the respiratory infections described in the current study, other ovine health issues of the flock, common in the area, include tick infestations and internal parasitic infestations with trematodes (*Fasciola* spp. and *Dicrocoelium* spp.) and cestodes. Also, the ground of the pastures was a flood zone, and during rainy weather, the ground became muddy, acting on the prevalence of foot rot.

The entire flock was subjected to deworming twice every year, during spring and fall, with albendazole, administered orally and ivermectin, injected subcutaneously, and the lambs were also dewormed during summer, in order to maintain parasitic infestations under control. Prophylactic measures included vaccinations against anthrax during spring, against anaerobic diseases during fall, and against contagious agalactia every six months. To prevent anaerobic diseases, lambs were first vaccinated at 4-6 weeks of age, and received a booster after 4 weeks.

The first cases of respiratory infections on the farm were recorded in June 2020. The symptoms included coughing, nasal discharge, severe weight loss and death, and affected mainly lambs, aged 3 to 6 months. Out of a batch of approximately 200 lambs, 80% expressed the disease, and the recorded mortality rate was 5 %. Only a few cases were recorded among adult animals, with mild symptoms and no mortality. The lambs that died of the disease were subjected to necropsy, and samples were collected from lung tissue and thoracic lymph nodes for bacteriological examination. According to the results of antibiotic susceptibility tests, the affected group was successfully treated with Enrofloxacin oral solution, 5 mg/ kg/ day, for 6 days.

A recurrence of respiratory symptoms was recorded among the animals on the flock, approximately 3 months after the initial treatment, during the fall of 2021. Both young and adult animals were affected, showing signs of respiratory distress, coughing and weight loss. No mortality was recorded during this period; however, a few more severely affected animals were slaughtered for diagnostic purposes. Bacteriological examinations were performed on samples collected from bronchial secretions, lungs, liver, spleen and thoracic lymph nodes during necropsy, and on nasal swab and palatine tonsils swab samples collected from clinically affected live animals. The samples were cultured on Columbia agar with 5% defibrinated sheep blood and incubated at 37°C for 20-24 hours. The morphological features of the isolated strains were examined microscopically on Gram stained slides, and catalase and oxidase tests were performed using conventional methods. The identification of the isolates was performed using the Api 20 E and Api 20 NE biochemical tests (Biomerieux), with the interpretation of the results performed according to the producer's instructions. Antimicrobial susceptibility was investigated by disc diffusion method, using Liofilchem antimicrobial discs, and the results were interpreted using Liofilchem and EUCAST standards. The entire flock of sheep was placed under treatment with Enrofloxacin oral solution, 5 mg/ kg/ day, for 6 days, as indicated by the results of the antibiotic susceptibility tests. All the animals present on the farm were housed in enclosed sheds and the antibiotic was administered via drinking water, limiting the animals' access to any untreated water source in order to ensure the ingestion of the appropriate dose of medication.

RESULTS AND DISCUSSIONS

Post mortem examinations of the carcasses revealed various degrees of pulmonary consolidation, pulmonary edema (Figure 1), congestion, atelectasis, and in some cases, abscesses were present in the lung tissue. Pleural effusion was present in the majority of the examined carcasses.

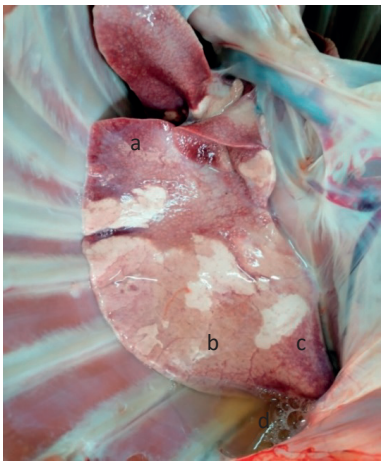

Figure 1. Necropsy examination – lung of an adult sheep, showing congestion (a), edema (b) and marbling (c). A large amount of yellow, serous fluid is present in the pleural space (d).

Bacteriological examinations of palatine tonsil swabs and nasal swabs constantly revealed the presence of medium sized, gray, transparent, non-hemolytic colonies, which appeared as Gram negative cocobacili upon microscopic examination.

The isolated strain was identified as *Pasteurella* spp. via biochemical tests.

The biochemical characteristics of the isolate are detailed in Table 1.

A *Pasteurella* spp. strain, with identical biochemical characteristics, was also isolated in pure culture from the pulmonary abscesses of two lambs, aged 3 and 4 months, which had died during the first outbreak of respiratory infections.

Table 1. Biochemical characteristics of the *Pasteurella* spp. isolated from palatine tonsil swabs and nasal swabs of affected sheep

NO3	TRP	GLU	ADH	URE	ESC	GEL	PNPG	GLU	ARA	MNE	MAN	NAG	MAL	GNT	CAP	ADI	MLT	CIT	PAC
+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-

Legend: NO3 – potassium nitrate, TRP – L-tryptophane, GLU – D-glucose (fermentation), ADH – L-arginine, URE – urea, ESC – esculin ferric citrate, GEL – gelatin, PNPG - 4-nitrophenyl-βD-galactopyranoside, GLU – D-glucose (assimilation), ARA – L-arabinose, MNE – D-mannose, MAN – D-mannitol, NAG – N-acetyl-glucosamine, MAL – D-maltose, GNT – potassium gluconate, CAP – capric acid, ADI – adipic acid, MLT – malic acid, CIT – trisodium citrate, PAC – phenylacetic acid, "+" - positive result, "-" - negative result.

P. multocida was isolated only from lung tissue samples from lambs and adult sheep.

On Columbia blood agar, the *P. multocida* colonies appeared grayish and non-hemolytic, slightly smaller and more transparent than the *Pasteurella* spp. colonies (Figure 2).

Both bacterial strains were catalase positive and oxidase negative.

The identity of the *P. multocida* isolate was confirmed by the results of two biochemical tests, Api 20 E and Api 20 NE, the results of which are presented in Table 2.

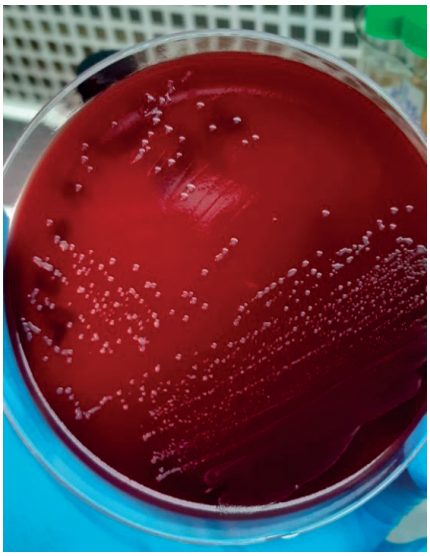

Figure 2. *Pasteurella multocida* colonies on Columbia blood agar

Table 2. Biochemical characteristics of the *Pasteurella multocida* isolated from lung tissue samples of an adult sheep

API 20 NE	NO3	TRP	GLU	ADH	URE	ESC	GEL	PNG	GLU	ARA	MNE	MAN	NAG	MAL	GNT	CAP	ADI	MLT	CIT	PAC
	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
API 20 E	ONPG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA
	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-

Legend: Legend: NO3 – potassium nitrate, TRP – L-tryptophane, GLU – D-glucose (fermentation), ADH – L-arginine, URE – urea, ESC – esculin ferric citrate, GEL – gelatin, PNPG - 4-nitrophenyl-βD-galactopyranoside, GLU – D-glucose (assimilation), ARA – L-arabinose, MNE – D-mannose, MAN – D-mannitol, NAG – N-acetyl-glucosamine, MAL – D-maltose, GNT – potassium gluconate, CAP – capric acid, ADI – adipic acid, MLT – malic acid, CIT – trisodium citrate, PAC – phenylacetic acid, ONPG - 2-nitrophenyl-βD-galactopyranoside, LDC – L-lysine, ODC – L-ornithine, H2S – H_2S production, TDA – L-tryptophane, IND – indole production, VP – acetoin production, INO – inositol, SOR – D-sorbitol, RHA – L-rhamnose, SAC – D-saccharose, MEL – D-melibiose, AMY – amygdaline, "+" - positive result, "-" - negative result.

Antibiotic susceptibility tests revealed that both isolates were sensitive to the majority of the antimicrobials used in the essay (Table 3), including enrofloxacin, the selected antibiotic for the treatment of the flock.

Table 3. Antibiotic susceptibility results for *Pasteurella* spp. and *Pasteurella multocida*

Antibiotic	***Pasteurella spp.***	***Pasteurella multocida***
Enrofloxacin	Susceptible	Susceptible
Trimethoprim + Sulfametoxazole	Susceptible	Susceptible
Gentamycin	Susceptible	Susceptible
Norfloxacin	Susceptible	Susceptible
Spectinomycin	Susceptible	Susceptible
Doxycycline	Susceptible	Susceptible
Ampicillin	Susceptible	Susceptible
Amoxicillin	Susceptible	Susceptible
Erythromycin	Intermediately susceptible	Intermediately susceptible
Tetracycline	Intermediately susceptible	Susceptible
Lincomycin	Resistant	Resistant
Colistin sulfate	Resistant	Resistant

The initial therapeutic approach, which targeted only the affected animals, was successful at the time in improving the clinical status of the lambs, and limiting mortality. However, the disease was not eradicated from the flock, possibly due to subclinically infected animals, which continued to spread the bacteria. After the second course of treatment, which included all of the animals on the farm, a complete remission of the respiratory symptoms was achieved.

Ovine pathology caused by members of the *Pasteurella* genus has been reported in a number of other studies. A study carried out in Iran reported a prevalence of 3.71% of *P. multocida* infections among pneumonia cases in sheep and goats (Valadan et al., 2014). In Ethiopia and Iraq researchers detected the presence of *P. multocida* and *Mannheimia haemolytica* in nasal swab samples and lung tissue specimens of pneumonic sheep, using the polymerase chain reaction (Deressa et al., 2010; Othman et al., 2014). The results of a study preformed on Icelandic sheep suggest that at least two groups of *P. multocida* coexist in sheep: a genetically homogenous group consisting of upper respiratory tract commensals, and a genetically heterogeneous group representing the cause of ovine pneumonia (Einarsdottir et al., 2016). Regarding antibiotic susceptibility, studies on *P. multocida* and *M. haemolytica* strains isolated from small ruminants revealed the majority of the isolates to be multidrug resistant; however, most strains were susceptible to enrofloxacin (Sarangi et al., 2015).

Further research is required to assess the pathogenicity and immunogenic properties of the isolated *Pasteurella* strains and whether they could be considered as candidates for the production of an auto-vaccine.

CONCLUSIONS

The current study presents an etiological approach over an emerging respiratory infection in a sheep flock. Based on clinical signs and post-mortem examinations, a suspicion of *Pasteurella* induced pneumonia was issued. Samples collected from the flock were subjected to bacteriological examinations, and two strains belonging to the *Pasteurella* genus were isolated. The two isolated were identified as P. spp. and *P. multocida.* Both strains were susceptible to enrofloxacin, and the antibiotic was used successfully for therapeutic purposes. Given the history of the respiratory disease in the herd, induced by *Pasteurella* species, the health of the animals remains under threat of recurrence, whenever the associated risk factors will intervene.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXVIII (2), 2022
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

OCCURRENCE OF PARASITIC AND *Malassezia* OTITIS EXTERNA IN DOGS AND CATS: A RETROSPECTIVE STUDY IN A PRIVATE PRACTICE IN SOUTHERN ROMANIA

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Abstract

Parasites and fungi cause otitis externa in dogs and cats and have an important impact on the welfare of the affected animals. Moreover, their control is often problematic. Here we present a retrospective study on the occurrence of otitis externa in client owned dogs (n=179) and cats (n=157) that were presented in a veterinary practice during a one-year period. All animals were subjected to the routine clinical examination followed by direct otoscopy and microscopic evaluation of ear exudates with and without Diff-quick staining..Of the investigated dogs and cats, 16.75% and 19.75%, respectively were diagnosed with otitis externa. Of the dogs, 10.05% (18/179) were positive for mixed Malassezia and Otodectes infestations, 5.58% (10/179) only with Malassezia, and 1.11% (2/179) with only Otodectes. In cats, 8.28% (13/157) of the animals were diagnosed with Malassezia and Otodectes mixed infestations, 11.46% (18/157) of cats had only Malassezia otitis, and none had only parasitic infestation. Overall, of the 30 dogs and 31 cats diagnosed with otitis externa, 43.33% and 32.25%, respectively, were presented for ear related problems. In conclusion, these findings showed a positive correlation between Malassezia and Otodectic otitis in dogs, while in cats Malassezia otitis externa was detected in all parasitic infestations of the ear. Additionally, the study underlines the importance of ear examination and microscopic evaluation for a proper therapeutic protocol.

Key words*: otitis externa, dogs, cats, Southern Romania.*

INTRODUCTION

Otitis externa is an inflammation of the external ear canal distal to the tympanic membrane; the ear pinna may or may not be involved (August, 1988). It is one of the most common reasons for small animals (especially dogs) to be presented to the veterinarian (Bruyette & Lorenz, 1993; Jacobson, 2002). Otitis externa can exhibit an acute or chronic evolution and can be unilateral or bilateral clinical condition. As response to chronic inflammation, changes that occur in the external ear canal may include different pathologies, such as glandular hyperplasia and /or dilation, epithelial hyperplasia, and hyperkeratosis (Huang & McNeil, 2009; Njaa & Tabacca, 2012).

Malassezia yeast is a common causative agent of otitis externa in dogs (Weisbroth et al., 1974; Murphy, 2001). Moreover, it is reported that some dogs appear to develop an allergic response to *Malassezia* spp., which leads to significant pruritus and discomfort (Mitrea, 2011; Bajwa, 2019; Brame, & Cain, 2021).

Several parasites have been associated with otitis externa, especially *Otodectes* but also *Demodex* and *Sarcoptes* (Powell et al, 1980). Among them, the ear mite *Otodectes cynotis* is quite common, being reported in the past for up to 50% of the otitis externa cases diagnosed in cats and 5% to 10% of the cases in dogs (August, 1988; Karen, 2022).

Ear mites may initiate otitis externa but remain undetected. One reason is the difficulty that may occur in demonstrating the mites. As few as two or three mites can cause otitis externa (Murphy, 2001; Jacobson, 2002; Karen, 2018). This has been explained by studies showing that ear mites can induce Arthus-type and immediate-type hyper-sensitivity reactions (Rosychuk, 1994; Brame & Cain, 2022). Another explanation is that the mites initiate the otitis externa and then leave the canal or are eliminated by secondary induced inflammation (Bruyette & Lorenz, 1993; Logas & Maxwell, 2021).

Causes of otitis externa could be primary or secondary, with a variety of predisposing and perpetuating factors that contribute to/or promote the disease. Collectively, these causes and factors are referred to as the primary secondary predisposing perpetuating (PSPP) classification system (Bruyette & Lorenz, 1993; Jacobson, 2002; Karen, 2022).

Primary causes of otitis externa create disease in a normal ear. They alter the environment in the ear, often allowing a secondary infection to develop. Primary causes include: allergy (adverse food reaction, atopic and contact dermatitis), parasites (*Otodectes, Demodex, Sarcoptes*), autoimmune/immune-mediated (pemphigus foliaceus, vasculitis, others) endocrine disease (hypothyroidism, hyper-adrenocorticism), epithelialization disorders (sebaceous adenitis, zinc-responsive dermatitis), foreign bodies glandular disorders (sebaceous gland hyperplasia), fungal (*Aspergillus*) viral (distemper), miscellaneous (proliferative necrotizing otitis of cats, juvenile cellulitis) (Powell et al., 1980; Brame & Cain, 2021).

Secondary causes of otitis externa create disease in an abnormal ear. These are often chronic/recurrent problems when the primary cause is not addressed. Secondary causes include bacteria (*Staphylococcus, Streptococcus, Enterococcus, Pseudomonas, Proteus*, etc), yeast (*Malassezia*) medication reactions, overcleaning (August, 1988; Rosychuk, 1994; Zaman et al., 2010; Brame & Cain, 2021; Logas & Maxwell, 2021).

Perpetuating factors occur due to otic inflammation and may be severe in chronic cases. They include changes of the epithelium (failure or alteration of migration; migration of the epithelium of the ear canal provides a natural cleaning mechanism in normal ears) of the ear canal (stenosis, oedema, proliferative changes), of the tympanum (rupture), of the glandular tissue (sebaceous hyperplasia), as well as pericartilaginous fibrosis or calcification middle ear disease (Powell et al., 1980; Zaman et al., 2010).

Predisposing factors increase the risk for developing otitis externa. These include: conformation (pendulous pinna, stenotic canals, hairy concave pinna, excessive hair in canals), excessive moisture (environment, swimming), obstructive ear disease (neoplasia, polyp, feline apocrine cystadenomatosis), primary otitis media, systemic disease (immune suppression, catabolic states), treatment effects (changes in normal flora, trauma) (Powell et al., 1980; Jacobson, 2002; Bajwa, 2019).

Malassezia spp. infections are a common secondary cause of otitis or inflammation of the ear canal (Mitrea, 2011). It is a yeast that normally lives in the ears of cats and dogs but overgrows when the environment of the ear canal changes. This change is a result of increased fatty secretions and moisture, making it easier for the *Malassezia* to multiply. Some primary, or underlying, causes of ear infection include allergies (atopic dermatitis or food hypersensitivity) and narrowed ear canals. Studies have shown that bacteria can be found in combination with a *Malassezia* otitis (Jacobson, 2002; Bajwa, 2019; Logas & Maxwell, 2021).

Otitis externa is a multifactorial disease with a complicated management that severely impacts animal wellbeing (August, 1988). In small animal practices it can be one of the most frustrating diseases to manage. Understanding how primary and secondary factors intervene in the pathogenesis of this disease is key to increasing the success rate of treatment (Rosychuk, 1994).

Therefore, the purpose of this study was to evaluate the occurrence of yeast and parasitic otitis in dogs and cats, and to better understand the correlation between them, as a documented base of treatment and better control the disease.

MATERIALS AND METHODS

Clinical examination and sampling

A number of 179 dogs and 157 cats were enrolled in this study during a 1-year period. During this time, a total of 457 animals (241 cats and 216 dogs) were referred to the clinic. The examined animals were kept both outdoor and indoor, and were admitted to the clinic for various reasons, most common being vaccinations and yearly check-ups but also for otic pruritus, head rubbing, headshaking and other ear related symptoms.

For all animals a thorough clinical examination of the ear pinnae canals was carried out using an otoscope whenever possible.

Samples of cerumen and discharges were collected from both ears for further microscopic ear mite detection, as well as for modified Wright Giemsa staining for detection of *Malassezia* yeasts.

Laboratory examination

For ear mite detection, from the collected cerumen and otic discharge smears were prepared with the addition of a small quantity of mineral oil on a microscopic glass slide (Ionita & Mitrea, 2013). A clean glass coverslip was put on the top surface of the smear and examined microscopically (40x and 100x obj.) on the same day, for the presence of adult mites, nymphs, larvae, or eggs (Figure 1).

For Malassezia yeast detection, smears were performed from collected cerumen and otic discharge, and were modified Wright Giemsa (Diff-Quick) stained. After staining, the smears were examined using a 100x oil objective (Figure 2).

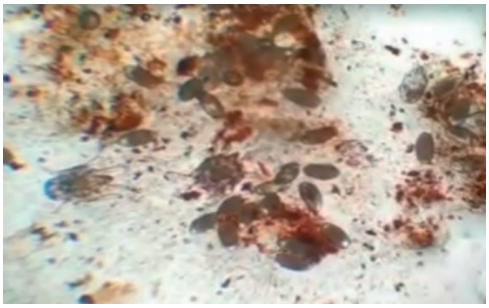

Figure 1. *Otodectes cynotis* infestation – adults, eggs – in a cerumen smear of a cat (original)

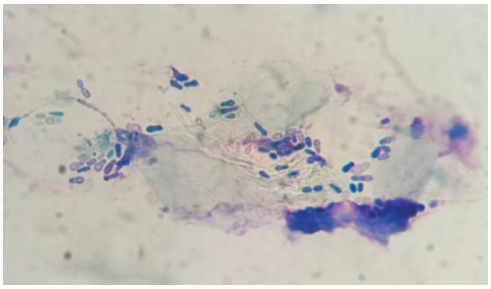

Figure 2. *Malassezia* yeast infection found in a cat with external otitis (original)

RESULTS AND DISCUSSIONS

A total number of 179 dogs (88 males, 91 females), of different breeds and age varying from 1 month to 10 years (average: 24 months; Standard Deviation [SD]: 29.17), with clinical signs (n=17; 9.49%) and without signs of external otitis, were examined.

Of the investigated dogs, 30 (16.75%) were diagnosed with external otitis after clinical examination. Their age varied from 2 to 10 years (mean age of 29.73 months; SD: 30.45). The laboratory examination revealed that out of the 30 dogs diagnosed with otitis, 2 animals were positive for single *Otodectes cynotis* infestation, 10 animals positive for single *Malassezia* yeast infection and 18 with mixed *Otodectes* and *Malassezia* infestations (Table 1).

Overall, the prevalence of *Malassezia* spp. was 15.6% (n=28) and for *O. cynotis,* 11.2% (n=20). Single infections with either *Malassezia* or *Otodectes* were present in 6.7% (n=12) of dogs whereas mixed infections were present in 10.05% (n=18) of dogs (Table 1, Figures 3 and 4).

Table 1. Distribution of causal agents in dogs and cats with external otitis

Animal Species	Total Cases	Single infection		Mixed infection
		Malassezia spp.	*Otodectes cynotis*	
Dog	30	10	2	18
Cat	31	18	0	13

With regards to cats, a total number of 157 cats (79 males, 78 females) of different breeds and age varying from 3 to 13 years old (average: 25 months; SD ±29.7) with signs (n=25; 15.92%) and without signs of external otitis. Of these, a number of 31 (19.75%) were diagnosed with external otitis after clinical examination. Their age varied from 3 months to 13 years (mean age of 32.3; SD: 17.54 months).

The laboratory examination revealed that among the cats diagnosed with otitis, 18 animals were positive for *Malassezia* infestation and 13 with mixed *Malassezia* and *Otodectes* infestations. None of the examined cats was infected only *Otodectes* (Table 1).

Overall, in the investigated cats, the prevalence of *Malassezia* was 19.75% (n=31), and of *Otodectes* 8.3% (n=13).

Single *Malassezia* infections were present in 11.5% (n=18) of cats.

There were no single *Otodectes* infections in the examined cats (Figures 3 and 4).

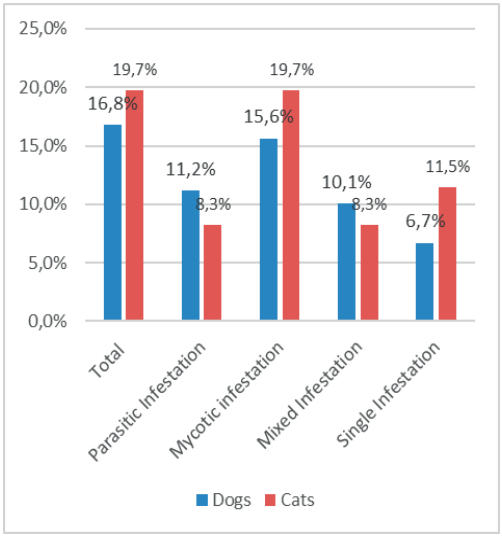

Figure 1 Comparative prevalence of pathogens in dogs and cats with external otitis

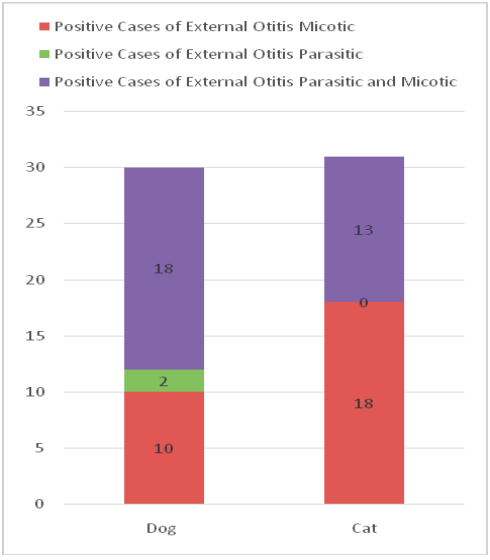

Figure 2. Distribution of the causative agent in positive dogs and cats for external otitis

DISCUSSIONS

This retrospective study that investigates the occurrence of otititis externa in client-owned dogs and cats emphasizes that 16.75% of the examined dogs and 19.75% of the cats were diagnosed with otitis externa.

