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SUMMARY

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COMPARATIVE ANATOMIC RESEARCH REGARDING THE RED DEER (*CERVUS ELAPHUS*) AND FALLOW DEER (*DAMA DAMA*) SKULLS

Cristian BELU, Gabriel PREDOI, Iulian DUMITRESCU, Bogdan GEORGESCU, Florica BĂRBUCEANU, Anca ȘEICARU, Petronela ROȘU, Gavrilă ZAGRAI, Mădălina DOBRILĂ, Theodora ȘTEFĂNESCU, Paul STOICULEASĂ, Oresti MIHELIS

University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, 105 Splaiul Independenței Street, District 5, 050097, Bucharest, Romania

Corresponding author email: cristbelu@yahoo.com

Abstract

The comparative morphology of the skulls is a field of anatomy that can provide answers to questions related to the relationships between species, helping to classify them and to place in a taxonomic group. Till now, a number of morphometric studies have established some differences in red deer and fallow deer skulls, considered as criteria for identification according to the geographical area to which the animals belong, depending on sex or age. The works containing comparative descriptions are very few and the details are absent. Our study presents a number of important aspects for differentiation, of which we list: particularities regarding the drawing of the bone sutures and the profile line in lateral view of the skull, the different conformation of the retro-glenoid process of the temporal bone, differences regarding the relative size of the alveolar process of the maxilla as well as the topography of the infraorbital hole etc. The data provided are useful when making the skulls expertise in order to identify this.

Key words*: skull, red deer, fallow deer.*

INTRODUCTION

The red deer (*Cervus elaphus*) and the fallow deer (*Dama dama*) are two valuable game species in the Romanian fauna. Both belong to the *Cervinae* subfamily. The two species are morphologically similar, though the red deer is larger than the fallow deer. However, size cannot be the only differentiating criterion between species, considering that sometimes a female red deer can be compared, weight wise, to a more well developed fallow deer (