Moreover, of the positive animals, only 43.33% of the dogs and 32.25% of the cats were brought by the owners for ear clinical signs.

Therefore, these findings highlight the need for thorough evaluation of the ear for every animal presented for a consultation.

Evaluation of cerumen and otic discharge with and without staining alongside otoscopic evaluation are important for correctly identifying the underlying disease.

Also, the result showed a positive correlation between parasitological infections of the ear and the presence of *Malassezia* yeast infections in both dog and cat.

In cats it was observed that *Malassezia* yeast infection accompanies all *Otodectes* infections.

As reported, animals with external otitis are not always presented by the owner due to specific clinical signs. In this study, the owners recognized ear- related clinical signs in 43.33% of the positive dogs and 32.25% of the positive cats, suggesting that owners are likely to overlook some ear problems (fig. 5).

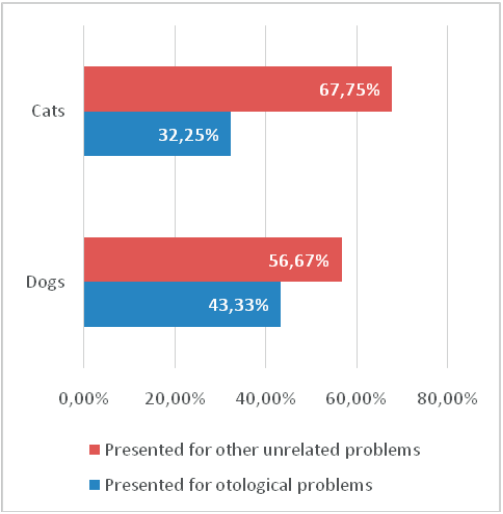

Figure 3. Percentage of animals that were presented to the clinic for otological problems

Additionally, it is worthy of mentioning that apart from mycotic and parasitic, many bacteria can accompany an external otitis. According to others studies, the most isolated bacteria from the ear canals of dogs affected by otitis is *Staphylococcus* spp (Bruyette, 1993). Other bacteria commonly associated with otitis include *Pseudomonas, Proteus, Enterococcus*, *Streptococcus,* and *Corynebacterium*. Some bacteria such as *Staphylococcus* and *Pseudomonas* may produce biofilm, which can

lead to persistence of infection despite adequate therapy, as the biofilm needs to be disrupted for any antimicrobial therapy to be effective in clearing the infection.

It is well known that otitis externa is an important pathology in dogs and cats and in most cases is caused by multiple factors. Given this fact, it is important to use microscopic evaluation in all animals with ear problems for determining the optimal therapeutic protocol. Therefore, an integrated approach, based on clinical, biological and medical features will assure a successful parasitological control (Mitrea, 2002).

CONCLUSIONS

The findings of the present study describe occurrence of parasitic and *Malassezia* otitis externa in dogs and cats, and show a positive correlation between *Malassezia* and *Otodectes* otitis in dogs, while in cats *Malassezia* otitis externa was detected in all parasitic infestations of the ear. Furthermore, the study underlines the importance of ear examination and microscopic evaluation for a proper therapeutic protocol.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXVIII (2), 2022
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

EAR CYTOLOGY - A KEY TEST IN THE DIAGNOSIS AND MANAGEMENT OF CANINE OTITIS EXTERNA

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Abstract

Otitis externa is a common condition in dog with a multifactorial etiopathogenesis including primary factors along with predisposing and perpetuating factors. Therefore, a successfull therapy of otitis externa should target the identification and correction of primary causes as possible as, not only the elimination of secondary infections. In practice, ear swab cytology is considered to be the best choice for the diagnosis and initial treatment of otitis, recommended to be repeated for the therapy evaluation or adjustment. Our study included 20 dogs exhibiting various clinical forms of otitis externa, from mild acute to severe chronic otitis with often associated pruritus and smelly discharge, came to be investigated at the Dermatology Service of Veterinary Medicine Faculty from Bucharest. Cytology showed variable proportion of desquamated keratinocytes, lipid droplets and debris of cerumen, leukocytes, especially degenerated neutrophils and free or phagocytized bacterial and yeast elements. The patients have been reevaluated at every 2-3 weeks after initiating therapy and finally, clinical signs resolution together with decreased cellularity in cytological preps were considered good indicators for patient recovery.

Key words*: cytology, dogs, external otitis.*

INTRODUCTION

Otitis externa (OE) is a common, multifactorial disorder found in 10-20% of dogs and frequently challenging to manage because of treatment failure resulting in progressive or relapsing changes (Cordero, 2015). Successfull therapy of OE requires the identification and correction, as possible as, the *primary causes* (allergies, foreign bodies, ectoparasites, seborrhea, immune-mediated diseases, hypothyroidism) along with the *concurrent perpetuating factors* (bacterial or yeast overgrowth/ infection, excessive moisture, aggresive ear cleaning, wrong medication).

OE is seen in 50-80% of atopic dogs and clinical symptoms may be quite variable including erythema, papules, pustules, crusting, scaling, excoriation, ulceration, lichenification and hyperpigmentation of the pinna, stenosis of ear canal, aural hematomas, malodorous ear exudates with grainy-black, waxy-brown, purulent or mucoid appearance and commonly associated pruritus or pain. OE may be additionally accompanied by other skin sites involvement or systemic illness (such as fever, depression, adenopathy) and can get complicated by otitis media in up to 82% of dogs with chronic disease (Gotthelf, 2005; Medleau, 2006).

The routine evaluation of OE patients consists in detailed history, physical examination and ear swab cytology. Cytology is considered to be the best choice for the diagnosis of secondary infection/overgrowth and initial treatment of otitis that should be repeated at every recheck examination for monitoring response to therapy or medication adjustement (Bajwa, 2019). Instead, culture and sensitivity test is rarely necessary, usually recommeded only for recurrent or resistant otitis even if it cannot accurately determine sensitivity to topical antimicrobials because of susceptibility differences between free and biofilm-forming bacteria (Ghibaudo, 2018; Hensel, 2021) .

Basically, cytological data refer to the presence and abundance of bacteria, yeast and leukocytes, usually neutrophils. The presence

of leucocytes and abundant resident bacteria or yeast is a reliable indicator of true infections which need long-term, high-dose therapy, while the disappearance of leukocytes in otic smears after therapy is considered to be a strong indication of clinical resolution (Gotthelf, 2005). Ear cytology may also reveal ear microbial overgrowth characterized by the presence of abundant or diverse microbiota with lack of leukocytes, being often incriminated in local inflammation that could be ameliorated using only topical therapy. In this regard, a recent study reported that most of canine OE (78,3%) are caused by microbial overgrowth, with predominantly bacterial and less frequently fungal and mixed ear pathogens (Tang et al., 2020).

The most isolated species from otitis were *Staphylococcus pseudintermedius*, *Streptococcus* spp., *Pseudomonas aeruginosa*, *Corynebacterium auriscanis*, *Malassezia pachydermatis* and the newly reported anaerobic organism *Finegoldia magna*, all being recognized as opportunistic pathogens (Kiss et al., 1997; Henneveld et al., 2012; Tang et al., 2020). In clinically affected ears there was also identified a reduced bacterial diversity (dysbiosis) compared to healthy dogs. Surprisingly, allergic dogs were found typically to display a skin and ear dysbiosis with possible implications in increased susceptibility to clinical infections (Tang et al., 2020).

MATERIALS AND METHODS

Patients: 20 dogs of different breeds and ages exhibiting clinical signs of otitis externa were examined at the Dermatology Service of Veterinary Medicine Faculty from Bucharest, in the past year. The affected dogs were 12 males and 8 females with the mean age of 6 years (between 1-12 years) and of the following breeds: Cocker spaniel, German Shepherd, Rottweiler, Labrador, French bulldog, Caniche, Bishon, Pekingese, Dachshund and mixed-breeds. As clinical findings, most patients displayed highly pruritic, bilateral erythemato-ceruminous otitis with greasy or waxy exudate of rancid smel, while five dogs were found with suppurative otitis expressing a malodorous discharge.

Laboratory investigations consisted primarily in ear swab cytology and cultures with susceptibility testing only for chronic or reccurent cases.

Ear cytology sampling. The exudate from each ear (even if of unilateral otitis) was collected from the deeper horizontal canal using a cotton-tipped applicator.Thereafter, the swab was firmly rolled onto 2 microscope slides and stained with Romanowsky (MGG) and Gram stain to be microscopically examined using high-dry (40X) and oil-immersion (100X) objectives. Each specimen was evaluated for the number and morphology of bacteria (cocci, rods), yeast (peanut-shaped *Malassezia* spp.) and leukocytes.

Culture and susceptibility testing. The ear exudates were initially cultured in Mueller-Hinton broth and the 24 h-cultures were used for antibiotic sensitivity testing by the disc diffusion method using a routine antibiotic panel.The plates were incubated at 37°C for 24-48 h.

In all cases, clinical, cytological, microbiological and therapeutic data were recorded and correlated.

RESULTS AND DISCUSSIONS

History and clinical findings

Based on history and physical examination, in most cases we identified highly pruritic, mild acute to severe chronic erythemato-ceruminous otitis, often displaying erythema, lichenification and hyperpigmentation of the pinna with greasy or waxy discharge of rancid smel (Figures 1, 2, 3).

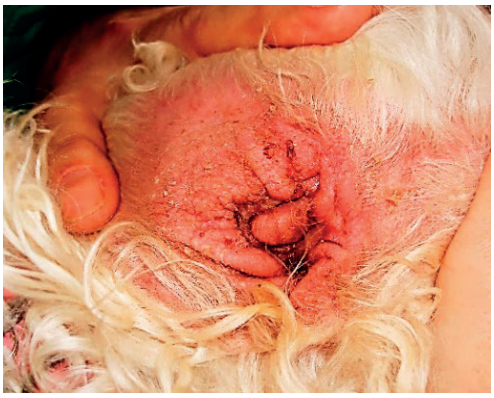

Figure 1. Erythematous otitis in an atopic dog

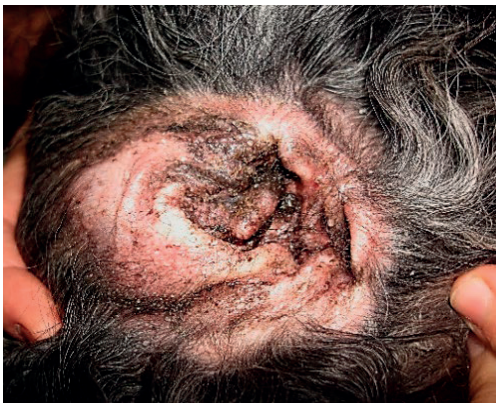

Figure 2. Ceruminous otitis with *Malassezia* (excessive brown waxy discharge)

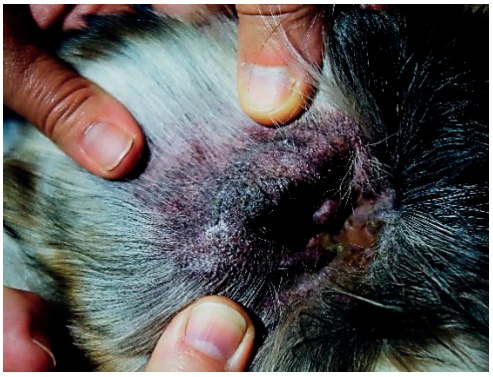

Figure 3. Severe erythemato-ceruminous otitis (pinnal lichenification and hyperpigmentation)

Five dogs were found with persistent or reccurent suppurative otitis characterised by erythema, oedema, ulceration and pain, with mucopurulent or black-colored exudate (Figures 4, 5).

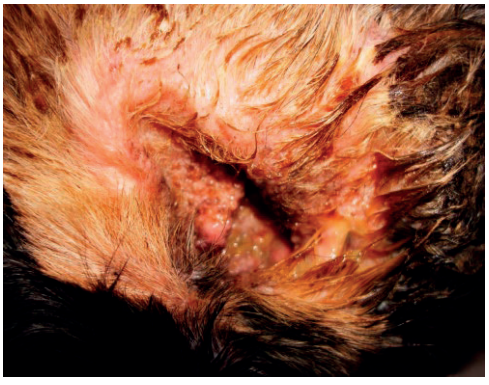

Figure 4. Chronic suppurative otitis with *Pseudomonas* (mucopurulent exudate)

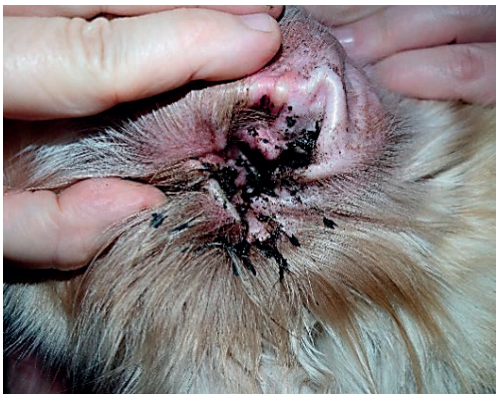

Figure 5. Reccurent otitis with *Pseudomonas* (black-colored exudate)

History data also indicated atopy, food allergy and primary seborrhea as predominant underlying causes of canine OE. In 2 cases, aggresive ear cleaning and overtreatment with oral and topical medication were found to be responsive for persistent, nonodorous exsudative OE. In few cases, OE were associated with facial and interdigital pyoderma and even gingivitis, especially in older patients with *Pseudomonas* infections.

Cytology data

In cytology, the most affected ears contained abundant and more or less diverse microbiota with few or no leukocytes coresponding to microbial overgrowth. Generally, the relative number of resident organisms considered to be normal is of 2 yeasts and 5 bacteria per high-dry field (40x), while more than 5 yeasts and 25 bacteria per field may be interpreted as abnormal, but in correlation with severity of clinical symptoms, past episodes of otitis and previous therapy response (Gotthelf, 2005).

Morphologically, in the most smears we identified a mixed population of rods and cocci (Figure 6) or a combination of rods, cocci and *Malassezia* yeasts (Figure 7), representing normal resident organisms of the ear canal.

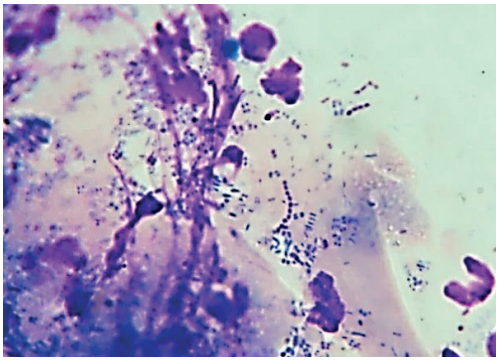

Figure 6. Mixed infection with bacterial rods and cocci together with few degenerate neutrophils (MGG stain, oil immersion objective)

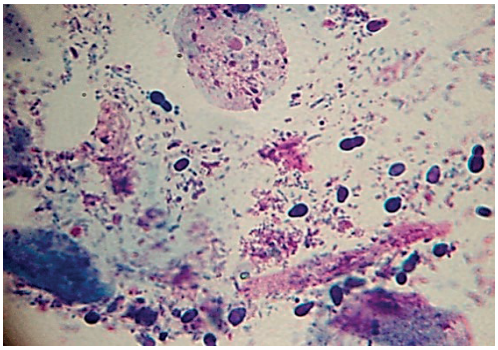

Figure 7. Mixed microbial overgrowth with rods and coccoid bacteria together with *Malassezia* yeasts without any leukocytes (MGG stain, oil immersion objective)

Gram stain was useful to distinguish Gram positive rods (*Corynebacterium* spp.) from Gram negative rods (*Pseudomonas aeruginosa, Proteus mirabilis*) which usually are more resistant to multiple antibiotics (Figure 8).

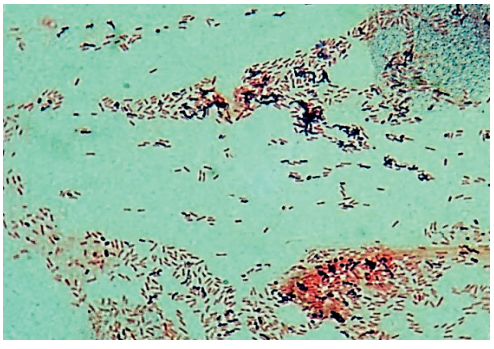

Figure 8. Chronic otitis with severe dysbiosis caused by mixed Gram negative and positive rods (Gram stain, oil immersion objective)

In the five cases of chronic suppurative otitis, cytology showed predominantly rods with variable number of degenerate neutrophils, indicating true infections with severe dysbiosis. In these cases, cytology was also helpful in biofilms detection, looking like an amorphous matrix of variable thickness entrapping microbial cells, leukocytes and other cell debris (Figure 9). Biofilms generally inhibit antimicrobial penetration, being notoriously difficult to eradicate by usual therapeutic schemes (Ghibaudo, 2018; Hensel, 2021).

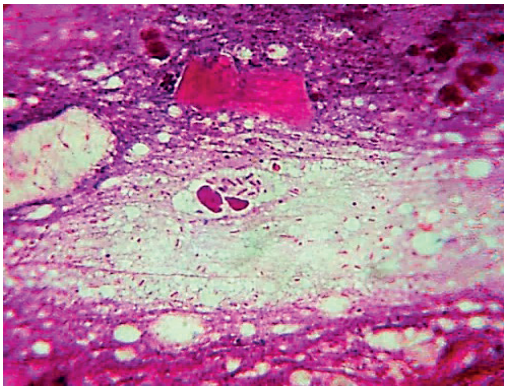

Figure 9. *Pseudomonas* otitis: free and phagocytized rods in a neutrophil within a purple filamentous matrix (biofilm) potentially implicated in antimicrobial resistance (Gram stain, oil immersion objective)

We have also noticed a marked dysbiosis with large numbers of cocci (*Staphylococcus* spp.) in an allergic dog with chronic ceruminous otitis (Figure 10).

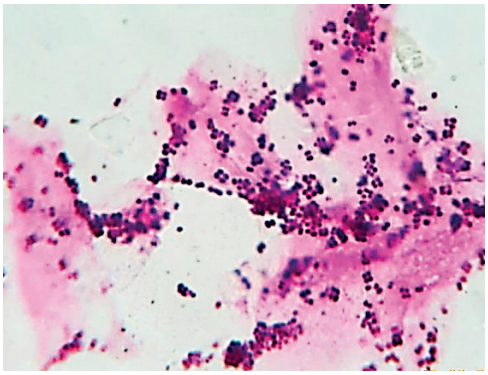

Figure 10. Chronic otitis (bacterial overgrowth) with severe dysbiosis implying Gram positive cocci of *Staphylococcus* spp. (Gram stain, oil immersion objective)

All these patients required cultures and susceptibility testing towards using a long-term, systemic antibiotherapy.
In six cases of *Malassezia* otitis, the ear smears exclusively contained relatively plentiful peanut-shaped elements of *Malassezia pachydermatis* free or adherent to corneocytes, without any neutrophils indicating an yeast overcolonization (Figure 11). *Malassezia* organisms is also commonly found in ear swabs even up to 49% of normal dogs (Cowell et al., 1999).

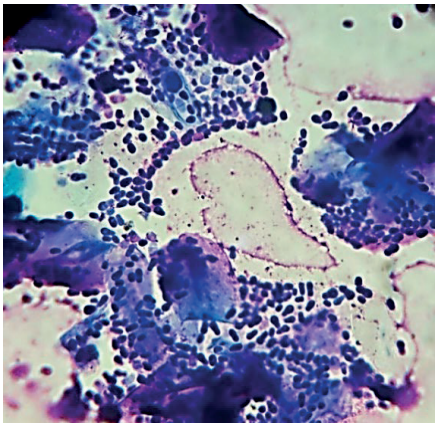

Figure 11. *Malassezia* otitis (yeast overgrowth) with large numbers of budding yeast organisms, free or adherent to corneocytes without any leukocytes (MGG stain, oil immersion objective)

Another particular aspect of ear smears consisted in an intensive desquamation of keratinocytes together with polymorphic resident flora (cocci and rods), secondary to aggresive ear cleaning (Figure 12).

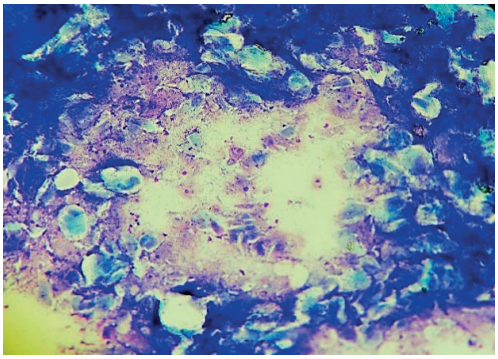

Figure 12. Intensive desquamation of epithelial cells and basophilic debris due to aggresive ear cleaning (MGG stain, magnification 40X)

Occasionally, in ear exudate from a young dog with demodicosis, we have found few adults of *Demodex canis* partially covered by desquamated corneocytes, although *Demodex* is considered a normal inhabitant of the external ear canal (Figure 13). Sometimes, *Demodex* mites may be responsible for chronic ceruminous OE (Hensel, 2021; Medleau, 2006).

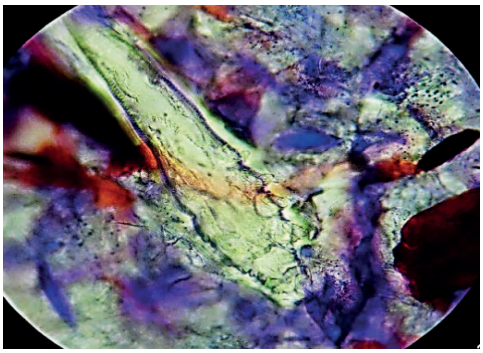

Figure 13. *Demodex* mite in ear exudate from a young dog with demodicosis (MGG stain, oil immersion objective)

Therapeutic data

Most ceruminous otitis clinically evolving for 1-3 weeks and expressing mixed bacterial/yeast overgrowth in cytology were treated with only an ear cleanser with antiseptic properties (Epi-Otic, Otodine). More severe or reccurent ceruminous otitis additionally needed a topical antimicrobial and anti-inflammatory therapy after ear cleaning (Easotic-Virbac, Surolan-Elanco).
Suppurative otitis showing predominantly rods in cytology and cultures (*Pseudomonas, Proteus)* were more difficult to control, combining an ear cleanser with Tris-EDTA (Otodine), topical antimicrobial and antiinflammatory products (Aurizon, Easotic) together with 1-2 systemic antibiotics selected by sensitivity testing (amoxiclav, gentamicin, enrofloxacin) which have been used alternatively for 8-12 weeks to prevent resistance.
Malassezia otitis commonly responded to acid ear cleaners (Epi-Otic, MalAcetic, boric acid) for 2-6 weeks, but in an allergic patient with reccurent otitis we have used topical antifungal medication (Posatex) for 3 weeks to keep it under control.

It also should be mentioned that prescribing of commercial hypoallergenic diets, antihistamines in combination with glucocorticoids together with essential fatty acids supplements visibly helped to ear recovery in allergic patients .

Demodicosis treatment with oral ivermectine and spot-on moxidectin (Advocate) led to otitis and dermatitis remission in Bulldog puppy .

The patients were reevaluated clinically and cytologically at every 2-3 weeks of treatment for therapy monitoring or adjustment.

Clinical improvement was correlated with negative cytology that has been recorded after 2-12 weeks of therapy. Generally, polimicrobial infections/overgrowth have responded better than the monomicrobial ones which needed longer and combined therapy to be controlled.

CONCLUSIONS

Ear cytology has shown to be the most reliable and rapid test for routine diagnosis of otitis externa and therapy monitoring even more accurate than cultures, especially recommended in persistent or reccurent cases.

In most dogs, otitis externa was caused by mixed bacterial overgrowth with underlying allergy or seborrhea. Chronic or recurrent otitis were usually associated with severe ear microbial dysbiosis. Correction of the underlying disorders had a significant impact on ear recovery.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXVIII (2), 2022
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

A COMPARISON OF ANTIBIOTIC RESISTANCE AND MULTIPLE ANTIBIOTIC RESISTANCE INDEX IN WILD BOARS FROM COVASNA AND CLUJ COUNTIES

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Abstract

Due to the global decline of bacterial antimicrobial sensitivity, international organizations such as Word Health Organization, Food and Agriculture Organization, and World Organization for Animal Health consider it necessary to implement an integrative approach to the fight against antibiotic resistance in the light of the One Health concept. The purpose of the study was to investigate the degree of interchangeability of the antibiotic resistance of the isolated microbiome from wild boars and their living environment. The samples (n=24) were collected from two hunting funds in Covasna and Cluj counties. The identification of microorganisms was performed using the Vitek®2 System and, subsequently, their susceptibility was evaluated using the agar diffusion method. The isolated strains *were identified as Pseudomonas spp., Pseudomonas aeruginosa, Pseudomonas luteola, Brevibacterium spp., Aerococcus viridans, Aeromonas sobria, Campylobacter coli, Campylobacter lari, Staphylococcus aureus, and Streptococcus suis. The highest multiple antibiotic resistance index (MAR 0.666) was identified in strains isolated from Cluj County, while the lowest (MAR 0.111) was obtained in strains isolated from Covasna County. The results of this study further acknowledge the bacterial resistance in wildlife.*

Key words*: multidrug resistance, wild boar, bacterial microbiome, One Health.*

INTRODUCTION

Nowadays antibiotic resistance is a complex global and extraordinarily complicated problem with a major impact on human, animal, and environmental health (Malik & Bhattacharyya 2019; Boyd et al., 2021). The identification of the antimicrobial potential of some substances and their routine administration for the prophylaxis or treatment of some infections have revolutionized modern medicine and often changed the therapeutic paradigm. Thus, substances with antimicrobial potential have become very important in the development of complex medical approaches (Munita & Arias, 2019). Antibiotics are natural substances, produced by microorganisms or artificially synthesized, with selective toxicity to bacteria, so they can destroy the microorganism without affecting the host (Rossiter et al., 2017; Seal et al., 2018). However, excessive and irrational use of these substances has led to the development of antibiotic resistance (Ventola, 2015).

Antibiotic resistance was first observed in 1940, in an *Echerichia coli* strain, resistant to penicillin, later identifying that the penicillinases were responsible for this phenomenon. In 1942 the same phenomenon was observed in four clinical isolates of *S. aureus* (Rammelkamp & Stolzer, 1961; Boyd et al., 2021). The use of antibiotics in human and veterinary medicine as well as in agriculture to prevent disease or as a growth promoter in mammals and birds, together with their use in farmed fish has led to contamination of water and soil with antibiotic-resistant microorganisms. The pattern of antibiotic resistance varies widely between regions and countries and is directly correlated with their degree of use (Sahoo et al., 2012; Manyi-Loh et al., 2018). The phenomenon of antibiotic resistance has long been known, but the role of wildlife as natural reservoirs in this

process is not fully elucidated. However, it is considered that both birds, wild rodents, and mammals in the vicinity of human settlements are potentially coming into contact with household waste and improperly stored faeces may be contaminated from these sources with resistant microorganisms. Due to the large territory, they cross, their role in the distribution of resistant microorganisms must also be considered (Radhouani et al., 2014).

Human density and proximity have a direct influence on the wild environment and vice versa. Thus, the identification of bacteria and the verification of antibiotic resistance in wild animals offers the possibility of monitoring microorganisms with pathogenic potential. Due to the rapid and continuous adaptation of bacteria, the importance of knowing the types of antibiotics, which have low efficacy, is of utmost importance.

The study aimed to investigate the interchangeability degree of the microbiome antibiotic resistance isolated from wild boars as well as their habitat with potential resistant flora to antimicrobials used in farm animal therapy or in the treatment of bacterial diseases in humans and also defining the antibiotic resistance profile (MAR index) of wild boar bacteriome.

MATERIALS AND METHODS

The samples (n=24) were collected from two hunting funds in Covasna and Cluj counties. The identification of bacterial strains was performed by standard microbiological methods adopted from the Clinical and Laboratory Standards Institute (CLSI) guideline. For initial microbiological analyses, the samples were inoculated in nutrient broth and MacConkey agar in aerobic conditions at 37°C for 24 hours. The cultures were repeatedly passed through nutrient agar to obtain isolated colonies. Macroscopic aspects such as colour, size, transparency of the colonies were the basis for their differentiation, followed by repeated microscopic examinations using Gram staining. The identification of microorganisms was performed using the Vitek®2 System. The antimicrobial sensitivity patterns of the isolated strains were evaluated using the standard Kirby-Bauer disk diffusion method according to the CLSI guidelines. The strains were tested towards 9 antimicrobials: amoxicillin/clavulanic acid (AMC, 20/10 µg; Oxoid, UK), doxycycline (DO, 30 µg; Oxoid, UK), enrofloxacin (ENR, 5 µg; KRKA, Slovenia), florfenicol (FFC, 30 µg; Oxoid, UK), penicillin (P, 10UI; Oxoid, UK), oxytetracycline (OT, 30 µg; Oxoid, UK), cephalexin (CEP, 30 µg; Oxoid, UK), neomycin (N, 30 µg; Oxoid, UK), and tylosin (TY, 30 µg; Oxoid, UK). Based on the growth inhibition zone diameters (mm), the bacterial strains were recorded as resistant (R), intermediate (I), and susceptible (S). For further analysis, intermediate and resistant pattern isolates were grouped as resistant. The multiple antibiotic resistance index was recorded according to the procedure described by Krumperman (Krumperman, 1983), so for the calculation of the MAR index, the total number of antibiotics to which the isolate was resistant / the total number of antibiotics tested was considered. According to Krumperman, values lower than 0.2 are considered low risk, while values higher than 0.2 indicate a high risk (Krumperman 1983). For each antibiotic, the MAR index was calculated, interpreted as the number of isolates resistant to the selected antibiotics, divided by the sum of the number of antibiotics used, multiplied by the number of isolates (Tambekar 2006; Pall et al., 2021). Classification of multidrug resistance (MDR) was carried out according to Magiorakos et al. (2012). MDR was considered as the resistance to at least one agent in three or more antimicrobial categories (Magiorakos et al., 2012). The results of the study were analysed with GraphPad Prism 5.00 software (GraphPad Software Inc., La Jolla, CA, USA) and Microsoft Excel. The data were presented as average values, and Excel was used for the graphical construction.

RESULTS AND DISCUSSIONS

Out of 24 (n=24) pharyngeal exudate samples collected from wild boars from Covasna and Cluj counties, 20 different single bacterial colonies were obtained. The isolated strains were identified as *Pseudomonas* spp., *Pseudomonas aeruginosa, Pseudomonas luteola, Brevibacterium* spp., *Aerococcus*

viridans, Aeromonas sobria, Campylobacter coli, Campylobacter lari, Staphylococcus aureus, Streptococcus suis. Most isolated bacteria were *Pseudomonas aeruginosa* (20%), *Pseudomonas luteola*, (15%), *S. aureus* (15%), and *Streptococcus suis* (15%) (Fig. 1).

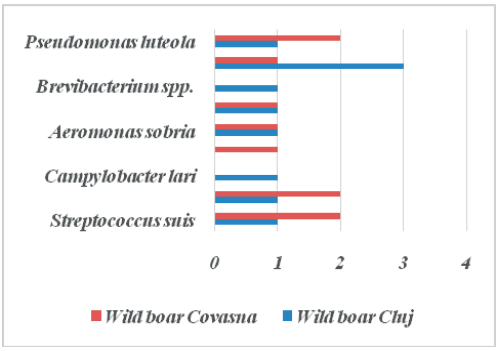

Figure 1. Isolated pathogens form wild boar from Covasna and Cluj counties

Similar to other studies (Costa & Iraola, 2019; Ruiz-Roldán et al., 2020) conducted on samples collected from wild boars, our study identified bacterial species belonging to the genus Pseudomonas such as *P. aeruginosa* and *P. luteola* and the genus Campylobacter namely *Campylobacter coli* and *Campylobacter lari.*
Other bacterial strains, such as *Brevibacterium* spp., *Aerococcus viridans,* and *Aeromonas sobria*, were also isolated. The presence of *Brevibacterium* spp. can be explained by the nutritional habits of wild boars, which often consume insects, birds, and other small mammals, from which strains of *Brevibacterium* spp. are frequently isolated (Giorgio et al., 2018). *Aerococccus viridans* is frequently isolated from domestic animals (Liu et al., 2015; Moreno et al., 2016; Nguyen et al., 2021), therefore the proximity of human settlements explains the identification of this bacterial species. *Aeromonas sobria* was isolated from freshwater, stagnant water, and wastewater (Miyagi et al., 2016), so contamination of wild boars could be achieved by consuming water from these sources (Fernández-Bravo & Figueras, 2020).
The isolated strains were evaluated for their level of antibiotic resistance, the results are indicated in Tables 1 and 2. Of the total isolated strains, 35% showed resistance to amoxicillin/clavulanic acid, penicillin, and tylosin, 40% to tetracyclines, 30% to neomycin, 20% to cephalexin and florfenicol, and 15% to Enrofloxacin.
In the present study, the resistance to Tetracyclines was the highest (40%) followed by penicillin (35%), Amoxicillin/Clavulanic acid, (35%), and Tylosin (35%), lower percentages were observed for Enrofloxacin (15%), Cephalexin and Florfenicol both with 20%. Five of the isolates were susceptible to all tested antimicrobials, and three of the isolates indicated resistance to a single antibiotic.
A MAR index value ≥0.2 was observed in 100% of the resistant pathogens. The MAR index calculated for isolated strains was between 0.33 and 0.88. The highest value of the MAR index was obtained in *P. aeruginosa, S. aureus*, and *Streptococcus suis* strains isolated from both countries. The lowest value of the MAR index was obtained in *P. luteola, Campilobacter coli, A. viridans, A. sobria* strains, predominantly isolated from Cluj County (Table 3). The most obvious similarity is the increased resistance of bacterial strains to antimicrobials such as, amoxicillin/clavulanic acid, penicillin, and tyrosine, which have been used for a long time and excessively in both human and veterinary medicine.

Table 1. The antimicrobial sensitivity patterns of the isolated strains from wild boars from Covasna County

Drugs	*P. luteola*	*P.luteola*	*P. aeruginosa*	*S. suis*	*S. suis*	*C. coli*	*A. sobria*	*A. viridans*	*S. aureus*	*S. aureus*
AMC	R	**S**	R	R	R	S	S	S	S	R
FFC	**S**	S	S	S	R	S	S	S	S	S
CEP	**S**	S	R	S	S	S	S	S	I	I
DO	S	S	S	S	S	S	S	S	S	S
ENR	S	S	S	S	S	I	S	S	S	S
N	R	S	S	S	R	S	S	S	S	I
OT	R	S	S	S	S	S	S	S	S	S
P	S	S	R	R	S	S	S	S	S	R
TY	S	R	R	R	S	S	S	S	S	S

AMC - amoxicillin/clavulanic acid, DO – doxycycline, ENR -enrofloxacin, FFC- florfenicol, P- penicillin, O – oxytetracycline, CEP – cephalexin, N- neomycin, TY- tylosin; R-resistant, I-intermediate, S- sensitive

Table 2. The antimicrobial sensitivity patterns of the isolated strains from wild boars from Cluj County

Drugs	*P. luteola*	*P. aeruginosa*	*P. aeruginosa*	*P. aeruginosa*	*A. viridans*	*A. sobria*	*C. lari*	*Brevibacterium spp.*	*S. aureus*	*S. suis*
AMC	S	R	R	S	S	S	S	S	S	S
FFC	R	R	R	S	S	S	S	S	S	S
CEP	S	S	R	S	S	S	S	S	S	S
DO	S	I	I	I	S	S	S	S	S	S
ENR	I	S	S	S	S	S	I	S	S	S
N	S	S	S	R	S	S	I	S	I	S
OT	S	I	I	I	S	S	S	S	S	I
P	S	R	R	R	S	S	S	S	R	R
TY	R	R	S	R	S	S	S	S	S	R

Table 3. Multiple antibiotic resistance (MAR) index of antibiotics against isolated

Antimicrobial class	Drug	Total number of resistant strains	MAR index of the tested antibiotics
Beta lactamase	Amoxicillin + Clavulanic acid	7	0.7
Cephalosporins	Cephalexin	4	0.44
Fluoroquinolones	Enrofloxacin	3	0.33
Phenicols	Florfenicol	4	0.44
Penicillins	Penicillin	7	0.77
Aminoglycosides	Neomycin	6	0.66
Tetracyclines	Doxycycline Oxytetracycline	8	0.88
Macrolide	Tylosin	7	0.77

At the same time, in the case of samples collected from both counties, low resistance to enrofloxacin was observed. The differences between the results obtained from the two counties appeared for the other antimicrobial substances such as florfenicol, neomycin, and oxytetracycline. These differences can be explained by the irrational use of antibiotics and the induction of different antibiotic resistance depending on the region but also by the natural resistance of some bacterial species to the mechanisms of action of some antimicrobials. Thus, we can state that the groups of antibiotics to which resistance has been identified (beta-lactamase, penicillin, macrolide, tetracyclines, and aminoglycosides) and those with efficacy (phenicols, cephalosporins, and fluoroquinolones) are the same for the isolated bacterial strains from the two counties. The values of the MAR index show a higher level of antibiotic resistance in Cluj County compared to Covasna County. The reason for this result can be correlated with the larger space development of human settlements, which implicitly increases the possibility of contamination with antibiotic residues leading to easier spread in the environment of bacterial strains resistant to antimicrobials. Identifying possible sources of the spread of antimicrobial-resistant bacterial strains in wildlife is of immeasurable importance to public health. Wild animals have a major implication in the biological mechanisms of the spread of antibiotic resistance genes (Radhouani et al., 2014).

The patterns of antibiotic resistance identified in our study indicate that the prevalence of antimicrobial resistance is increased in bacteria isolated from wildlife in the two areas studied. This reflects a high level of exposure of these wild species to antimicrobials, and an increased percentage of resistant bacteria in the areas where these animals live and feed. These results can be interpreted in terms of anthropogenic impact in these areas. The results of the study reflect the presence of antimicrobial-resistant bacterial strains that are considered with the highest priority of critical importance by the World Health Organization (WHO, 2019).

CONCLUSIONS

Continuous monitoring of the prevalence and antimicrobial resistance of bacterial strains isolated from wild boar is important for the

purpose of epidemiological surveillance of different territories. These assessments are also important for public health. Our study confirms the role of wild boars as natural reservoirs of resistant bacterial strains. The value of the MAR index indicates the existence of bacterial species resistant to several groups of antimicrobial substances in the natural environment. The results of the antibiotic resistance pattern together with the MAR index reveal a higher antibiotic resistance level in the samples collected near the developed human settlements.

In order to prevent the spread of antibiotic resistance, rational use of antibiotics is extremely important, only for therapeutic purposes, at the doses and intervals necessary to ensure maximum effectiveness, both in human and veterinary medicine. In order to monitor antibiotic resistance, it is important to periodically investigate bacterial susceptibility on samples collected from the human population, domestic and wild animal populations, and the environment.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXVIII (2), 2022
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

POTENTIAL BIOMARKERS FOR TESTICULAR CANCER IN DOGS – GROUNDWORK FOR INNOVATIVE SCREENING PROGRAMS: A REVIEW

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Abstract

Testicular cancer is a frequently encountered pathological entity, which is distinguished by wide morpho-histological variability. Only recent research efforts were able to highlight, throughout advanced diagnosis techniques, certain biochemical, immunological and hormonal characteristics. In men, testicular cancer is one of the most common malignant oncopathologies, the usually affected age group being 25-35 years. Statistically, the increasing prevalence in humans can be correlated with veterinary in-field reports, an increase in canine testicular cancers being incriminated. Practically, 90% of the tumours with genital localization, in dogs involve the testicular parenchyma, this being the second most common site of neoplastic evolution in intact males. The aim of this paper was to review scientific papers published, regarding potential biomarkers for testicular cancer in dogs such as the anti-Müllerian hormone, insulin-like factor 3, α-fetoprotein, lactate dehydrogenase, c-KIT, CD-30, placental alkaline phosphatase and cytokeratins expression. Furthermore, this paper intends to lay the foundation for further research in order to establish proper screening protocols for testicular cancer similar to those used in human medicine.

Key words*: testicular cancer biomarkers, dogs, anti-Müllerian hormone, insulin-like factor 3, α-fetoprotein.*

INTRODUCTION

Testicular neoplasia has become increasingly common in veterinary medicine (Foster, 2012). The dynamics of oncological cases was multimodally justified taking into account all predisposing factors including environmental changes and genetic traits (Grieco et al., 2008; Nødtvedt et al., 2011). Nine out of ten tumours with genital location in dogs affect the testicular parenchyma (Nødtvedt et al., 2011; Manuali et al., 2020). The high prevalence reported in both human and veterinary medicine backs up the importance of the research in gynaecological oncology field. Due to the availability of advanced diagnostic techniques and thanks to latest technological advances, the heterogenicity of the testicular tumours was studied and cited. Immunological, biochemical and hormonal depiction can contribute to future establishment of screening programs and novel diagnosis methods.

Testicular tumours can be divided into 2 main categories: stromal sex cord tumors and germ cell tumors. In the first category, we include sertolinoma and Leydig cell tumors, the latter being considered the most common testicular tumor in canids, rats and mice (Creasy et al., 2012; Kudo et al., 2019). As for the tumors originating from the germinal epithelium of the seminiferous tubules we include: seminomas, teratomas, embryonal carcinoma (EC) and yolk sac tumours. In addition, histological findings showed subsequent evolution of mixed tumours and also primary tumors without specificity for the testicular parenchyma (Yu et al., 2009).

A retrospective study on the prevalence of testicular tumors which gathered 476 cases in total showed a detection rate of 16.8% (80/476) for neoplasia in male dogs, 94.1% (80/85) being localized in the genital area. The calculated prevalence for each tumor subtype in this case was: 34.4% seminoma, 26% interstitial cell tumors, 22.9% mixed germ cell and stromal tumors and 16.6% tumors with Sertoli cells (Liao et al., 2009). Research in certain geographical areas has shown similar data. In central Italy, Umbria region, 1969 individuals developed some kind of tumors. Out of the total, 388 (6.42%) had testicular

localization, the histopathological diagnosis incriminating a more frequent evolution of interstitial cell tumors, 50% of the total, 194 cases, respectively. Most of the cases presented a unique tumoral subtype (82.5%), only 63 males being diagnosed with mixed cell tumors. (Manuali et al., 2020).

Nascimento et. al. (2020) in the mass study which gathered 3,323 biopsies from male dogs, during a 19 years period, revealed a prevalence of 11.2% for testicular neoplasia. Seminomas were most commonly involved (40% rate), Leydig cell tumors (29.1%), Sertoli cell tumors (27.7%) and only 3.2% rate for mixed germ cell-sex cord stromal tumors.

The constant noted in both veterinary and human medicine studies is the relation between cryptorchidism and testicular neoplasms. The retained testes are prone to develop sertolinomas due to the higher temperature, which will lead to the destruction of all cells except Sertoli cells. This statement is backed up by several research papers, which focused on the depiction of testes tumors found in dog populations all over the world (Nascimento et. al. 2020; Gazin et al. 2022). Actually, the least diagnosed tumor in cryptorchid testes was the interstitial cell tumor, according to Gazin et al. (2022) and Liao et al. (2009).

According to some authors, the inguinal retention offers an intermediate thermal regime, between the abdominal and the intrascrotal one, predisposing to the development of seminomas (Ciaputa et al., 2012).

Studies on the molecularity and immunohistochemical features have highlighted the importance of discovering new markers for early diagnosis of testicular cancer. Cytokeratin, c-KIT, CD30, epithelial membrane antigen, α-inhibin, and (placental alkaline phosphatase) PLAP are some of the markers commonly researched or used, in human medicine, for the diagnosis of testicular tumors (Yu et al., 2009). Another example would be the evaluation of the expression of the Ki-67 gene to establish the character of malignancy, based on the relationship between Ki-67 and the number of mitotically active cells. In veterinary medicine, it could be considered a marker for metastatic evolution and it was used in order to assess the prognosis of different oncological cases. Ki-67 discriminated malignant from benign mammary gland tumors in bitches (Kudo et al., 2019).

Serum biomarkers such as α-fetoprotein (AFP), human chorionic gonadotrophin (hCG) or lactate dehydrogenase (LDH) are often included in staging and prognostic evaluation protocols, being used as screening factors for testicular neoplasms in men (Leão et al., 2020). Another possible biomarker could be the antimüllerian hormone (AMH) for which there is preliminary data on its applicability in the diagnosis of cryptorchidism (Walter, 2020). Also, establishing variations in insulin-like 3 (INSL 3) in patients with Leydig cell tumors could represent a non-invasive serum marker to be used in the diagnosis of testicular pathologies.

Adapting these markers for veterinary medicine can allow us to formulate the premises for innovative screening programs for testicular neoplasia in canids.

Therefore, the purpose of this work was to review the latest data extracted from research papers aiming to assess the potential biomarkers with a defined role in the early diagnosis and prognosis of testicular tumors. Thus, this research is based on the analysis of the current information related to the previously studied biomarkers. Topics such as the role of AMH for the diagnosis of sertolinomas and the serum INSL 3 levels in Leydig cell tumor cases will be subjects to debate. In addition, the differential diagnosis of non-seminomatous tumors based on AFP and LDH, the distinction between classic seminoma from spermatocytic seminomas based on C KIT and PLAP and the use of CD 30 as a high specificity biomarker will also be discussed in the present subchapters.

ANTIMULERIAN HORMONE AND SERTOLINOMAS

AMH is a glycoprotein produced by Sertoli cells in males and by granular follicular cells in females. Its primary role is to arrest the development of the Müllerian ducts in male embryogenesis, in females these ducts represent the origin of the external ovarian epithelium, salpinx, uterus, cervix and cranial portion of the vagina (Walter, 2020).

AMH secretory dynamics also differ between sexes. Ovarian granulosa cells will produce

small amounts of AMH until puberty. This will inhibit aromatase activity and will reduce the ability of androgens to convert to oestrogens. Having a well-established role in the ovarian follicle formation, its concentrations will be higher in women with polycystic ovaries and lower during menopause. (Dólleman et al., 2014; Hagen et al., 2014; Walter, 2020). In human medicine it is actively used to assess oocyte reserve (Sahmay et al., 2014), being also an important tool in the case of premature birth prevention (Stegmann et al., 2015). It was also used for the evaluation of ovarian function postoperatively, after chemotherapy (Lind et al., 2015), and may also assess the adverse effects of endocrine disorders, such as hypothyroidism, on the ovarian reserve (Kuroda et al., 2015). In men, AMH values were assessed for the diagnosis of testicular atrophy, Sertoli cell tumors or to evaluate possible sex development issues (Walter, 2020).

AMH being the specific protein most rapidly expressed by Sertoli cells, allows us to detect it even in foetuses or puppies up to 45 days of age (Banco et al., 2012). As sexual maturity is reached, serum values decrease. Therefore, high AMH levels in an adult can reveal the development of sertolinoma.

The use of immuno-enzymatic kits, in patients with Sertoli tumor both preoperatively and postoperatively, highlighted the dynamics of this hormone. Preoperatively, the recorded AMH values were much higher than those obtained from dogs without testicular pathologies, compared to those of the same post-orchiectomy patients (Ano et al., 2014).

A study, which involved 20 dogs with testicular masses, tried to establish the reference values for AMH, by comparison to a control group. Thus, they set the level of 10 ng/ml as the physiological maximum for intact dogs without testicular pathology, stating that patients with sertolinoma or mixed tumors had AMH values > 22 mg/ml (Holst et al., 2015).

Advanced immunohistochemical research highlighted the potential of AMH in the diagnosis of several testicular pathologies. By collecting samples from foetuses, new-borns, puppies aged between 43 and 180 days, 6 adult dogs and 24 dogs with sertolinoma, it was possible to highlight the degree of expression of AMH genes according to age, development stage, and presence or absence of testicular masses. Thus, AMH was expressed in the cytoplasm of Sertoli cells in both foetuses and new-borns, the percentage of labelled cells being 71-100%. Individuals up to 120 days were also intensely positive, with AMH expression becoming absent in dogs between 120 and 180 days. The adults included in the study did not express AMH in Sertoli cells, unless they had Sertoli cells tumors. Since the results for AMH expression are extremely variable between age groups, the relation between intense production of this glycoprotein and certain stages of cell differentiation can be considered. However, the potential of AMH in the diagnosis of Sertoli cell tumors has been formulated, detailed research on larger groups of individuals remains necessary (Banco et al., 2012).

INSL 3 IN LEYDIG CELL TUMORS

INSL 3, a peptide from the relaxin family, is one of the most innovative markers described in both human and veterinary medicine (Rossato et al., 2011). INSL 3's potentially high specificity for the diagnosis of Leydig cell tumors is motivated by the origin of this molecule. This peptide is produced exclusively by Leydig cells within the testes. This fact supports the premises for high value screening protocols focused around INSL 3 serum concentrations. The applicability of INSL 3 has also been stated for early diagnosis of canine cryptorchidism (Hannan et al., 2015), but due to its characteristic physiology, further research is considered necessary. However, the diagnostic value of this hormone has not been yet fully elucidated.

INSL 3 has been characterized as a reliable indicator of Leydig cell functionality. Unlike testosterone, INSL 3 is produced constantly without proving a pulsating release pattern. It is not influenced by other hormonal factors and it is not a subject of the hypothalamic-pituitary-gonadal axis regulation. Small differences between the expression capacity of INSL 3 in the testicular tissue and its serum values were cited, due to its independence related to the modulating mechanisms already mentioned. Practically, INSL 3, once produced by active

Leydig cells, will be directly released into the tissues and bloodstream (Ivell et al., 2013).
Currently, there is a lack of data on the direct association of INSL 3 values and testicular tumor pathology. Gene expression patterns for INSL 3 have been studied throughout immunohistochemistry in human medicine. In a study that included individuals with benign and malignant forms of Leydig cell tumors, it was shown that INSL 3 was expressed in all tissue samples collected from diagnosed leydigomas. However, no differences in peptide expression rates were observed in malignant samples compared to those with benign tumors. The authors note the potential of this marker and point out the need to establish correlations between serum values of INSL 3 and various testicular pathologies (Rossato et al., 2011).

POTENTIAL MARKERS FOR NON-SEMINOMATOUS TUMORS

In human medicine, the markers intensively used in the diagnosis and screening of testicular tumors are represented by: hCG, AFP and lactate dehydrogenase. They are considered to have satisfactory specificity and sensitivity, which is why they are often used not only to identify an ongoing neoplastic process, but also to monitor therapeutic success. The 3 stated markers are expressed in 60-80% of non-seminomatous tumors. The techniques involved also have another advantage related to their lack of invasivity (Pedrazzoli et al., 2021).
LDH is a glycolytic enzyme present in all tissues, but mostly in the muscles, brain and liver. Its widespread distribution in the body causes modified LDH values to produce many false positive results (Liao et al., 2009). LDH levels change in many conditions such as: pulmonary thromboembolism, muscle damage, myocardial infarction, thalassemia or haemolysis. These aspects underline the importance of interpreting the obtained results in a holistic manner, taking into account all clinical aspects and other objective parameters. However, according to the meta-analysis provided by the literature, elevated LDH values occur in 40-60% of cases of testicular tumors (Liao et al. 2009; Pedrazzoli et al., 2021).
Unlike hCG, which cannot be extrapolated to veterinary medicine for this purpose, AFP has the potential to be included in diagnosis protocols for testicular tumors (Pedrazzoli et al., 2021). AFP has been previously used in canids to detect multiple liver diseases and hepatic carcinoma (Yamada et al., 1999). AFP is an oncofoetal protein expressed in the embryonic sac, gastrointestinal tract and liver. Its role in the human body is not fully understood. However, reported data shows that in non-seminomatous tumors, it becomes detectable serologically, while in seminoma cases it is not produced (Pedrazzoli et al., 2021). Statistically, 60-70% of non-seminomatous tumors in men were correlated with serum detection of AFP (Pedrazzoli et al., 2021).
Although they do not excel in terms of specificity or sensitivity, they can be useful tools in the diagnosis of testicular tumors, the main advantage being their lack of invasivity.

C KIT AND PLAP – HIGHLY SENSITIVE MARKER USED FOR DIFFERENTIATING CLASSIC AND SPERMATOCYTIC SEMINOMA

The immunohistochemical technique is frequently used in human medicine in order to differentiate tumoral subtypes, as it is also a viable technique for testicular tumors diagnosis. Various established markers have recently been proposed for insertion in novel diagnosis protocols in the veterinary field. c-KIT is a proto-oncogene responsible for coding the tyrosine kinase receptor KIT, a transmembrane receptor. The before mentioned receptor is found in many different cell types, including germ cells, Purkinje cells from the cerebellum, precursors of the hematopoietic cells and melanocytes (Webster et al. 2006; Lennartsson et al. 2012; Gil da Costa et al., 2015).
The primordial germ cells will express KIT, followed by the progressive migration and proliferation until they reach the embryonic testicular tissue where they interact with the Sertoli cells expressing stem cell factor (SCF), which will condition their differentiation until gonocyte stage. The KIT – SCF interaction is essential for cellular differentiation and maturation processes (Grieco et al. 2010).
Right before birth, gonocytes will evolve to the pre-spermatogonia stage. Subsequently, during

puberty and adult life, KIT – SCF interactions will directly induce progressive differentiation towards the spermatogonia stage.
In males, KIT is present in Leydig cells as well, possibly playing a modulator role in the testosterone synthesis (Grieco et al., 2010). Unlike other markers present in the early stages of spermatogenesis, such as PLAP, which is only detected until prespermatogonia stage, KIT's expression is maintained for a longer period of time, along the different maturing stages (Rajpert-De Meyts et al, 2003; Grieco et al., 2010). Both markers are used for the immunohistochemical analysis of seminoma in males, c-KIT and PLAP's simoultaneous immunoreactivity being characteristic for this kind of testicular tumor. (Stoop et al., 2008).
Both markers, c-KIT and PLAP are often included in human medicine protocols in order to differentiate classic seminoma from spermatocytic seminoma. Grieco et al. (2010) showed possible similarities with human medicine research results, namely that the simultaneously expression of KIT and PLAP concurs with classic seminoma diagnosis. It was also highlighted that both markers have high specificity, KIT not being expressed by the Sertoli cells in neither cases, being identified exclusively in Leydig cells and spermatogonia (Grieco et al., 2010).
Although they have depicted the dynamics of the mechanism through which PLAP and KIT are expressed, the latter being indentified even after the primary stage of differentiation of the germ cell, at which point PLAP diminishes, Hohšteter et al. (2014) backed a different hypothesis from that of Bush et al. (2011) and Thorvaldsen et al. (2012). In the study conducted on 52 canids he was able to demonstrate, by determining the expression of KIT and PLAP through immunohistochemistry, that the majority of seminoma cases in dogs are classical seminoma, resembling the incidence reported in men, opposing Thorvaldsen and Bush's statement on the predominance spermatocytic seminoma.
Yu et al. (2009) tested the applicability of multiple tumoral markers, including PLAP and c-KIT on a number of 35 individuals diagnosed with seminoma and sertolinoma. In this case, it was concluded that c-KIT remains a highly sensitive marker for seminoma diagnosis, PLAP being unable to highlight the development of this specific tumoral type. On the other hand, in some sertolinoma cases included in the study PLAP showed increased reactivity.
Markers such as PLAP and c-KIT offer unexploited potential, possibly being able to identify the tumor type and consequently indicate the proper diagnosis and therapeutic protocol. Unfortunately, the insufficient data in the veterinary medicine field does not allow the precise establishment of protocols that include advanced immunohistochemical determinations centered around these two factors.
Even so, the possible implications in the oncologic diagnostic remain under debate, more information being needed in order to accurately assess the applicability, sensibility and specificity of the two factors.

CD 30 - HIGH SPECIFICITY MARKER

CD 30 is a glycoprotein integrated in the tumor necrosis factor superfamily. Its main site for expression is considered to be the surface cells of EC. One of the proposed applications for this marker was to differentiate seminomas from EC (Leroy et al., 2002). However, CD 30's potential asks for more research based on other possible physio-immunological aspects that can indicate other medical involvements.
CD 30's expression has been highlighted in a series of cells with certain malignant characteristics such as cells extracted from highly anaplastic lymphomas or Hodgkin's lymphoma (van der Weyden et al., 2017).
The expression dynamics of this possible valuable immunohistochemical marker is explained by the defined reactivity sites. Some certain activated B and T lymphocytes are the main actors in CD 30 expression. Thus, it could be shown that for certain cases of lymphoma, their expression in the incriminated cells becomes defining for diagnosis (van der Weyden et al., 2017).
Gopalan et al. (2009), during their stem cell markers research have proved that CD 30 could be a useful tool for testicular mixed germ cell tumor diagnosis. Their findings showed that 98% of the EC were positive for CD 30 expression, with some staining variations that need to be further investigated.

Unlike other markers, CD 30 can be used also as an indicator for therapeutic success. This fact has been showed by Albany et al. (2018) during their clinical trial on human patients with CD 30 expressing germ cell tumors and stromal cord tumors.

CD 30 has also been considered a good prognostic factor. Human patients with CD 30 ‑ expressing EC have worse progression ‑ free survival rates and overall survival rates than those with CD 30 ‑ negative tumors (Albany et al., 2018).

Considering the premises based on CD30 characteristic overexpression in some tumoral subtypes, it can be stated that the incriminated component can work as both a diagnosis and therapeutic target (van der Weyden et al., 2017).

Veterinary research efforts have not focused so much on the study of this biomarker. One of the few studies on testicular tumors that included immunohistochemical testing of CD 30 showed its poor rate of expression in both classical and spermatocytic seminoma. Backing up Leroy's suggestions, Yu et al. (2009) pointed again the role in differential diagnosis between seminomas and EC, stating that CD 30 was not expressed by any tissue sample originating from either classic or spermatocytic seminoma.

In fact, the link between CD 30 and EC, already being established in the literature, allowed the authors to suggest that the low expression of CD 30 in a study group demonstrates the low incidence of this tumor type. Hohšteter et al. (2014), obtained low or absent reactivity for CD 30 in dogs. He motivated his findings by not identifying any EC within the individuals included. Judging by its high specificity, the low positivity encountered in some seminomas, was explained by the possible abnormal transformation of the neoplastic cells into EC. This cellular drift has been previously described in human medicine (Hitmair et al., 1996).

According to Yu et al. (2009) CD 30 was not identified in Sertoli cell tumors either, which is consistent with the idea that CD 30 is highly specific for EC.

CONCLUSIONS

Given the many possible applications in the diagnosis, prognosis and even monitoring of the therapeutic success in testicular tumors, the value of these markers needs to be emphasized. Extrapolating these markers from human medicine may help establish new screening protocols for animal testicular oncopathologies. Corroborating the ascending trend and the high values of prevalence noted for testicular neoplasms in veterinary medicine, we can state the importance of the continuous research efforts focused on this subject.

Therefore, this area requires extensive research with a consequent increase in data that veterinary medicine has on tumor markers and their possible implications.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXVIII (2), 2022
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

CHANGES OF METABOLIC LIVER PARAMETERS ASSOCIATED WITH GENERAL ANESTHESIA IN DOGS AND CATS – A REVIEW

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Abstract

The aim of this systematic review is to examine the effects of different anesthetic drugs and protocols on hepatic functions. The liver is a very important organ since it is directly involved in biotransformation of the drugs used in anesthesia. Comprehensive understanding of anesthetic drugs and their effects on hepatic functions remains fundamental to a successful anesthesia. Understanding the connections that exist between anesthetic drugs and liver function remains essential for a safe anesthesia both during the surgery and in the post-operative period. Almost all anesthetic drugs depend on hepatic biotransformation into their metabolites that can be transported and excreted by the kidneys. If the liver enzymes are altered, the biotransformation of the drugs will be decreased and the recovery from the anesthesia prolonged

Key words*: anesthesia, biotransformation, enzymes, liver.*

INTRODUCTION

Through this study, the effects of different anesthetic drugs and protocols on liver function will be described.

The liver is located between the diaphragm and the abdominal viscera. The blood that arrives from the gastrointestinal tract is full of proteins, carbohydrates, fat and other exogenous particles (drugs, bacteria). From the total cardiac output, 25-30% flows through the liver via dual blood supply: the hepatic artery and the portal vein. The main function of the liver is to participate to nutrients digestion by breaking them down to more absorbable substances. The other functions of the liver that are more important for the anesthesist are the homeostasis of glucose, production of proteins needed for coagulation, production of albumin and other proteins needed to maintain the plasma oncotic pressure, biotransformation and excretion of drugs and ammonia removal and blood urea nitrogen (BUN) production (Tranquilli, 2013; Dugdale 2020). The functional unit of the liver is the hepatic lobule. These lobules are hexagonally shaped and are organised around the central vein. Histologically, the hepatocytes can be divided into three different areas based on their localisation. First area is located at the periphery, closer to the portal canals and arterial blood vessels. The hepatocytes in zone one receives the highest amount of oxygen and also is the area where most oxidative processes occur. Zone two is a transitional zone between zone one and zone three. Zone three is closer to the central vein, hence the hepatocytes receive less amount of oxygen. These hepatocytes contain a large number of organelles responsible for drug deactivation and metabolism (Pawlina, 2018).

BIOTRANSFORMATION AND ELIMINATION OF XENOBITICS

Xenobiotic is a general term used to describe a chemical substance that is foreign to the animal body. There are a lot of substances that can be included here, such as drugs, pesticides, food additives (Patterson et al., 2010). The enzymatic system that is involved in the biotransformation of drugs is mainly localized in the liver, although there are also other organs capable of biotransformation. The majority of anesthetic drugs are removed from the blood by

hepatocytes and excreted in the bile or urine. Drugs usually undergo a two-phase process in order to became an easily excreted substance. In phase one, the drugs undergo an oxidation reaction so that one or more hydroxyl groups are attached to the molecule. By doing this, the more lipophilic compounds are converted intro hydrophilic compounds so that they can be excreted via bile or urine. The main class of enzymes responsive for these reactions belongs to the family of cytochrome P450 monooxygenases (Reece et al., 2015). Ketamine has a reversible and competitive influence on the CYP 3A family, but also these effects may be due to the metabolite, norketamine (Meneguz et al.,1999). Because there are a lot of enzymes in the P450 family, it is very important to know which enzyme reacts with different drugs. In one investigation, the metabolism of ropivacaine was studied. In general, local anesthetics are administrated with other agents, including general anesthesia. When there are metabolized by the same P450, some agents may influence the plasma concentration of ropivacaine (Oda et al.,1995). Differences between CYP 450 exist not only among species, but also among breeds and genders. One drug take into consideration is propofol. Greyhounds have a slower drug clearance and longer recovery compared with any other breeds. This may be to the fact that the enzyme activity are almost three times lower in greyhounds compared with beagle microsomes (Hay Kraus et al., 2000). Comparing the activity of CYP 450 in cats and dogs shown that cats have a significant lower activity compared to the dogs (Van Beusekom et al., 2010). Phase two reaction is responsible for making the molecule more water-soluble the facilitate the excretion. This reaction is obtained through conjugation to a glucuronide or sulfate molecule. These reactions occur mostly in the cytosol (Reece et al., 2015). The glucose homeostasis is a complex process that is maintained by different organs such as the pancreatic islet cells, liver and peripheral tissues. Glycogen is the form of glucose that is stored in the muscle and liver cells. It is synthesized when the blood glucose levels are high and serves as a source of glucose for the body when the levels of blood glucose are low. Glycogenesis is the process of glycogen synthesis while glycogenolysis is the process in which the glycogen is transformed into glucose. Abnormalities in these processes can lead to hypoglycaemia, a common sign of severe hepatic disfunction Hypoglycaemia can also be iatrogenic induced by indicating the owners to withdraw water and food before anesthesia for a longer period that necessary in neonatal/juvenile patients. Elevated liver enzymes are the sometimes the first sign of hepatobiliary disease. The typical biochemical panel evaluates the following enzymes: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatise (ALP) and gamma- glutamyl transpeptidase (GGT). Based on their localisation, these enzymes are classified in “hepatocellular leakage enzymes” (ALT and AST) and “cholestatic enzymes” (ALP and GGT). If plasma membrane damages are present due to hepatic inflammation, the enzymes leak into the perisinusoidal space and then into the systemic circulation, causing elevation of ALT and AST. An elevated ALP is one of the most common reported abnormalities in dogs. These enzymes have a high sensibility but a low specificity to hepatic disease. If there is also a concurrent increase of GGT, the changes of an existing hepatic disease or cholestasis increase (Center, 1992; Comazzi, 2004). Changes in this parameters can appear after general anesthesia, but some factors must be taken into consideration: age of the patient, duration and type of the surgery (Turcu et al., 2021). The liver is the place where several coagulation factors, such as fibirnogen, prothormbin, factors V, VII, IX, X, XI, XII and XIII, are synthesized or activated (Weiss et al, 2011). The amount of urea nitrogen in the blood is indicated by the BUN value. The ammonia that results from protein catabolism from the gastrointestinal tract is carried to the hepatocytes through the portal circulation where is metabolized to urea through the urea cycle. Then, the blood urea nitrogen is filtered at the glomerulus and excreted by the kidneys. A decrease in BUN may indicate a hepatic dysfunction or the fact that the blood is shunting the liver. This condition is called portosystemic shunt (PSS). In patients affected by PSS hyperammonaemia occurs. Although there are many toxins involved in the pathogenesis of hepatic encephalopathy (HE), ammonia is the only factor that can be

measures, so the most important. (Ettinger, 2010; Tivers, 2014)

PHARMACOLOGICAL AGENTS USED FOR GENERAL ANESTHESIA

Phenotiazines- Acepromazine is the most widely used phenothiazine agent in veterinary medicine due to its sedative and anxiolytic effects. Note that acepromazine has no analgesic properties and should always be associated with other agents that have these properties. It is metabolised in the liver and excreted in the urine (Clarke et al, 2013). No antagonist is available, as such caution must be taken when using acepromazine in patients with hepatobiliary disease (Johson et al., 2022). One of the major side effects of acepromazine is a dose related fall in the arterial blood pressure due to the vasodilatation. Hypothermia is also a common side effect due to the vasodilatation.

Benzodiazepines are another class of substances that are used in anesthesia. Drugs of this group are used to provide antianxiety action, sedation, *muscle* relaxation, anticonvulsant effects. Benzodiazepines exert their sedative effect by depression of the limbic system and also act at the GABA (gamma-aminobutyric acid) receptors throughout the central nervous system. GABA is an inhibitor neurotransmitter and by acting at this level, benzodiazepines inhibit the transmitter of the neuronal potential (Olkkol et al, 2008). Flumazenil, a competitive antagonist, is available if profound or prolonged sedation is present. Midazolam and diazepam have minimally adverse effects on cardiovascular system and is well tolerated by both healthy and sick animals. However, there are differences between the 2 agents: midazolam is water-soluble and can be administered by intramuscular injection with less local irritation (Jones et al., 1979). One study revealed fulminant hepatic necrosis after oral administration of diazepam in cats. Clinical, the cats presented with lethargy, anorexia and became jaundiced. Biochemical tests showed increased in ALT and AST (Center et al., 1996). The are many pathologies that affect the liver, from mild elevation of the enzymes, to fulminant liver failure. However, many liver conditions require sedation or general anesthesia for surgical interventions. These interventions can vary from liver biopsy to surgical repair of portal venous shunt (Johnson et al., 2022).

The first *α_2 adrenoreceptor agonist* used since 1968 is xylazine, but since then, new potent and highly selective α_2 agonists have been developed. These new molecules are detomidine, medetomidine, dexmedetomidine and romifidine. Most α_2 adrenoreceptor agonists are metabolized by the liver and excreted by the kidneys. The main effects of these substances are sedation and analgesia. The sedation is dose related, but when the sedation reaches the maximum effect, increasing the dose will only increase the duration of the sedation. In combination with an opioid, they produce deep sedation and decrease the dose of inhalant anesthetic. They also produce analgesia through both spinal and central action (Khan et al 1999; Murrell, 2005). The major side effects of the α_2 adrenoreceptor agonists are on the cardiovascular system. In all species they produce profound bradycardia due to the suppression of the cardiac centre and mediated through the vague nerve. However, most studies demonstrated that hepatic blood flow is well-maintained (Johnson et al., 2022).

Opioids are powerful drugs used for pain management. In the central nervous system there are 3 types of receptors: mu, delta and kappa. Based on the affinity of the opioids to those receptors, these can be divided as following:

- Agonist drugs: they have a high affinity to the mu receptors. In this category we can include: morphine, fentanyl, methadone, hydromorphone.
- Partial agonist drugs: they do not have a full affinity to the mu receptors. Here we care include buprenorphine.
- Mixed agonist-antagonist: they act as an agonist to some receptors and as antagonist to other receptors. Butorphanol is the opioid included in this category.
- Antagonist: can reverse the effects of both mu and kappa agonists. Here we can include naloxone (Duke-Novakovski et al, 2016).

The main organ that helps to metabolise the opioids is the liver. After the liver metabolism, the opioids enter the systemic circulation.

The 2 main enzymatic systems that help the opioid metabolism are P450-CYP 450 and UDP (UGT)- glucuronosyltansferases (Mercadante, 2015). Among opioids, remifentanil is a very unique substance. Remifentanil is a potent synthetic mu agonist. Compared to fentanyl, remifentanil is an ultrashort acting opioid, with rapid control of the depth of the anesthesia. It does not accumulate in the body even after a prolong infusion, and has a half-life less than 6 minutes. Considering these properties, it is an excellent choice for total intravenous venous anesthesia (Mercadante, 2015). Like all other opioids, remifentanil shares the same pharmacodynamic properties: dose-related analgesia, central nervous system, respiratory and cardiac depression. Unlike other full mu agonist opioids, remifentanil don not cause histamine release. What makes this opioid special is the ester linkage, making it susceptible to metabolism by hydrolysis by the esterases in the tissues and blood. The pharmacokinetic properties of remifentanil are independent of hepatic and renal function (Stroumpos et al, 2010).

Ketamine is a dissociative anesthetic that produce the dissociative anesthesia. This state is characterized by a dissociation of the thalamocortical and *limbic* system that cause a change of the awareness (Tranquilli et al., 2013). Ketamine is a N-methyl-D-aspartate (NMDA) antagonist, which means that ketamine has analgesic properties. Unlike benzodiazepines, it does not have action on GABA receptors, hence there is no hypnotic effect (Clarke et al, 2013). Hydroxylation and conjugation are the 2 main paths on which ketamine is metabolised. One of the metabolites, norketamine, is also active, this being the reason for the prolong effects (Bettschart-Wolfensberger et al., 1996).

Inhalation agents are widely use in veterinary medicine and very well tolerated by the animals because these substances are removed from the body via lungs. For a safely delivery of the inhalation agent, a special machine is required (Tranquilli et al., 2013). Historically speaking, during the years there were a lot of substances that were used for the purpose anesthesia. In our days, the primarily inhalation agents used in veterinary medicine are isoflurane and sevoflurane. Although the lungs are the main organ on which they act, studies demonstrated that there some degree of hepatotoxicity associated with inhalation agents.

Halothane was the most commonly known anesthetic agent. Developed in 1956, rapidly become one of the most used volatile agents (Safari et al., 2014). In 1969, the National Institutes of Health developed a study on 250 000 cases of halothane administration. This study was designed to examine the possible association of halothane and anesthesia and postoperative massive hepatic necrosis in 34 hospitals throughout a period of 4 years (Moses et al, 1968). There are 2 types of liver reactions associated with halothane administration: the first type 1, mild hepatitis, associated with elevation of liver enzymes (AST and ALT). the values remain elevated for a period of 2 weeks and then resole without treatment (Dabbagh, 2011; Safari et al, 2014). The second form of hepatic injury associated with halothane exposure was severe hepatitis with massive hepatic necrosis. (Safari et al., 2014). The characteristic manifestations of this form are progressive jaundice, hepatic coma and shrinkage of the liver. The etiology of this rare condition has a lot of prone factors, including other drug administration (Trey et al., 1968). After halothane, more modern volatile agents were introduced, including enflurane, isoflurane, desflurane and sevoflurane. A number of studies have demonstrated the effect of inhalant agents on the liver and hepatic function. For example, a case of fulminant hepatic necrosis was observed after inhalator anesthesia with sevoflurane. Right after surgery, no signs were observed, but 20 hours post-operative, the hepatic enzymes were strongly increased and the patient become jaundice. During the next period of time, the patient progressively developed severe hepatic insufficiency, with renal, respiratory and cardiocirculatory failure. Followed all the exams performed post-mortem, the cause of death may be related with sevoflurane exposure (Turillazzi et al., 2007). Although severe hepatic failure was observed after sevoflurane exposure, no apparent renal effects were observed after long duration low flow of sevoflurane or isoflurane (Kharasch et al., 2001). To decrease the minimal alveolar

concentration (MAC) of inhalation agents, different agents were used as bolus or as continuous rate infusion (CRI). One study was conducted to determine how the combination of morphine and cannabidiol (CBD) influence the sevoflurane MAC. The results of the study showed that CBD alone did not reduce MAC sevoflurane and did not enhance the MAC sparring effect associated with morphine used (Orden et al., 2021). A similar study was conducted by Akashi in 2020 on a group of six healthy dogs. The study evaluated the sevoflurane MAC sparring effect after an CRI of dexmedetomidine and/or remifentanil. By administrating this combination, the requirement of sevoflurane administration was significantly reduced (Akashi et al, 2020).

In recent years, there was a growing interest for experimental administrating the halogenated anesthetics intravenously (IV). The major advantage of administrating the volatile agents IV was that the anesthesia was induced faster than administrating through the lungs (Eger et al, 1995). Tests conducted on 15% isoflurane lipid nanoemulsion for general anesthesia showed that there was no evidence of acute renal or hepatic injury. The selected laboratory tests (creatine-kinase, creatinine, BUN and ALT) remained within normal limits 90 minutes after the infusion (Natalini et al., 2017). The goal of another study was to evaluate the renal and hepatic function when 8% sevoflurane lipidic emulsion was administrated intravenously compared to inhaled sevoflurane anesthesia. The following parameters were measured before and after the anesthesia: AST, ALT, lactate dehydrogenase, alkaline phosphatase, total bilirubin and gamma-glutamyl transferase). After all the date were analyzed, the conclusion was that there were no significant differences between the data (Natalini et al., 2016).

CONCLUSIONS

It is very important to create an anesthetic protocol specially designed for the patient. Knowing how each substance can change the liver parameters can help develop a tailored protocol for each patient.

Every substance depends more or less on the hepatic metabolism and the side effect of each substance needs to be well known. Phenothiazines are metabolized in the liver, but because its prolong effects after administration, must be used with caution. Benzodiazepines and opioids rely heavily on liver metabolism. A unique and appropriate opioid that can be used in patients with severe hepatic failure is remifentanil because it is not metabolised by the liver.

α_2 adrenoreceptor agonists do not directly affect the liver, but can cause damage through the cardiovascular effects.

The inhalant agents, isoflurane and sevoflurane, rely on lungs for metabolism and excretion, but some degree of hepatic injury was observed. In case of halothane, the degree of hepatic lesions was far more severe.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXVIII (2), 2022
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

ASSESSMENT OF THE ANTIBIOTIC RESISTANCE PROFILE IN MASTITIC MILK OF DAIRY COWS, DEPENDENT ON THERAPY AND CLINICAL CONDITION

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Abstract

Mastitis remains a major challenge for the global dairy industry, despite the widespread implementation of control strategies. Escherichia coli and Streptococcus uberis are the most common agents of mastitis in cattle and a significant problem for the dairy industry. The aim of this study was to establish the prevalence of bacteria involved in mastitis of dairy cows and to establish the antibiotic resistance profile, which frequently complicates the therapy. In order to achieve these objectives, samples (n=25) were collected from cattle diagnosed with clinical mastitis and subclinical mastitis detected by biochemical assays. The samples were processed by standard microbiological methods and the results indicated an increased prevalence of Staphylococcus spp. and of microorganisms of the Enterobacteriaceae family. Isolated bacterial strains have shown significant resistance to antibiotics, especially to amoxicillin, while ciprofloxacin has proven the most effective. Thus, the early detection and correct treatment of clinical and subclinical mastitis is an important challenge for the economy and for the public health.

Key words*: mastitis, E. coli, antimicrobial resistance, dairy cows.*

INTRODUCTION

Mastitis is one of the most common disease of dairy cattle associated with significant economic losses. With the advent of improved breeds and intensive breeding systems, the incidence of subclinical and clinical mastitis has increased. Cows' mastitis, as inflammatory processes of the mammary gland, are produced by different causative agents, in most cases they are microbial agents, that can be classified into three groups: specific microorganisms that cause exclusively mastitis (*Sreptococcus agalactiae*, etc.); microorganisms that affect the whole body and can also be located in the mammary gland (*Mycobacterium bovis, Mycobacterium tuberculosis, Brucella abortus, Echerichia coli*, *Pasteurella multocida*, *Micoplasma* spp., etc.); and occasional pathogens that can cause accidental mastitis (*Corynebacterium pyogenes, Pseudomonas aeruginosa Staphylococcus epidermidis*, *Staphylococcus aureus, Actinomyces* spp., *Arcanobacterium pyogenes, Proteus* sp., *Prototheca* spp., *various yeasts,* etc.) (Dworecka-Kaszak et al., 2012; Cheng and Hang 2020). The incidence of the isolation of different bacterial species involved in the etiology of mastitis is varied and depending on several factors, among which the conditions of exploitation, the applied therapy and the physiological period of the mammary gland are of particular importance (Quinn et al., 2015; Lakew et al., 2019). Numerous observations have shown that the lactating mammary gland is more susceptible to *Streptococcus agalactiae* and *Staphylococcus aureus*, while the in dry period the mamary gland is more susceptible to *Streptococcus dysgalactiae, Streptococcus uberis, Actinobacillus pyogenes* infections (Belay et al., 2022, Lakew et al., 2019). The main component of pathogenicity *S. agalactiae* is the virulence, however, it can be also pathogenic through the secretion of hemolysin, streptokinase, hyaluronidase, protease and deoxyribonuclease. Bacterial proliferation is rapid and occurs in the epithelium of the galactophorous ducts, rarely being accompanied by penetration into the canalicular and acinar epithelium. As a result, neutrophils

are transferred from the blood capillaries to the galactophorous ducts, sometimes associated with a neutrophilic reaction (Burvenich et al. 2007).

The inflammatory process blocks the lumen of the galactophorous ducts and causes alveolar involution, replacement of secretory tissue with fibrous connective tissue, atrophy, hypogalaxy and agalaxia. Mastitis are typically chronic or subclinical, with occasional acute outbreaks and the pathogenic process being limited to the mammary gland. *Streptococcus dysgalactiae* has been isolated from the tonsils, rumen and genitals of cows as well as from the skin of the mamary gland or from their mammary secretions. It usually produces subclinical forms of mastitis. *Streptococcus uberis* usually produces subclinical mastitis and persists for long periods of time (Radsak et al., 2000).

The aim of this paper was to evaluate the interrelationship of subclinical mastitis and the microbiome status for diagnosis, prognosis and therapy. In this regard, milk samples were collected and analyzed from cattle diagnosed with mastitis to establish the bacterial prevalence involved in such pathological processes. In addition to the isolation and identification of bacterial species involved in the appearance of mastitis, the antibiotic resistance profile was also evaluated in order to achieve an appropriate prognosis and therapeutic protocols.

The results of these tests can be used to monitor, prevent and treat correctly and early pathological processes in the mammary gland.

MATERIALS AND METHODS

The milk samples (n=15) from private Fleckvieh herd were collected after screening quarters using the R-Mastitest. Milk samples from healthy cows (n=5) were also collected. For initial microbiological analyses, the samples were inoculated in nutrient broth, Chapman and MacConkey agar (both from Oxoid) in aerobic conditions at 37°C for 24 hours. Bacterial strains identification were performed by standard microbiological methods adopted from the Clinical and Laboratory Standards Institute (CLSI) guideline. The identification of microorganisms was performed using Api Staph 20 and Api 20E identification galleries.

The antimicrobial sensitivity patterns of the isolated strains were evaluated using the standard Kirby-Bauer disk diffusion method according to the CLSI guidelines. The strains were tested towards 8 antimicrobials: amoxicillin/clavulanic acid (AMC, 20/10 µg) amoxicillin (AX, 10 µg), streptomycin (S 10 µg), cefoperazone (CFP, 30 µg), ciprofloxacin (CIP, 5 µg), oxytetracycline (OT 30 µg), penicillin (P, 10UI), neomycin (N, 30 µg); all purchased from Oxoid. The results were assessed based on the growth inhibition zone diameters (mm), and were calculated also the multiple antibiotic resistance index according to Krumperman (Krumperman 1983). According to the standard values of CLSI the tested strains were classified as sensitive (S), Intermediate resistant (I) and resistant (R).

RESULTS AND DISCUSSIONS

Of a total of 15 (n = 15) milk samples collected from cows diagnostised with subclinical mastitis 16 different single bacterial colonies were obtained. The isolated *strains* were identified as *Staphylococcus xylosus* (15.38%), *Staphylococcus lentus* (23.07%), *Shigella* spp. (7.69%), *Actinobacter bauman* (7.69%), *Cryseomonas luteola* (7.69%), *Enterobacter cloacae* (30.76%), *Echericia vulneris* (7.69%) (Fig. 1).

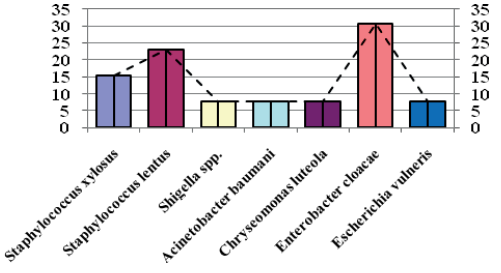

Figure 1. Percentage distribution of strains isolated from cow's milk with mastitis

From healthy cows *Staphylococcus xylosus* (30%), *Staphylococcus sciuri* (60%) and *Staphylococcus lentus* (10%) were isolated (Fig. 2).

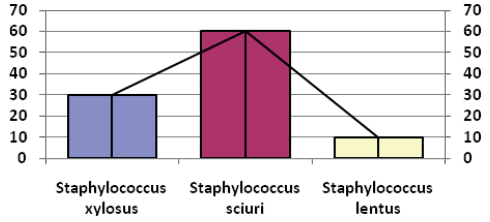

Figure 2. *Staphylococcus strains* isolated from healthy cow's milk (%)

Subclinical mastitis caused by intra-mammary infection with coagulase-negative staphylococci is one of the most common causes of dairy cows. The control of this infection is often complicated due to the increased number of bacterial species that are isolated in these cases. A study of 11 flocks, testing milk samples from affected quarters at one-month intervals and identifying bacteria isolated by biochemical tests, showed that persistent intra-mammary infection were characteristic of quarters infected with *Staphylococcus epidermidis, Staphylococcus chromogenes*, and *Staphylococcus simulans*. No differences were established between coagulase-negative staphylococcal species in relation to daily milk production, somatic cell count and month of lactation in cows with subclinical mastitis. *S. epidermidis* has been identified mainly in pluriparous cows with intra-mammary infection, while *S. chromogenes* has been isolated mainly from primiparous cows with intra-mammary infection (Thorberg et al., 2009). The isolated strains were evaluated for their level of antibiotic resistance, the results are indicated in Table 1. Of the total isolated strains, 93.75% showed total resistance to Amoxicillin/Clavulanic acid, 25% to Penicillin 31.25% to Neomycin and Streptomycin, 12.5% to Amoxicillin and Oxytetracycline, and 68.75% to Cefoperazone. The MAR index can be used to facilitate the interpretation of the results. This represents the ratio between the antibiotics to which the tested strain is resistant and the total antibiotics used for the antibiogram. The closer the MAR value is to 1, the more resistant the strain is to the antibiotics used for evaluation (Fig. 3).

The prevalence of more than 60% of bacteria with a MAR index greater than 0.4 is closely related to the therapeutic history of the study animals. Antimicrobials are often used in the treatment of the mastitis in large ruminants, so the possibility of antibiotic resistance should be considered. According to the results of our study in all isolated strains the MAR index was 1. Our results are not in line with the studies performed by Oliveira and coworkers (2012) who indicate a low degree of antibiotic resistance in *Staphylococcus aureus* strains isolated from milk samples collected from cows with clinical and subclinical mastitis. Resistance to one or two classes of antibiotics was observed in 24.1% of strains, and a single strain showed multiple resistance to a wide range of antibiotics. Our results indicate resistance of the strains to several classes of antibiotics. None of the isolates from clinical mastitis showed complete resistance to ciprofloxacin. The diameter of the inhibition zone was between 12-17 mm (accoding to CLSI ≥21 mm sensible; 16-20 mm intermediate resistant and ≤15 mm resistant). Intermediate resistant pathogens cannot be considered susceptible to the antibiotic tested (Ali et al., 2010). The results of the study conducted by Saini coworkers (2012) revealed the usefulness of monitoring the use of antibiotics and their resistance patterns, in order to be able to manage the antibiotic resistance. It can thus be deduced that the antibiotics with which there is an increased resistance are those often used for the treatment of different diseases in cattle, thus determining the resistance of the strains carried by these animals. The evaluation of the antibiotic resistance pattern in correlation with the epidemiological investigation can guide the therapy in order to obtain the expected results. Of the strains resistant to more than one antibiotic, most were resistant to the combinations of amoxicillin/clavulanic acid, neomycin, streptomycin ad cefoperazone. A MAR index value ≥0.2 was observed in 100% of the resistant pathogens.

Table 1. The antimicrobial sensitivity patterns of the isolated strains

Nr.crt	AMC	P	N	S	AX	CEP	CIP	OT
S1	I	I	R	I	I	R	I	I
S2	R	I	R	R	I	R	I	I
S3	R	I	I	R	I	R	I	R
S4	R	I	I	I	I	I	I	I
S5	R	I	R	R	I	I	I	R
S6	R	I	I	R	I	R	I	I
S7	R	R	R	I	I	R	I	I
S8	R	R	I	R	I	R	I	I
S9	R	R	R	R	I	R	I	R
S10	R	8	I	R	I	R	I	I
S11	R	R	I	R	I	R	I	I
S12	R	I	R	R	I	R	I	I
S13	R	I	I	R	I	I	I	I
S14	R	I	I	R	I	I	I	I
S15	R	I	R	R	R	I	I	I
S16	R	I	R	R	R	I	I	I

Legend: I – intermediate resistant, R- resistant, S1-S4 *Staphylococcus xylosus,* S5-S7 *Staphylococcus lentus,* S8, *Shigella spp.* S9 *Actinobacter bauman,* S10 *Cryseomonas luteola,* S11-S15 *Enterobacter cloacae,* S16 *Echericia vulneris* (7.69%)
AMC - amoxicillin/clavulanic acid, AX– amoxicillin, S- streptomycin, CFP-cefoperazone, CIP- ciprofloxacin, OT-oxytetracycline, P-penicillin, N-neomycin

Table 3. Multiple antibiotic resistance (MAR) index of antibiotics against isolated

Drug	Total number of rezistent strains	MAR index of the tested antibiotics
Amoxicillin+Clavulanic acid	16	1
Penicillin	16	1
Neomycin	16	1
Streptomycin	16	1
Amoxicillin	16	1
Cefoperazone	16	1
Ciprofoxacin	16	1
Oxytetracyclin	16	1

Studies performed in the Louisiana, USA, in order to determine the prevalence of mastitis in dairy cows that have reached sexual maturity, showed that intra-mammary infection were present in 97% of animals and 75% of quarters. *Staphylococcus hyicus, Staphylococcus aureus* and *Staphylococcus chromogenes* were the most common isolated bacterial strains (Nickerson, 2009). About 28% of the animals and 16% of the quarters had clinical mastitis. Other researchers indicated the importance of *Staphylococcus aureus* in mamamry gland infections in dairy cows. Both lactating and dry cows are included in the *Staphylococcus aureus* infection control program. The number of cases of mastitis reported to be cured following *Staphylococcus aureus* infection varies considerably (Barkema et al., 2006.

From an economic point of view, the choice of treatment protocol must be fully justified. It is important to carefully select antibiotics, as well as the correct administration and compliance with waiting periods. One of the biggest public health problems in the world is antibiotic resistance. Due to improper treatment, antimicrobial-resistant microorganisms can spread very easily in nature. Treatment failures can occur due to resistance to effective broad-spectrum antibiotics (Ali et al., 2010). These treatment failures have a major impact on animal welfare and also lead to significant economic losses.

CONCLUSIONS

The varied and potentially pathogenic microflora isolated from cows with subclinical mastitis may have implications for the production of gastrointestinal disorders in dairy products consumers. In addition, the antibiotic resistance present in these bacterial strains may play a role in spreading to other animals and humans. Isolated species may indicate fecal pollution (*Shigella* spp., *Escherichia vulneris,*

Enterobacter cloacae) suggesting the need for better shelter hygiene. as well as the mammary gland before milking. Total or partial resistance (resistant colonies) to several antibiotics is worrying, suggesting the harmless use of antibiotics in the territory either to control possible clinical mastitis or to treat conditions other than mastitis in individuals in the experimental group. The treatment must be individualized according to the bacterial species and its degree of resistance according to the antibiogram, at the same time as the application of non-specific measures for the prophylaxis of mastitis.

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ANIMAL PRODUCTION, PUBLIC HEALTH AND FOOD QUALITY CONTROL

Scientific Works. Series C. Veterinary Medicine. Vol. LXVIII (2), 2022
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

FRESHNESS INDICATORS, PHOSPHORUS CONTENT AND HUMIDITY CORRELATIONS IN ANGLERFISH AND MONKFISH SAMPLES *(Lophius spp.)* FROM THE NORTH SEA AND ATLANTIC OCEAN

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Abstract

The paper aimed to present the correlations between the freshness indicators (pH, TVB-N) and water content, phosphorus content and humidity, protein content and TVB-N (mg %) in wild Anglerfish/ Monkfish captured from the North Sea and Atlantic Ocean during naval expeditions aboard the research ship Walther Herwig III[rd]. It is based on the research data obtained at Max Rubner Institute Department of Safety and Quality of Milk and Fish Products Hamburg between 2013 and 2014. The processed data were the following indicators: pH, TVB-N, phosphates content, percent of water, salt, protein, fat and ashes. Correlations were made using chi square test. Angler- and Monkfish (Lophius spp.) samples analysed (n = 77), presented a reasonable positive correlation (R^2 = 0.44) between the pH values and the percentage of water, a very weak positive correlation (R^2 = 0.1) between TVB-N and protein, a high negative correlation (R^2 = - 0.7018) between the phosphate and water content and a strong negative correlation (R^2 = - 0.7961) between the protein and the water content.

Key words*: Lophius spp., freshness indicators, proximate analysis, phosphorus content, humidity.*

INTRODUCTION

Anglerfish (*Lophius piscatorius*) and Monkfish (*L. americanus*) are belonging to the *Lophiidae* Family. They have massive heads, that are broad, flat and depressed, making the rest of the body (normally called the tail) seem like an appendage.

The Anglerfish is a demersal fish, well adapted to its environment, living in coastal regions and at high depths. In northern latitudes, the Anglerfish spawns between April and June mainly north, W and S of the British Isles at a depth beyond 400 meters.

Seven species of *Lophius* are known worldwide, six within the Atlantic Ocean and only one within the Northwest Pacific. The genus supports valuable fisheries (except for *Lophius vaillanti*), most for an extended time, though the exploitation of *Lophius gastrophysus* along the coast of Brazil is comparatively recent (Fariña *et al.*, 2008).

The primary goal of this study was to research the composition of *Lophius* spp. tail muscle which is the main part of the body that is usually destined to human consumption.

MATERIALS AND METHODS

Lophius spp. live in many seas. The foremost important areas of distribution include the N Atlantic and the Mediterranean. They include the American waters from Newfoundland to Brazil, and also the Gulf of Guinea to the New Zealand zone. The fish genus *Lophius* is exploited worldwide. *Lophius* spp. is caught by demersal trawlers with trawls or long lines, mostly gill nets. They are usually prepared for sale on board where the head is removed, the fish is skinned and gutted.

This species is widely found in coastal waters of the N-E Atlantic, from the Barents Sea to the Strait of Gibraltar, the Mediterranean Sea and the Black Sea. The greatest capture volumes within the last decade are reported by France, UK, Ireland and Spain (International Council for the Exploration of the Sea, 2021; Norwegian Institute of Marine Research, 2021).

According to International Council for the Exploration of the Sea (ICES) the main fisheries areas for Black-bellied Anglerfish *(L. budegassa*) and White Anglerfish (*L. piscatorius*) are divisions 7b.-k, 8.a-b and 8d (W and S-W Ireland, Bay of Biscay), 8c and 9a (Cantabrian Sea, Atlantic Iberian waters); sub-areas 1 and 2 (N-E Arctic); sub-areas 4 and 6, and in Division 3.a (North Sea, Rockall and W of Scotland, Skagerrak and Kattegat) (International Council for the Exploration of the Sea, 2018).

L. piscatorius and L. budegassa are mainly caught by Spanish and Portuguese bottom-trawlers and net fisheries (gill net and trammelnets). As with *L. piscatorius, L. budegassa* is a crucial target species for the artisanal fleets and a by-catch for the trawl fleets targeting hake or crustaceans. Since 2010, Spanish landings of *L. piscatorius* were on the average 83% of total landings of the stock. Reported values for *L. budegassa* represented < 1% (on average) of the full landings of the stock. The total allowable catch advice for the Celtic Seas, West of Scotland, Bay of Biscay and Iberian Sea is 34,275 t in 2022, while the most recent estimated stocks were around 45,651 t (International Council for the Exploration of the Sea, 2021).

The stocks assessed are also distributed over a large area of the Northeast Atlantic shelf from ICES Division IIIa to IXa: here are currently 4 stocks of Anglerfish (*Lophius piscatorius* and *L. budegassa* in Divisions VIIb-k and VIIIa,b,d and *L. piscatorius* and *L. budegassa* in Divisions VIIIc and IXa)(International Council for the Exploration of the Sea, 2021).

The *Lophius* spp. samples we analysed in Germany come from the N-E Atlantic (North Sea) and were mainly cached during the fishing expeditions 366 and 372 respectively of Walther Herwig III[rd] the main research and exploration ship of Thünen Institute Hamburg (Figure 1). Fish weight was between 857 g and 6.34 kg. The medium size for commercial landings from this area is 1 m or 20 kg (ICES, 2020). Three species of Anglerfish dominate in the fishery. On the German market may be found the White Anglerfish (*Lophius piscatorius*), which might reach a length of up to 2 meters and a weight of up to 40 kilograms. The commercial weight of obtainable tails is sometimes between 1 and 3 kg.

Figure 1. Walther Herwig III[rd] research ship (Thünen Institute of Sea Fisheries)

Black-bellied Anglerfish (*Lophius budegassa*), grows up to 70 centimeters long.

Mainly on the American Atlantic Coast is located the home of the American Anglerfish (*Lophius americanus*), which might reach up to 1.2 meters and is known as the Monkfish (Figure 2).

Figure 2. Monkfish (*Lophius americanus*) specimen

European landings are limited in volume (between 9,000 and 10,000 metric tons catch weight per year). The market share in Germany in 2013 was around 0.5%. Due to of the meat quality, which is extremely valued by connoisseurs, good prices may be achieved for *Lophius* spp.

Some are sold with head on but mainly without it, also skinned, because the appearance of the Anglerfish sometimes it is not commercially offered as an entire fish (Figure 3). They are mainly sold as fresh (whole with head on/ tail skinless fillets/ whole skin-on tails) or deep frozen as tail skinless fillets/ whole skin-on tails (Figure 4).

The marketed meat, hosts a fine aroma and it is ideal for roasting with a fine inherent flavour of the lean, white meat, which is nearly boneless (Figure 5).

Figure 3. Whole Anglerfish (*Lophius piscatorius*)

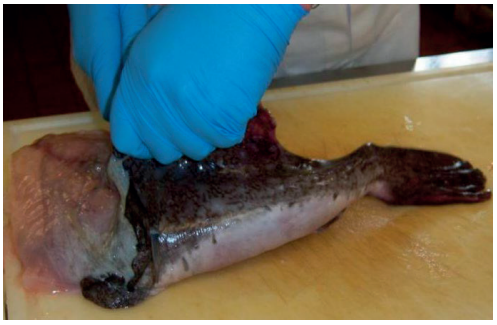

Figure 4. Anglerfish (*Lophius piscatorius*) edible portion skinning

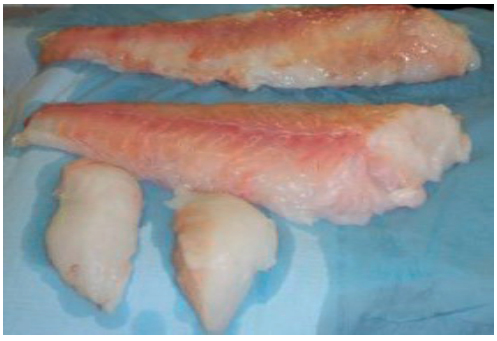

Figure 5. Economically valuable part of *Lophius* spp.

Both lipid quantity and quality of fish are subject to changes because of their age and size and environmental conditions like season and feeding (Karl *et al.*, 2018). Moreover, both fat content and the fatty acid composition are species specific (Borresen, 1992; Hamre *et al.,* 2003; Iverson *et al.,* 2002; Lenas *et al.,* 2011). *Lophius* spp. belong to the semi-fatty (3–6% fat) fish species (Karl *et al.*, 2013). They store their lipids mainly within the muscle tissue, but only little information is accessible on the distribution within the edible part. However, the flesh quality is correlated to the fat, water and protein content and to its distribution within the muscle tissue. The lipid distribution is additionally a very important factor for the values of contaminants eventually present within edible parts with fat content. Determination in parts of the fillets and/or in parts of entire fish can yield different contamination levels, depending on the respective lipid content (Karl & Lahrssen-Wiederholt, 2013). This work provides information on the amount of fat, water and protein within the muscle tissue of *Lophius* spp. from the North Sea fishing areas. Additionally, we establish a relationship between the lipid and water content, the share of TVB-N and protein content, the water content and pH, the protein and the water content respectively (Figures 7 to 10) using available muscle flesh thawed samples (n=77).

Previous chemical analysis

Proximate composition, fatty acids profiles and other nutritional values were evaluated for fillets of *Lophius* spp. Crude protein (N × 6.25) and total lipid content were determined on the edible portions of fish by the methods descrybed within the AOAC manual (AOAC, 1975). Moisture content was determined by drying the samples in an air circulation oven for 8 h at 100°C. Samples for ash determination were heated in a muffle furnace at 550°C for 6 h to constant weight. (Jhaveri *et al.*, 1984).

Moisture, total lipid and protein contents ranged from 828 to 845, 3.0 to 3.7 and 135 to 170 g/ kg muscle, respectively (Prego *et al.*, 2012). Other previous research that associated the chemical composition and nutritional value of Anglerfish is scarce (Table 1) or has focused on proximate and amino acid composition (Jhaveri *et al.*, 1984), fatty acid composition (Sirot *et al.*, 2008), and assessment of α-tocopherol and essential minerals (Jhaveri et al., 1984; Carvalho *et al.*, 2005).

MATERIALS AND METHODS

Fish samples were collected from the N-E Atlantic (correspondent German Bight of the North Sea) and were cached from a bottom trawl catch during the fishing expeditions

no.366 and no.372 of Walther Herwig III[rd] (Figure 6).

Table 1. Nutritional values and energy of 100g Anglerfish and Monkfish (edible portions) medium values compared to lobster (Fish Information Center, 2021; Pehrsson *et al.*, 2016; Strobel *et al.*, 2012).

Proximate composition	Anglerfish	Monkfish	Lobster
Fat	4.6 g	1.5 g	2.0 g
Protein	20.3 g	14.9 g	16.0 g
Sodium	180 mg	180 mg	270 mg
Calories	88 kcal	76 kcal	89 kcal
Iodine in raw liver	96 µg	100 µg	185 µg
Omega-3 fatty acids	0.1 g	0.13 g	79 mg

Between 26.07.2013-20.08.2013 and 23.01.2014 - 23.02.2014 respectively and previously 77 Angler- and Monkfish (*Lophius* spp.) of various size were collected by the above mentioned German fishing research vessel (German Oceanographic Data Center, 2013; 2014).

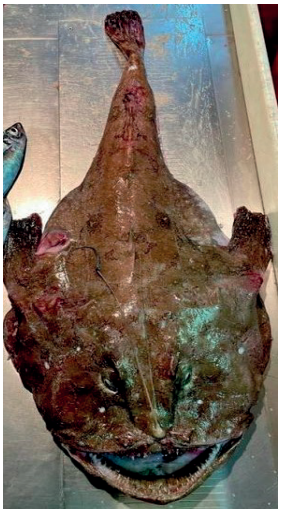

Figure 6. Anglerfish (*Lophius piscatorius*) caught in the North Sea

Individuals were weighed, lengths were measured and then were manually filleted. The skin was removed and muscles were weighed (FAO, 1995). Tail muscle meat of *Lophius* spp. was minced and homogenised. Corresponding left and right tail parts of every individual were combined and deep frozen. Samples were stored at –20ºC until further analysis.

Proximate composition (water, ash, protein and lipid content in %) of every muscle part homogenate was measured. Total sum, as percentage, was in the range of 98-102%.

Water content was indirectly determined after drying an aliquot of the homogenised tail muscle flesh for 12 h at 105ºC.

Protein nitrogen was measured by Dumas method (Miller *et al.,* 2007) using a LECO TruSpecN (LECO Instruments GmbH, Germany).

Percent of protein was calculated by multiplying % N by 6.25 (AOAC, 2005).

Ash content was determined according to Antonacopoulos (1973).

Lipid content was determined by modifying the Smedes method (1999) (according to Karl *et al.,* 2012). After double extraction from 5 g sample with cyclohexane and isopropanol, the fat was transferred within the cyclohexane phase, after adding water and stirring by means of an Ultra-Turrax instrument (IKA-Werke, Germany). Centrifugation and phases separation was followed by gravimetric measurement of fat after separation and concentration from the cyclohexane phase. Organic phase was separated from the aqueous layer and evaporated. Lipid content was determined after drying the remained residue for 1 h at 105ºC.

Total phosphorus-determination

The total phosphorus content was determined photometrically from the nitric acid extract of the ash in line with a modified official German method § 64 LFBG to detect and measure phosphorus in meat (German Food & Feed Code, 2008).

Total volatile basic nitrogen (TVB-N)

A perchloric acid extract was prepared using 20 g minced and homogenised fish tail muscle flesh with 180 mL 6% (w/w) perchloric acid. The filtered acid extract was used for the determination of total volatile basic nitrogen (TVB-N)

Determination of pH values

After mincing and homogenizing, the sample was diluted 1:1 with deionised water, stirred and the pH was determined by means of a pH-electrode (Oehlenschläger *et al.*, 2002).

Quality assurance and statistical analysis

For the analytical internal quality control the matrix meat MUVA Reference material food supplements no.752 (MUVA GmbH, Kempten,

Germany) was used. Certified values were given for ash, moisture, lipid and nitrogen. All analysed components' values showed excellent agreement with the certified values.
Statistical analysis according to MUVA Kempten Statistical Protocol was conducted (https://www.muva.de/fileadmin//user_upload/ Statistisches-Protokoll.pdf). Pairs of variables were subjected to t tests ($p < 0.05$).

RESULTS AND DISCUSSIONS

Proximate composition of the tail muscle flesh of *Lophius* spp. (Monkfish and Anglerfish samples analysed) is compiled into Tables 2 and 3. The sizes of the fish are typical for bottom trawl catches of *Lophiidae* in this area. The *Lophius* spp. samples covered a larger length and weight range of 32–76 cm and 857g–6.34 kg, respectively.

Table 2. Results of proximal analysis in the tail muscle samples from Monkfish (*Lophius* spp.) caught in the Atlantic Ocean (n=13)

Component	I st catch (n=3)	II nd catch (n=10)	Min.	Max.
Moisture %	83 ± 0.7	84.8 ± 0.9	81.84	86.8
Protein%	16 ± 0.5	15.32 ± 0.8	14.2	15.9
Ash %	1.3 ± 0.3	1.05 ± 0.1	0.9	1.66
Lipids %	0.5	0.4	0.4	0.5
pH	6.5 ± 0.12	6.8 ± 0.13	6.4	6.9
NaCl%	0.5± 0.4	0.4 ± 0.1	0.3	1
TVB-N <mg/100g>	11.3 ± 2.7	10.9 ± 1.6	7.1	14.3
Phosphate g P_2O_5/kg	4 ± 0.7	3.2 ± 0.3	3	4.5

Table 3. Proximate composition of Anglerfish, caught in the North Sea (n=64)

Catch nr.	Water %	Protein %	Ash %	Lipids %	Phosphate g P_2O_5/ kg	NaCl %
1 (n=10)	84.56± 0.74	15.40±0. 64	1.01± 0.04	0.34	3.23± 0.13	0.30± 0.08
2 (n=10)	89.57± 0.78	8.88± 0.78	1.20± 0.17	0.25	1.16± 0.28	0.48± 0.16
3 (n=10)	88.92± 0.89	10.41±0. 72	0.69± 0.07	0.38	2.23± 0.24	0.09± 0.02
4 (n=10)	90.22± 0.81	9.23± 0.46	0.92± 0.05	0.21	1.53± 0.21	0.14± 0.02
5 (n=10)	86.73± 1.62	12.47±1. 21	1.62± 0.04	0.39	1.92± 0.4	1.20± 0.07
6 (n=4)	84.87± 0.11	14.08	1.02± 0.03	0.42	3.6± 0.08	0.19± 0.01
7 (n=10)	88.74± 1.75	12.89±1. 61	1.00± 0.03	0.24	2.35± 0.19	0.39± 0.10
Min.	83.49	7.88	0.78	0.21	0.86	0.11
Max.	89.87	16.24	1.58	0.42	3.69	1.28

Lophius spp. samples were collected from fishing grounds of the Atlantic Ocean and North Sea to review the composition of the muscle tissue. Little information is available on the composition and nutritional values of *Lophius* spp.
Overall lipid, water, protein and ash contents were calculated as arithmetic means (averages) ± standard deviations (SD) of the respective values analysed from the edible tail muscle parts. Proximate composition of the *Lophius* spp. edible part analysed during the present study (Tables 2 and 3) was generally within the range previously reported. A number of 30 analysed Anglerfish samples presented a higher percentage in moisture (88.92 to 90.22%), thus all other components of dry matter had smaller amounts (protein from 9 to 10%; lipids from 0.21 to 0.38%; ash from 0.69 to 1.20%). The high water percent could not be correlated with an increased salt or phosphorus values.
Lowest mean lipid content of 0.21% was correlated with high water percentage and low protein percentage respectively. The higher lipid content corresponded to the lowest water content.
Protein content of both species stayed within the range of 9-16%. Lowest protein value was equal to 7.88%. Conversely, the highest protein content was 16.5%, while the phosphate ranged from 1.23 to 4.5 g P_2O_5/ kg.
Ash content ranged between 0.55 and 1.69%.
Prego *et al.* (2012) analysed the proximate composition of Anglerfish (n=5): according with his results the values found were: moisture content varied from 82.8 to 84.5%, total lipids from 0.3 to 0.37% and protein from 13.5 to 16.1%, in agreement with other results (Jhaveri *et al.*, 1984; Barros-Velázquez *et al.*, 2008; García-Soto *et al.*, 2011).
Previously, Jahveri *et al.* (1985) had reported in edible portions of Monkfish: 83.29 ± 0.63% moisture, 15.85 ± 0.54% protein, 1.21 ± 0.04% ash and 0.53 ± 0.09% lipids.
Composition differences could not be associated with the dimensions of the fish, although in other fish species, the maturation stage may affect the muscle structure (Karl *et al.,* 2013).

Water and lipid content of the muscle tissue

For both species, a negative correlation between the water and lipid content of the muscle tissue was found ($r = -0.7071$, see

Figure 10). Previously, Karl *et al.* (2018) have reported the identical tendency in other fish species.

The average composition (fat, ash, protein and water) of the thawed *Lophius* spp. samples, the pH of the muscle and also the phosphorus content, given as P_2O_5, are found in Tables 2 and 3.

Crude protein content of tail muscle flesh ranged between 8.8 and 16%, results being similar compared to the protein content reported in other studies (which ranged between 13.5 and 16%).

Water content of the sampled fillets ranged between 81.8 and 91.5% compared to available literature data which showed the proportion of water in fillets at around 83-84%.

Fat contents were comparable with those from other studies, varying between 0.2 and 0.5% (Jhaveri *et al.*, 1984; Barros-Velázquez *et al.*, 2008; García-Soto *et al.*, 2011).

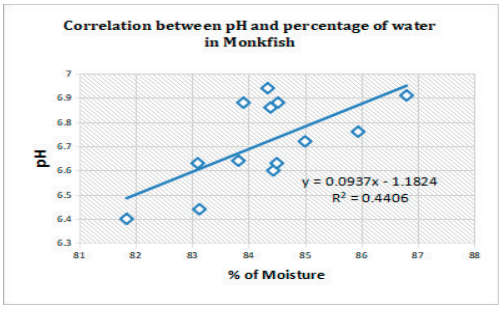

Figure 7. Scatter plot showing a reasonable positive correlation (R^2 = 0.4406) between the pH-values (Y) and the water content (X) in Monkfish, caught in Atlantic Ocean (n = 13)

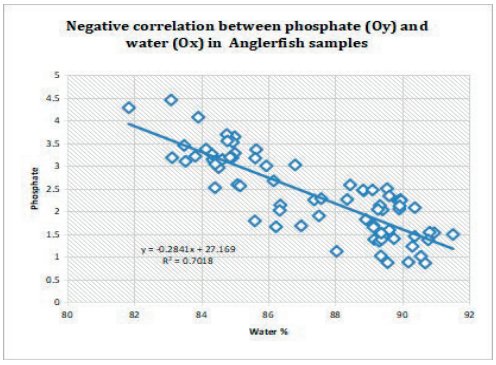

Figure 8. Scatter plot showing a high negative correlation (R^2 = - 0.7018) between the the phosphate (g P_2O_5/ kg) content and water percent in Anglerfish, caught in the North Sea (n = 64)

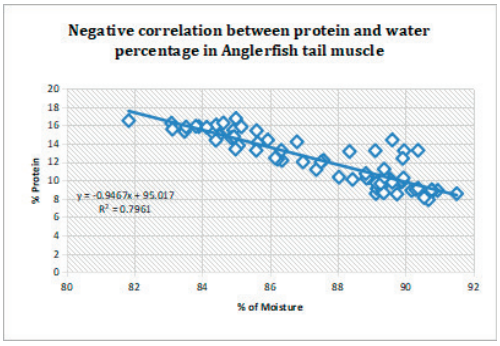

Figure 9. Scatter plot showing a strong negative correlation (R^2 = - 0.7961) between the protein (Y) and the water content (X) in Anglerfish, caught in the North Sea, (n = 64)

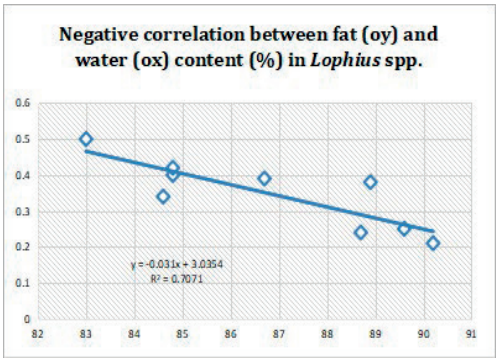

Figure 10. Scatter plot showing a negative correlation (R^2 = - 0.7071) between the fat (Y) and the water content (X) medium values in Anglerfish, caught in the North Sea (n = 77)

Most analysed samples had low pH-values <7.0 (pH=6.4–6.7). Fresh fish muscle has pH values <6.5, and the critical pH is 6.8-7.0 (Lin *et al.*, 2015).

The low pH value of analysed *Lophius* spp. samples corresponds well to literature data reported for fresh *Lophiidae* in which liver samples of Anglerfish had a variation of pH from 6.33 to 6.78 when stored in slurry ice one week.

Composition of *Lophius* spp. tail muscle flesh should be compared to other demersal fish species, therefore further research is required. Feed composition and season of capture may influence the composition of fish (Hamre *et al.*, 2003; Yildiz *et al.*, 2008).

The total volatile basic nitrogen (TVB-N) is a widely used instrument to assess the degree of freshness of marine fish species and legal limits are set for unprocessed fishery products. In

freshly caught fish TVB-N equals to 4–5 mg N⁄100 g values which may be experimentally increased up to over 200 mg N ⁄ 100 g or even 500 mg N/ 100g (Ruiz-Capillas and Horner, 1999, Azam *et al,* 2005).
All analysed thawed samples during this study had low TVB-N contents ranging between 7.1 and 14.3 mg N ⁄ 100 g (Table 2). Differences between the TVB-N values measured in this study were not significant.

CONCLUSIONS

The results indicate a good freshness of the fish and proper maintenance of the cold chain.
Differences in muscle composition between the individuals were noticeable. The obtained data confirmed a large variation within the muscle composition of the various *Lophius* spp. samples analysed (Table 3).
Consequently, when analysing control and/or monitoring samples, large differences in the water, protein and lipid content of the edible parts of *Lophius* spp. can be expected even in specimens fished simultaneously.
In 30 of analysed samples low protein and high water content along with elevated pH value were detected while the literature offers little information on the water content, protein and pH of the edible muscle meat of *Lophiidae*.
Due to high negative correlation between the amount of phosphate and the percentage of water in analysed samples (Figure 8) we conclude that differences in tail muscle structure of *Lophius* spp. could be induced by different diet composition which the fish encountered in the wild.

ACKNOWLEDGEMENTS

We would like to thank German Academic Exchange Service (DAAD) and Max Rubner Institute (MRI). Special gratitude to Mr. Marcel Balaci (Josef Möbius Bau AG), Professor Claudia Sala and Associate Professor Laurențiu Tudor.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXVIII (2), 2022
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

STUDY REGARDING THE TRIGGERING FACTORS OF *Apis mellifera carpatica* SWARMING PHENOMENON

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Abstract

Swarming is the natural phenomenon that involves the reproduction of the bee species. The name swarm comes from one of the phases of the multiplication process that takes place in the form of a multitude of flying bees, with one or more queens and drones, forming in the air a globular shape, "swarm", on their way from the hive to the temporary or permanent destination. The study follows the importance of the favoring and the determining factors of Apis mellifera carpatica bees swarming, in order to develop good management strategies and describes the stages of the swarming phenomenon, from the preparation for swarming to leaving the hive. The main factors that led to the swarming of the four bee families were the overcrowding in the hive, the blockage of the hive with honey and pollen and the poor management of the apiary.

Key words: *apiary, favoring and determining factors, swarming.*

INTRODUCTION

Swarming is one of the most complex events that occur in the world of bees. Studying the stages of the swarming phenomenon and its triggering factors is especially important for the development of new management strategies for the apiary (Zacepins et al., 2015). For beekeepers, the swarming phenomenon represents on the one hand a loss, given the economic implications of the phenomenon, but also, being a frequently used way of breeding bee colonies. It may be also associated with risks for diseases (Dumitru et al., 2020; Zacepins et al., 2021).

Swarming does not have to take place every year. It corresponds to the need to propagate the species and to fill the gaps caused by the natural mortality. Swarming requires a long preparation and it happens only in a context of abundance and euphoria (Getz et al., 1982).

In Romania, bee colonies reach a maximum of development in mid-June. The queen's egg laying is constantly progressing since January, when the days start to become longer. The decline begins in July, when the days decrease in duration. In mid-October, the queen ceases to lay eggs. The preparation for winter period begins with the last hatching taking place in November (Antonescu, 1979).

The first stage of the swarming begins with the rearing of the queens. The queen cells are built nine or ten days before the swarm leaves. During the following days, the queen lays eggs in these cells (Bencsik et al., 2005).

In the second stage of the swarming, the colony splits into two parts: one part of the colony flies away with the queen, and the other part stays behind and rears another queen (Gilley et al., 2005).

There is an ongoing need for further studies regarding this complex phenomenon, including its triggering factors. The beekeepers need to come with new ways to improve the management of the apiary, as bees begin to become endangered due to the excessive use of pesticides, the over industrialization of the agriculture and the continuous expansion of the urban areas (Gardi et al., 2015; Spivak et al., 2005).

Therefore, the aim of the study was to examine the phases of the swarming phenomenon, as well as to identify the factors involved in triggering the swarming of honey bees.

MATERIALS AND METHODS

The study was conducted in Negoesti, Calarasi County, between March 2019 and March 2021 and was carried out on seven bee colonies belonging to a stationary apiary. All the queen bees used in the study belong to the *Apis mellifera carpatica* species (Lipan (Buescu) et al., 2021).

During this study, the factors that led to the swarming of the bee families were identified and described. The activity of the apiary was recorded with the help of a beekeeper's notebook. Only natural swarms were taken into account and were analyzed. The letters A, B, C, D, E, F and G were used in order to identify the hives as easily as possible.

The hives were organized into two main categories: the first category consists in the type of the food that was given to the bee colonies at the end of the winter and the second one is based on different management actions. Hives A, C and D were given protein cakes at the end of the winter to stimulate the development of bee families and hives B, E, F and G were given caloric cakes.

The honey was not extracted from the hive E after the sunflower harvest, this action led to the blockage of the hive with honey and pollen, the lack of space being a favorable condition for triggering the swarming phenomenon. The queen cells that were previously identified in the hive G. were not broken when their presence was notified. The presence of swarming hives indicates the entry of bees into the swarming fever.

RESULTS AND DISCUSSIONS

There has been a faster development of the families that have been fed with proteic cakes. The egg-laying of the queens in the hives A, C and D has intensified and the number of nursing bees and worker bees has almost doubled. This has been observed during the periodic inspections of the apiary.

Figure 1. One day old eggs, workers bee and the queen bee (original)

The spring of 2019 has registered high temperatures compared with the previous years (30ºC on 25.03.2019). This shortened the period between the flowering of rapeseed and acacia crops, with the two of them overlapping. The intense harvesting of pollen and nectar led to the growth of the colonies.

The rainy days that followed led to a lack of activity in the hive and overcrowding. Consequently, the bee colony went into swarming fever.

On 13th of May the hive D swarmed, being followed two days later by hive A. After a few attempts the hive A was recovered. It should be noted that the bee families belonging to the hives A and D are those that entered the winter period stronger than those in the hives B, C, E, F and G.

In the first stage of the swarming, the bees began bearding, this behavior being followed by the fast flying in front of the beehive.

In the second stage of the swarming, the swarm chose to sit on a tree branch that was approximately 30 meters away from the hive. It should be noted that the cluster of bees sat on a high branch positioned at a distance of about 4 meters from the ground.

Figure 2. Primary swarm - hive A (original)

The swarm was recovered by placing it in an empty hive. This maneuver was performed by shaking the branch on which the swarm was located.

In the presence of abundant sources of food, the harvesting has intensified. The frames were filled with pollen and honey, limiting the space for laying eggs.

Figure 3. Cells filled with honey and pollen (original)

In order to demonstrate the role of the limited space on swarming, it has been decided to postpone the extraction of honey in the hive. This led to the blockage of the hive with honey and pollen. As a result, the bees began to build queen cells and went into swarm fever. The swarming started around 1pm and ended at 2.30 pm.

The last swarming that took place during the study was caused by keeping the queen cells filled with larvae intact in the hive G.

Figure 4. Queen cell with queen bee larvae (original)

After the pre-swarming behavior that was showcased in the early hours of the afternoon, the hive G swarmed. An attempt was made to track the swarm, but its recovery was not possible. An inspection of the hive followed, which revealed the presence of empty queen cells.

CONCLUSIONS

The main factors that led to the swarming of the four bee colonies were the overcrowding in the hive, the blocking of the hive with honey and pollen and the poor management of the apiary. It should be noted that no precise delimitation can be made between the determining factors and the predisposing factors, as they are closely linked.

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Scientific Works. Series C. Veterinary Medicine. Vol. LXVIII (2), 2022
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

MICROBIOLOGICAL CONTROL OF CULTURE MEDIA USED IN THE EVIDENCE OF FOOD-BORNE PATHOGENS AND THEIR PERFORMANCE PARAMETERS ON QUANTITATIVE AND QUALITATIVE METHODS

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Abstract

A culture medium contains basic elements (water, nutrients), to which are added various supplements capable of contributing to the growth of the bacteria of interest, and at the same time, inhibiting the association flora. This study aimed for testing and optimization of special supplemented culture media used for diagnosis of microbial origin food-borne pathogens. For this, performance criteria (productivity, selectivity, and specificity) of ten different culture media used for the identification and characterization of most common bacteria (i.e., Listeria monocytogenes, Salmonella spp., Escherichia coli and Staphylococcus aureus) involved in food poisoning, were investigated. For the evaluation of the performance parameters, artificial contamination was performed with Reference Materials (MR) represented by reference strains, on two levels of contamination (low and high) and blank samples. On the qualitative methods (detection of L. monocytogenes and Salmonella spp.), selectivity, specificity, accuracy, concordance and evaluation of the detection limit at 50% (LOD50) were tested; while, on the quantitative methods (enumeration of E. coli and S. aureus), repeatability, reproducibility, critical difference and measurement uncertainty were assessed. For the qualitative methods (detection of L. monocytogenes and Salmonella spp.), the evaluated parameters showed values between 82-100% and 0.429-0.564 cfu/25 g for LOD50, respectively. For the quantitative methods (enumeration of E. coli and S. aureus), the values obtained had a measurement uncertainty between 0.24-0.26 $\log_{10}$ cfu/g). The performance criteria (productivity, selectivity and specificity) of the culture media investigated were successfully achieved. These findings on the benefits of the addition of supplements for the culture media used to diagnose food poisoning provide further evidence of the importance of additional components with the role of enrichment, stimulation, inhibition, selection and highlighting of metabolic and enzymatic equipment.

Key words*: culture media, microbiological performance, quantitative and qualitative methods, food-borne pathogens.*

INTRODUCTION

Foodborne illness (also referred to as foodborne diseases) are a problem with a strong impact on public health. They are caused by the ingestion of food contaminated with microorganisms or their toxins. Toxins or microorganisms from food cause food poisoning only if they are present in large quantities and only if they are introduced into the body orally (Barzoi et al., 1999).

There are 5 factors that cause food poisoning: bacteria, toxins, parasites and viruses. Among the bacteria involved in food poisoning are *Salmonella* spp., *L. monocytogenes*, *E. coli* and *S. aureus*, the diagnosis being based on the isolation of the pathogen causing the disease.

The culture medium is defined as a sterile nutrient medium that allows the growth and study of a microorganism. The culture media have some general characteristics: to be sterile, to have specific nutrient support for the bacteria of interest, to have a certain pH, to fulfil the physiological characteristics of the bacteria and to identify safely and specifically the metabolic and enzymatic characteristics of the bacteria subject to microbiological determination (SR EN ISO 11133:2014/A2:2020).

The evolution of bacterial cultures began with the development of the first liquid culture medium by Louis Pasteur in 1860 and the first solid medium by Koch. The advent of selective media was an important step for microbiology, making it possible to inhibit unwanted bacteria without compromising the growth of bacteria of interest (Bonnet et al., 2020).

Over the years, various studies have been done on selective enrichment broths for *Salmonella*

bacteria: Rappaport-Vassiliadis (RV) and Muller-Kauffmann Tetrathionate-Novobiocin Broth (MKTTn), the study confirming that RV broth is the most suitable for enrichment (Hyeon et al., 2012).
Another important step was the introduction of rabbit plasma and bovine fibrinogen into Baird-Parker (BP) agar, a medium used for the enumeration of coagulase-positive staphylococci in food (Buyser et al., 1998).
This study aimed for testing and optimization of special supplemented culture media used for diagnosis of microbial origin food-borne pathogens.

MATERIALS AND METHODS

The research was carried out in the Laboratory of Food Microbiology Department of the Sanitary-Veterinary and Food Safety Vâlcea.
As research materials, were used samples previously analyzed in the laboratory (sausages, salami, prepared meat and sheep cheese) according to the standards in force: SR EN ISO 11290-1:2017, SR EN ISO 6579-1:2017/A1:2020, SR EN ISO 16649-2/2007, SR EN ISO 6888-2/2021, SR EN ISO/IEC 17025/2018, SR EN ISO 6887-1/2017, SR EN ISO 7218:2007/A1:2014, SR EN ISO 11133:2014/A1:2018, SR EN ISO 16140-3:2021.
Other research materials were reference strains (positive, negative, partially or completely inhibited) *Listeria monocytogenes*: ATCC (American type culture collection) 35152, TCS Biosciences Ltd, Botolph Claydon, England, *Listeria innocua*: ATCC 33090, Tody Laboratories Int., Bucharest, Romania, *Escherichia coli* ATCC 25922, Tody Laboratories Int., Bucharest, Romania, *Enterococcus faecalis* ATCC 29212, TCS Biosciences Ltd, Botolph Claydon, England, *Salmonella enteritidis* ATCC 13076, TCS Biosciences Ltd, Botolph Claydon, England, *Salmonella typhimurium* ATCC 14028, Tody Laboratories Int., Bucharest, Romania, *Klebsiella pneumoniae* ATCC 13883, Tody Laboratories Int., Bucharest, Romania, *Staphylococcus aureus* subsp. *aureus* ATCC 25923, Tody Laboratories Int., Bucharest, Romania, *Staphylococcus epidermidis* ATCC 12228, TCS Biosciences Ltd, Botolph Claydon, England and culture media Half Fraser broth (DF), Oxoid Ltd, Cheshire, England, Half Fraser Selective Supplement, Oxoid Ltd, Cheshire, England, Fraser broth (F), Oxoid Ltd, Cheshire, England, Fraser Selective Supplement, Condalab, Madrid, Spain, Agar Listeria according to Ottaviani and Agosti (ALOA), Oxoid Ltd, Cheshire, England, Selective Supplement, Oxoid, Differential Supplement, Oxoid Ltd, Cheshire, England (for *L. monocytogenes*), Rappaport-Vassiliadis Soya broth (RVS), Scharlau, Barcelona, Spain, Muller-Kauffmann Tetrathionate-Novobiocin broth (MKTTn), Oxoid Ltd, Cheshire, England, Novobiocin Selective Supplement, Oxoid Ltd, Cheshire, England, Iodine-iodine potassium, Scharlau, Barcelona, Spain, Xylose Lysine Deoxycholate agar (XLD), Oxoid Ltd, Cheshire, England, Brilliant Green agar (BGA), Biolife Italiana, Milan, Italy (for *Salmonella* spp.), Tryptone Bile X-Glucuronide agar (TBX), Oxoid Ltd, Cheshire, England (for *E. coli*), Giolitti-Cantoni broth (GC), Scharlau, Barcelona, Spain, Baird-Parker agar (BP), Biolife Italiana, Milan, Italy, Egg Yolk Tellurite Emulsion, Scharlau, Barcelona, Spain, Rabbit Plasma Fibrinogen (RPF) Supplement, Biolife Italiana, Milan, Italy (for coagulase-positive staphylococci).
On the qualitative methods selectivity, specificity, accuracy, concordance and evaluation of the detection limit at 50% (LOD50) were tested; while, on the quantitative methods repeatability, reproducibility, critical difference and measurement uncertainty were assessed (SR EN ISO 16140-3:2021).
Sixty samples (20 for high contamination level, 20 low level and 20 blank negative samples) were analysed by artificially contaminating a sample of sausages with *L. monocytogenes*, the reference strain ATCC 35152, TCS Biosciences Ltd, Botolph Claydon, England. The high level of contamination is represented by the 10^{-7} dilution of 1 Mc Farland ($3x10^{-8}$) and the low level by the 10^{-8} dilution of 1 Mc Farland ($3x10^{-8}$).
Sixty samples (20 for high contamination level, 20 low level and 20 blank negative samples) were analysed by artificially contaminating a sample of salami with *S. enteritidis*, the reference strain ATCC 13076, TCS Biosciences Ltd, Botolph Claydon, England.

The high level of contamination is represented by the 10^{-7} dilution of 1 Mc Farland ($3x10^{-8}$) and the low level by the 10^{-8} dilution of 1 Mc Farland ($3x10^{-8}$).

For the detection and enumeration of ß-glucuronidase-positive *E. coli* (artificially contaminating prepared meat with *E. coli* ATCC 25922, Tody Laboratories Int., Bucharest, Romania) and coagulase-positive staphylococci (artificially contaminating cheese with *S. aureus* subsp. *aureus* ATCC 25923, Tody Laboratories Int., Bucharest, Romania) 10 samples in duplicate by 2 analysts were analysed for each method.

RESULTS AND DISCUSSIONS

This study shows microbiological control of the following culture media: Demi-Fraser broth (DF), Fraser broth (F), Agar Listeria according to Ottaviani and Agosti (ALOA), Tryptone Bile X-Glucuronide agar (TBX), Rappaport-Vassiliadis Soya broth (RVS), Muller-Kauffmann Tetrathionate-Novobiocin broth (MKTTn), Xylose Lysine Deoxycholate agar (XLD), Brilliant Green Agar (BGA), Giolitti-Cantoni broth (GC), Baird-Parker agar (BP).

Based on the results obtained for *L. monocytogenes*, blank negative 0/20 detected, low level of contamination 17/20 detected and high level of contamination 20/20 detected, the performance parameters (Table 1) were evaluated, all of them fulfilled the requested conditions (Gasanov et al., 2005).

Table 1. Analyzed parameters for *L. monocytogenes*

Parameters	Low level of contamination (%)	High level of contamination (%)
Selectivity	85	100
Specificity	100	100
Accuracy	93	100
Concordance	100	100

As can be seen, LOD50 represents the highest dilution or the lowest concentration of *L. monocytogenes*, at which at least 50% of the samples are positive. At the low level of contamination 7 out of 10 samples tested were positive (70%), more than 50%. The high level of contamination is represented by the 10^{-6} dilution of 0.5 Mc Farland ($1.5x10^{-6}$) and the low level by the 10^{-6} dilution of 0,5 Mc Farland diluted 1/3. For the statistical calculation of the LOD50, the literature guidelines of Wilrich (2009) were used, the result being 0.564 cfu/ 25 g (Table 2).

Table 2. LOD50 for *Lysteria monocytogenes*

High level of contamination (4 cfu/ml)		Low level of contamination (1 cfu/ml)		BLANK NEGATIVE	
Samples	cfu/ml	Samples	cfu/ml	Samples	cfu/ml
1	6	1	0	1	0
2	4	2	3	2	0
3	5	3	1	3	0
4	4	4	2	4	0
5	5	5	0	5	0
6	3	6	2	6	0
7	5	7	3	7	0
8	6	8	1	8	0
9	4	9	0	9	0
10	5	10	2	10	0
4.7 cfu=5 ufc		1.4 cfu=1 ufc			

The evaluated performance criteria (productivity, selectivity and specificity) of the culture media, used for *L. monocytogenes* detection, were successfully achieved (Table 3).

Table 3. Performance of culture media for *Lysteria monocytogenes*

Culture media	**Productivity**	**Selectivity**	**Specificity**
Half Fraser	>10 (36) specific colonies on ALOA agar	total inhibition	-
Fraser	>10 (29) specific colonies on ALOA agar	total inhibition	-
Agar Listeria according to Ottaviani and Agosti	0.73	total inhibition	blue-green colonies without opaque halo

Based on the results obtained for *Salmonella* spp., blank negative 0/20 detected, low level of contamination 18/20 detected and high level of contamination 20/20 detected, the performance parameters (Table 4) were evaluated, all of them fulfilled the requested conditions.

Table 4. Analyzed parameters for *Salmonella* spp.

Parameters	Low level of contamination (%)	High level of contamination (%)
Selectivity	82	100
Specificity	100	100
Accuracy	95	100
Concordance	100	100

As can be seen, LOD50 represents the highest dilution or the lowest concentration of *Salmonella* spp., at which at least 50% of the samples are positive. At the low level of contamination 8 out of 10 samples tested were positive (80%), more than 50%. The high level of contamination is represented by the 10^{-6} dilution of 0.5 Mc Farland ($1.5x10^{-6}$) and the low level by the 10^{-6} dilution of 0,5 Mc Farland diluted 1/3. For the statistical calculation of the LOD50, the literature guidelines of Wilrich (2009) were used, the result being 0.429 cfu/ 25 g (Table 5).

Table 5. LOD50 for *Salmonella* spp.

High level of contamination (4 cfu/ml)		Low level of contamination (1 cfu/ml)		BLANK NEGATIVE	
Samples	cfu/ml	Samples	cfu/ml	Samples	cfu/ml
1	5	1	1	1	0
2	5	2	2	2	0
3	6	3	2	3	0
4	4	4	0	4	0
5	5	5	1	5	0
6	3	6	2	6	0
7	6	7	0	7	0
8	4	8	1	8	0
9	5	9	2	9	0
10	4	10	1	10	0
4.7 cfu=5 cfu		1.2 cfu=1 cfu			

The evaluated performance criteria (productivity, selectivity and specificity) of the culture media, used for *Salmonella* spp. detection, were successfully achieved (Table 6).

Table 6. Performance of culture media for *Salmonella* spp.

Culture media	Productivity	Selectivity	Specificity
Rappaport Vassiliadis Broth	>10 (74) specific colonies on XLD agar	<10 (5) white colonies on TSA	-
Muller-Kauffmann Tetrathionate-Novobiocin Broth	>10 (93) specific colonies on XLD agar	<10 (6) white colonies on TSA	-
Xylose Lysine Deoxycholate agar	good growth of specific colonies	total inhibition	yellow colonies
Brilliant Green Agar	good growth of specific colonies	total inhibition	yellow colonies

For the evaluation of repeatability (r) the standard deviation of repeatability (STDEVr), the coefficient of variation of repeatability (VCr), the limit of repeatability (Lr) and the conditional repeatability (rC) were determined. For the evaluation of reproducibility (R) the reproducibility standard deviation (STDEVR), reproducibility coefficient of variation (VCR), reproducibility coefficient of variation limit (LVCR), reproducibility limit (LR) and conditional reproducibility (RC) were determined. In addition to these two, the critical difference (CD) and the measurement uncertainty (U) were evaluated.

The results of the quantitative method for the detection and enumeration of ß-glucuronidase-positive *E. coli* met all proposed objectives: the coefficient of variation of repeatability meets the 20% limit, the value of conditioned repeatability is less than the repeatability limit, the standard deviation of repeatability is less than the standard deviation of reproducibility multiplied by the coefficient of 0.66, the coefficient of variation of reproducibility meets the 30% limit, the limit of the coefficient of variation of reproducibility is less than the reproducibility limit, the conditional reproducibility is less than the reproducibility limit and the difference between the result obtained in the first analysis and in the second analysis is less than the critical difference (Table 7).

Table 7. Results for *Escherichia coli*

STDEVr (log10 cfu/g)	0.08
VCr (%)	18
Lr (log10 cfu/g)	0.22
rC	0.13
STDEVR (log10 cfu/g)	0.13
VCR (%)	27
LVCR (log10 cfu/g)	0.13
LR (log10 cfu/g)	0.36
RC	0.31
CD (log10 cfu/g)	0.40
Ma1-Ma2 (log10 cfu/g)	0.14
U (log10 cfu/g)	0.26

The evaluated performance criteria (productivity, selectivity and specificity) of the culture media, used for *E. coli* enumeration, were successfully achieved (Table 8).

Table 8. Performance of culture media for *Escherichia coli*

Culture media	Productivity	Selectivity	Specificity
Tryptone Bile Agar	0.87	total inhibition	white colonies

Also, the results of the quantitative method for detection and enumeration of coagulase-positive staphylococci met all the proposed objectives: the coefficient of variation of repeatability meets the 20% limit, the value of conditional repeatability is less than the repeatability limit, the standard deviation of repeatability is less than or equal to the standard deviation of reproducibility multiplied by the coefficient 0.66, the coefficient of variation of reproducibility meets the 30% limit, the limit of the coefficient of variation of reproducibility is less than the reproducibility limit, the value of the coefficient of variation of conditional reproducibility is less than the reproducibility limit and the difference between the result obtained in the first analysis and that obtained in the second analysis is less than the critical difference (Table 9).

Table 9. Results for coagulase-positive staphylococci

STDEVr (log10 cfu/g)	0.08
VCr (%)	19
Lr (log10 cfu/g)	0.22
rC	0.08
STDEVR (log10 cfu/g)	0.12
VCR (%)	29
LVCR (log10 cfu/g)	0.13
LR (log10 cfu/g	0.34
RC	0.033
CD (log10 cfu/g)	0.41
Ma1-Ma2 (log10 cfu/g)	0.01
U (log10 cfu/g)	0.24

The evaluated performance criteria (productivity, selectivity and specificity) of the culture media, used for coagulase-positive staphylococci enumeration, were successfully achieved (Table 10).

Table 10. Performance of culture media for coagulase-positive staphylococci

Culture media	Productivity	Selectivity	Specificity
Giolitti-Cantoni Broth	>10 (31) specific colonies on BP agar	total inhibition	-
Baird-Parker Agar	0.72	total inhibition	black colonies without transparent halo

CONCLUSIONS

The results of the present study provide further evidence of the importance of additional components, added to the composition of special culture media, prepared with the role of enrichment, stimulation, inhibition, selection and highlighting of the metabolic and enzymatic equipment.

In addition, these results confirm that in order to achieve an accurate identification and a complex characterization of the metabolic and enzymatic behaviour of pathogenic bacteria involved in the production of food poisoning, analytical laboratories must acquire, depending on their experience, the most efficient culture media, additional supplements for stimulation, inhibition, selection and highlighting of specific enzymatic equipment.

It should be mentioned that in case of low contamination, with a low number of microorganisms, the use of standardized methods may produce negative results in their detection. It is therefore recommended to improve the diagnostic methods currently used.

ACKNOWLEDGEMENTS

This research work was carried out with the support of Sanitary-Veterinary and Food Safety Department Vâlcea.

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EXPERIMENTAL MEDICINE

Scientific Works. Series C. Veterinary Medicine. Vol. LXVIII (2), 2022
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

EVALUATION OF THE OSTEOGENESIS PROCESS AFTER SHEEP DENTAL EXTRACTION WITH THE PURPOSE OF CREATING AN IMPLANT BED FOR TESTING MEDICAL DEVICES

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Abstract

The essential conditions for a successful osseointegration of dental implants are bone support and the bone-implant contact area. The aim of this study was to evaluate an innovative treatment in sheep periimplantitis, carried out in three stages: preparation of the implant bed, fixation of the implants and induction of periimplantitis. In phase I was verified the process of osteogenesis following the premolars extraction.
Ten sheep were prepared by putting them on a diet before surgery and by giving them antibiotics. Under inhalatory anesthesia, a total of 8 premolars/animal were extracted by dislocating each one, followed by rotational movements in the perpendicular axis and straight traction to the outside. A hemostatic sponge was inserted into the tooth alveoli and the gum was sutured. After extraction, the sheep received anti-inflammatory and antibiotic treatment. During the monitoring period, the local and general clinical conditions were monitored, and bone regeneration was assessed by Computer Tomography.
Clinically, the evolution was favorable, with no signs of discomfort, with gum scarring in 7 days and weight gain of 24-41%. Imagistic, a degree of bone atrophy within physiological limits could be observed, and the density of the neoformation bone tissue showed an optimal regeneration.
Through the approached technique we provided a favorable environment for performing the next stages of the study, the osteogenesis process being validated both clinically and paraclinical. We also propose this method for flocks of sheep with dental diseases.

Keywords*: osteogenesis, sheep, tooth extraction.*

INTRODUCTION

Orthopedic and dental implants are in a continuous development with the aim of creating an optimal interaction between the body and the implanted material. Reaching this goal was achievable by changing the surface of the implants, the topography or the coating with different biomaterials. An ideal implantable material is biocompatible in terms of chemical composition, has an excellent ability to resist corrosion and wear in the physiological environment and has an elasticity similar to bone that could prevent bone resorption periimplant. The safety of an implant is certified by its ability to minimize adverse tissue reactions, which is very important in long-term clinical situations. In order to determine whether a new material complies with biocompatibility and mechanical stability requirements, it must be subjected to rigorous *in vitro* and *in vivo* testing prior to clinical use (Pearce, 2007). The results of *in vitro* examinations are not always easy to extrapolate in *in vivo* tests, as no cell culture system is able to accurately reproduce physiological conditions (Aguirre et al, 2021). For this reason, animal models are the ideal solution for assessing, over a long period of time, the biocompatibility, tissue response and mechanical function of an orthopedic or dental medical device (Davies, 2006). Depending on

the proposed objectives, when choosing a particular animal model, certain factors must be considered, namely: availability, cost of acquisition and maintenance, maneuverability. In the field of orthopedic research, animal models must have a good tolerance for surgery and the micro and the macrostructure of the bone must be as close as possible to the human one when it is desired to translate the obtained results (Muschler, 2010).

Factors worth considering when choosing an animal model for use in orthopedic research include blood reserves that support bone healing, sexual dimorphism, skeletal immaturity, or the influence of sex hormones (Kim, 2003; Meyer, 2001).

International standards have established the main species of animals suitable for testing orthopedic and dental devices which are represented by dogs, small ruminants, rabbits, pigs and rodents (ISO 10993-6:2016). Non-human primates are closest to human bone structure and dogs have been widely used in orthopedic and dental studies, but for ethical reasons, rodents, rabbits, pigs, and sheep are preferred. The latter has become increasingly popular in recent years as an animal model used in bone research due to their superior vertebrate quality and non-pet status. Sheep are readily available animal models that are inexpensive to maintain and have a high resistance to pain and respond very well to surgical procedures (Vlaminck, 2008).

In terms of macrostructure, adult sheep offer the advantage of having a human-like body weight, with skeletal support of suitable size for the placement of implants or human prostheses, which is not possible in the case of smaller animal species such as rabbits or rodents.

The dental anatomy of sheep significantly differs from that of humans. The incisors are separated from the rest of the teeth (premolars and molars) by an edentulous area of 3-5 cm. The premolars are relatively small, have a long and prominent hypsodont crown compared to the small mesial and distal root. They have the best accessibility when it is desired to extract them for testing dental implants. In the premolar area of the sheep's mandible, thin apical bone plates are present along with a well-developed neurovascular canal. As the mandible matures, these plaques will become thicker, providing solid support for the implants to be tested (May, 1964).

The aim of the study was to evaluate an innovative treatment in periimplantitis in sheep, carried out in three stages: preparation of the implant bed (I), fixation of the implants (II) and induction of periimplantitis (III), and in phase I of the process of osteogenesis following the extraction of the first 2 premolars was verified.

MATERIALS AND METHODS

All procedures during the study were performed at Băneasa Animal Facility (BAF), Preclinical Testing Unit, Cantacuzino National Medico-Military Institute for Research and Development (CI). Animal studies have been approved by the CI Ethics Committee and authorized by the competent authority, in accordance with the provisions of EU Directive 63/2010 on the rules for the care, use and protection of animals used for scientific purposes. BAF is also an authorized unit under current legislation as a user of animals used for scientific purposes.

Ten 4-year-old Ţigae sheep were included with an average weight of 45 kg at the beginning of the study. The animals came from a farm in Hungary and on arrival at the BAF, they were weighed and clinically examined by a veterinarian, who monitored their general condition and identified possible oral abnormalities. Throughout the quarantine period, the sheep were scored weekly for welfare, sheltered together, receiving water and fodder ad libitum.

To achieve the objectives of the study, the sheep were subjected to the procedure of extraction of the first 2 premolars, from each arch, maxilla and mandible, left-right, so that at the end of the procedure a total of 8 teeth were extracted. 12 hours before, the animals were weighed, their blood was collected for biochemical examination (pre-anesthetic profile), put on a total diet of water and food and received a preventive dose of antibiotic (Enrofloxacin FP 10%, Farmavet Group, Romania). Anesthesia was aimed at premedicating animals with Dexmedetomidine (Dexodomitor, 0.5 mg/ml, Orion Pharma Finland) and Ketamine (Ketabel 100 mg/ml, Bela-Pharma, Germany) IM, followed by

administration of Propofol (Propofol Fresenius, 10 mg/ml, Fresenius Kabi, Germany) IV and intubation. Maintenance of anesthesia was performed with Isoflurane (Anesteran, Rompharm Company, Romania) 3%. The sheep were placed in lateral recumbency on the surgical table and the oral cavity was disinfected on the outside with Betadine 3% (Figure 1).

Figure 1: Anesthetized sheep, ready for tooth extraction

From each half arch, the premolars were extracted by dislocating each with a dental elevator. By rotational movements in the axis of the tooth and traction perpendicular to the outside, with the pliers (Figure 2), the periodontal ligament was cut and thus the tooth was extracted. (Figure 3).

Figure 2: Dental instruments used for premolars extraction

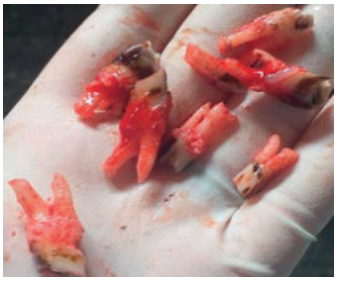

Figure 3: The tooths after extraction

A hemostatic sponge was inserted into the tooth socket (Figure 4) and the gum was sutured with 3/0 resorbable thread (Megasorb, Vetro Design, Romania) over it.

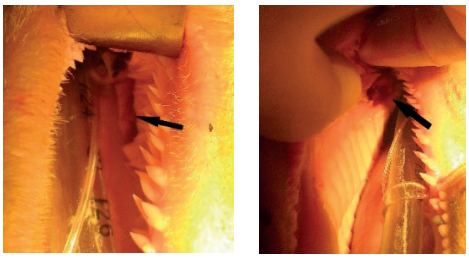

Figure 4: Tooth alveoli after the extraction

After extraction, the animals were infused with 0.9% saline and received anti-inflammatory treatment (Meloxicam 0.5 mg / kg – Melovem 5 mg/ml, Dopharma Romania) for 3 days and antibiotic (Enrofloxacin 2.5 mg/kg) for 5 days. During the monitoring period (3 months), the way of animals feeding was followed but also the body weight and the local clinical condition. The hepatic and renal profiles were monitored paraclinical, and bone regeneration was evaluated by computed tomography (CT) examination.

RESULTS AND DISCUSSIONS

Clinically, the animals performed favorably. Immediately after recovery from anesthesia, resuming the physiological process of chewing. The chewing of the fodder, both pellets and of the green mass, did not show any sign of oral discomfort. Bodyweight monitored on days 0, 45 and 90 showed exponential increases of up to 18% compared to day 0 (Figure 5). After extraction, the dental alveoli began the healing process, after the first 7 days, so that at the clinical examination on day 14, the dental alveoli, both the maxillary and the mandibular ones were in different stages of physiological refilling (Figure 6).

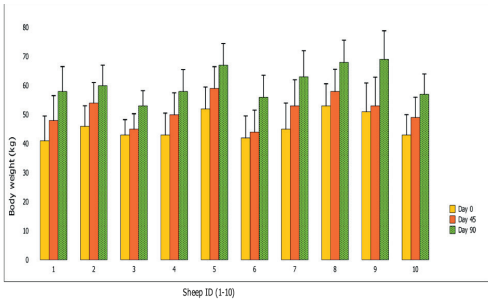

Figure 5: The average body weight/sheep

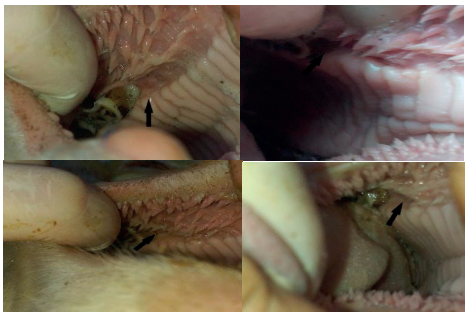

Figure 6: Dental alveola in different stages of healing (day 14)

The biochemical examination, performed on an Idexx VetTest 8008 device, on days 0, 45 and 90 followed any changes due to the anesthesia-induced on the day of extraction but also before performing the Computer Tomography examination. A slight increase in urea and ALT could be observed, on day 45, because of anesthesia from day 0, values that resolved on their own until the next operation, without additional drug support (Tabel 1).

Tabel 1: Biochemic profile of the sheep on day 0, 45 and 90

		Urea	Crea	ALT	ALK P	PT
D0	Sheep 1	6,1	62	37	77	81
D45		12,1	44	26	79	86
D90		6,8	53	31	48	66
D0	Sheep 2	5	53	26	79	86
D45		9,6	53	30	61	84
D90		3,9	53	29	16	38
D0	Sheep 3	6,8	62	10	169	75
D45		10,4	71	38	181	77
D90		6,8	71	19	91	79
D0	Sheep 4	6,8	53	28	161	74
D45		11,1	62	33	186	71
D90		5,4	44	30	74	57
D0	Sheep 5	6,8	44	41	116	83
D45		9,6	53	52	171	85
D90		6,1	53	20	78	93
D0	Sheep 6	6,8	53	22	134	80
D45		10	62	49	55	86
D90		3,2	35	20	78	93
D0	Sheep 7	7,9	53	30	150	80
D45		12,5	53	38	103	87
D90		7,5	62	34	107	75
D0	Sheep 8	6,4	53	39	132	80
D45		10	44	48	81	84
D90		4,6	44	34	57	69
D0	Sheep 9	6,8	44	32	76	80
D45		9,6	44	48	88	85
D90		3,9	35	39	37	57
D0	Sheep 10	6,8	35	31	258	77
D45		10,4	44	42	345	84
D90		6,1	62	39	207	76

Imaging examination - 90 days after the extraction, the tomography of the head was obtained under general anesthesia. Each sheep was positioned in ventrodorsal recumbency on the table of the CT scanner, and the position of the head was adjusted for symmetry. CT images, obtained with a conventional CT scanner (Philips, USA) of the 4Vet imaging center, Bucharest, Romania, showed a degree of bone atrophy within physiological limits. The density of neoformation bone tissue indicated an optimal regeneration, ensuring a uniform bone bed. Only in two cases were observed small pieces of the roots which were sequestered inside the alveolar bone (Figure 7).

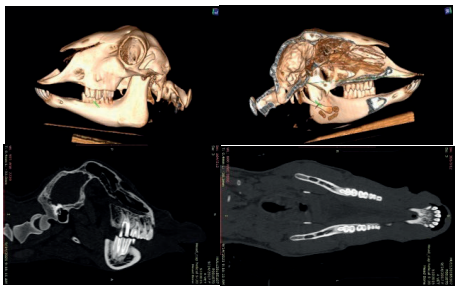

Figure 7: CT images which show a good bone regeneration

The osseointegration was originally defined as a direct structural and functional link between the living bone and the surface of an implant. It is now said that an implant is considered osseointegrated when there is no relative progressive movement between it and the bone with which it is in direct contact. Although the term “osseointegration’ was originally used to refer to titanium metal implants, the concept is currently applied to all biomaterials that have the ability to osseointegrate. (Marquezan et al, 2012).

Primary stability in implant placement is one of the most critical factors determining the outcome of implant therapy. The key factors in improving the primary stability of the implant are bone density (Trisi et al, 2011), surgical protocol, (Turkyilmaz, 2008), and implant type (Dos Santos, 2011). Primary stability is ensured by the mechanical friction between the outer surface of the implant and the osteotomy walls of the implant.

Sheep have a healing ability comparable to that of humans, making them potentially interesting

for studying bone remodeling (Martini, 2001). It is generally accepted that the chances of successful osseointegration of an implant into the oral area increase when a non-stress-free healing period can be provided, but the preparation of the implant bed is just as important as the implant itself. When choosing the technique of premolar extraction, it must take into account that sheep are ruminants, and their mandibular structures are constantly exposed to strong compressive forces, shear and continuous muscle activity (May, 1964). These elements can influence the process of tissue regeneration, through excess alveolar bone lesions, the bone support becomes improper to place an implant (Barzilay, 1993). Clinical, radiological, and CT parameters are generally used to assess the dentoalveolar reconstruction (Schouten, 2009) and the healing progress is assessed based on bone changes obtained from imaging examination. Although two-dimensional evaluation of dentoalveolar bone remodeling can be done by normal radiographs, such an analysis is insufficient to appreciate subtle changes in bone density (Kim et al, 2008). CT seems to be the best imagistic method for the morphological and qualitative analysis of bone and an additional advantage of CT measurement is that bone density can also be calculated (Brett, 2015). In our rough evaluation of the CT-based 3D reconstruction of the sheep's jaws, 90 days after extraction, the dental alveoli showed a large amount of bone augmentation and uniformity of the alveolar ridge.

Sheep suffer seasonal bone loss. While in mature sheep, the trabecular bone is denser and stronger than in humans, immature sheep have a weaker, lower-density trabecular bone, which is very flexible due to its high collagen content. For the research of implant-type medical devices, it is recommended to choose adult sheep to eliminate the risk of implant failure (Bonucci, 2014).

From a veterinary perspective, surgery, such as dental extractions, is necessary in case of dental abnormalities or premature loss of incisor teeth. These are major problems because the affected sheep cannot bite the pasture resulting in malnutrition, poor production, and weight loss as is the case with premolar and molar teeth whose excessive growth, wear or absence causes problems with chewing fibrous feed and subsequent weight loss.

Effectively functioning teeth are fundamental for optimal sheep production. Dental disorders are relatively common and can affect individual sheep or multiple sheep within a flock. A careful examination of the incisor teeth is a straightforward and essential part of any veterinary examination of sheep and provides valuable information on age and dental abnormalities. Examination of the cheek teeth is more difficult but should be attempted in any animal or flock with a history of weight loss, cud-staining, unusual mastication, or jaw swelling. By the method approached in this study, we observed an increase in weight gain and an obvious general good condition, so we can recommend intervening by tooth extraction when dental abnormalities are encountered that endanger the welfare of the animals and harm the breeders.

CONCLUSIONS

The extraction of the first 2 premolar teeth, left-right, top and bottom provided the uniform and compact bone support necessary for the next stages of the study. Osteointegration could be demonstrated both by clinical examination and by assessment of bone structure on CT. We also propose the technique applied for sheep herds in which there are dental diseases; it can serve as a starting point for the further development of comprehensive, valid, and practicable protocols for sheep welfare.

ACKNOWLEDGEMENTS

This work was supported by a grant of the Romanian National Authority for Scientific Research and Innovation, CCCDI-UEFISCDI, project number 89/2019 within PNCDI III. The authors contributed equally to the study. Thanks to the biochemist Gheorghiu Petronica, Ioniță Fabiola and Suhăianu Vladimir veterinarians for their support throughout the study, as well as the Pet Stuff and 4Vet team for their support in providing anesthesia and imaging, respectively.

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VETERINARY EDUCATION

Scientific Works. Series C. Veterinary Medicine. Vol. LXVIII (2), 2022
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

PLATFORMS AND APPLICATIONS USED IN TEACHING AND CONSOLIDATION OF VETERINARY PHARMACOLOGY

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Abstract

In the last three years, the economic, social and health situation of the whole world has been deeply tested and not at all predictable. The way of life imposed by Covid-19 changed the paradigm of many human activities, of which education could not be an exception.Taken by surprise, at a very low level of digitization of teaching-learning activities, Romania has suddenly switched to online education. Thus, the use of platforms such as Zoom Video Communications or Microsoft Teams for teaching and moreover, avoided the freezing of the school and university year.The use of applications in support of learning, such as books creator, random cards or random wheel, have also proved their usefulness for consolidating knowledge of veterinary pharmacology.

Key words*: teaching, consolidation, platforms, applications, pharmacology.*

INTRODUCTION

In the last three years, the economic, social and health situation of the whole world has been deeply tested and not at all predictable. The way of life imposed by Covid-19 changed the paradigm of many human activities, of which education could not be an exception.

Taken by surprise, at a very low level of digitization of teaching-learning activities, Romania has suddenly switched to online education. Thus, the use of platforms such as Zoom Video Communications or Microsoft Teams for teaching and moreover, avoided the freezing of the school and university year (Sofi-Karim et al., 2022).

Both Zoom and Teams served to create virtual classrooms and workshops where teachers and students could interact online to carry out their teaching-learning activities, despite the social distance imposed by the global epidemic.

These platforms have made it possible to make group video calls from your laptop, PC, tablet or smartphone, by using chat, recording courses, having at your disposal a virtual whiteboard an ideal tool for distance learning.

Thus, video conferencing has become a common medium for many companies and institutions throughout the pandemic given the fact that the vast majority of meetings, either business related or online courses, were conducted through them.

Given the circumstances of the past years the educational platforms and applications have become useful for any curricular area (Andron et al., 2021). This evolution has been adapted to suit the teaching-learning process of our faculty, including the Pharmacology Discipline (Karacsa et al., 2013).

MATERIALS AND METHODS

The Zoom platform was founded in 2011 by Eric Yuan, an engineer who, being a Cisco employee, started from the idea that the existing platforms were outdated and dull. With Zoom, video conferencing has become a common workplace for many companies in the context of quarantine, and most online courses and meetings have been conducted through it (Huddleston, 2019). Thus, Zoom has become one of the most important video conferencing software applications in the world, being used in all fields, both for online learning and for tele-working.

Second, Microsoft launched Teams in New York in March 2017 as a useful web application for the modern educational

environment, which in November 2019 numbered 20 million users and in March 2020 it reached 44 million daily users, largely due to the COVID-19 pandemic. Microsoft Teams allows real-time sharing and editing of Word, Excel, Power Point, audio/video files, or screen sharing for up to 10,000 people (Anderson, 2018).

Examples of useful applications for online learning, which allow you to send messages, send files and participate in fully digitized lessons, all with applicability in teaching medical subjects are, among others, book creator, flash cards and or random wheels (Shi et al., 2020).

We also used them in teaching-learning the contents of the subject Pharmacology in quarantine and after that, continuing the educational activity and avoiding the freezing of the academic year (Figure 1).

Figure 1. Keeping the enthusiasm in the pandemic (foto credit Andreea Fira and Andrei Rădulescu)

Both Zoom and Teams help create a virtual classroom, where teachers and students can interact online to carry out their educational activities, despite the social distance imposed by the global epidemic. It allows you to make group video calls from your laptop, PC, smartphone or tablet, by using chat, recording courses, and having a virtual whiteboard, an ideal option for distance learning.

Applications for teaching and learning (memorization) are just as useful. Of these, Book Creator, random cards and random weel have found their applicability in the activities carried out both at school and at home.

Book Creator allows you to create books in digital format that can then be assigned to a group or learning unit. It is an application used in educational institutions around the world for teaching all types of subjects. It allows its users to create materials that include images, text or video.

Flash cards are cards composed of two sides as follows: The front of the card may include a question or a notion whereas the back includes the answer or the definition/explanation. It is an application that generates collaborative interactions between teachers and pupils or students.

Random wheel is an application that allows the teacher to randomize the retrieval of a question from a data set by spinning / actuating a digital wheel.

All these applications can be easily accessed, created and customized according to the specifics of the teaching discipline and can easily be made available to groups of pupils or students (Lee et al., 2018).

RESULTS AND DISCUSSIONS

We have used the Zoom Meetings platform for more than 2 years given the pandemic conditions of COVID-19 - teaching regularly and continuously, while recording the sessions to be lately used as an educational source. As a result of this routine, we understood, together with the students, that the best features of this rather intuitive platform are reserved for paid versions, which can be quite expensive.

The free of charge policy and the huge number of individuals allowed to attain a meeting turned Microsoft Teams into an attractive alternative to Zoom. Experience has shown us the disadvantages of the platform, the expulsion of conference participants, confusing structural files, and limited flexibility.

In both cases, we were able to use chat as an additional means of interaction, with both platforms finally proving their usefulness in overcoming the pandemic crisis.

We mentioned that, in a ranking of digitization in European countries, prepared by the European Commission in 2020 (at the beginning of the pandemic), Romania ranks 26th out of 28 countries, with a percentage of only 49% of households connected to the Internet (Figure 2).

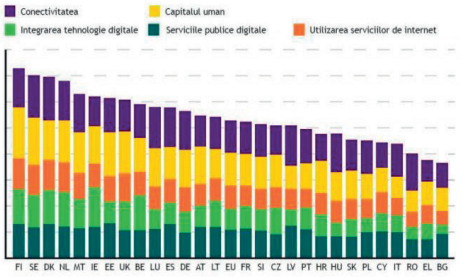

Figure 2. Digitization ranking in European countries in 2020 https://www.dendrio.com/noutati/fonduri-europene-pentru-digitalizare-profitati-de-oportunitate/

In this context, we resorted to the virtual learning model, then, as the epidemic showed signs of fading, migrated to a hybrid system. In both cases, the interactive educational applications have proven their worth and are still useful despite the end of the pandemic in February 2022. Together, we created a digital book using Book Creator to present the most commonly used medicinal products in the veterinary clinic for therapeutic purposes. We have named it a memorizer of medicinal products, starting from the intended purpose, that of facilitating the easier memorization of indications and therapeutic doses (Figure 3).

Figure 3. Book sheet made with Book Creator https://app.bookcreator.com/books/0DDxeeDGTP-NmJvLzUmamg/GbMTXJTbSniQ1geeTD6HTA

For flash cards there are countless options with the ability to create, customize and distribute. Several platforms offer the possibility of creating and using Quizlet, World wall, Twinkle, etc. As for us, we note and recommend the use of a set of cards that explain the abbreviations used in pharmacology (Figure 4).

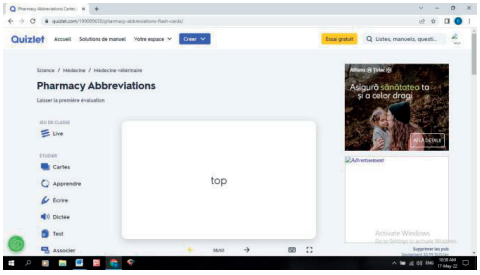

Figure 4. Set of cards with notions of pharmacology https://quizlet.com/190089650/pharmacy-abbreviations-flash-cards/

Random wheel can also be used in learning activities, as a way to extract the topic to be discussed/verified. Once all the clarifications have been made and all the unclear aspects have been clarified, the teacher can exclude the subject from the range of subjects. The wheel can be spin again thus choosing another topic of discussion for another student (Figure 5).

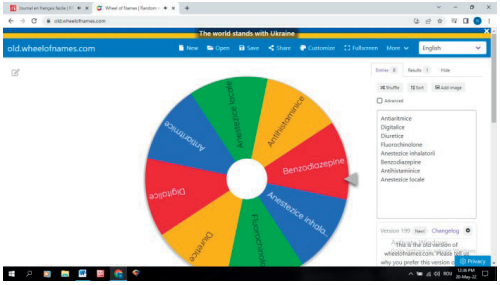

Figure 5 Random wheel https://old.wheelofnames.com/

All these applications are used for better learning, interaction and training of students in the learning-assessment approach taken by the Pharmacology Discipline of our faculty. By the end the pandemic most individuals enhanced their digital experience through the use of online platforms and applications.
Despite the return to the traditional teaching approach, we can still apply the experience acquired throughout recent years to improve our learning process. With the pandemic in mind, we continue our veterinary medical education.

CONCLUSIONS

For the past three years, veterinary medical education has experienced virtual and hybrid

models.Institutional digitization has been improved with the skills of teachers and students to use digital Internet solutions.

The use of Zoom, Teams and institutional platforms created the framework for continuing the teaching-learning-assessment activity at a time when the risk of stop education activities was discussed.

Book Creator, Flash Cards, Random Wheel applications used by teachers and students increase the level of attractiveness and interaction in educational activities.

In our turn, we have used and are using these applications in the process of teaching the contents to the Pharmacology Discipline of the Faculty of Veterinary Medicine.

Educational platforms and applications continue to prove their usefulness even when it comes to returning to physical learning.

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ISSN 2065 – 1295
ISSN-L 2065 – 1295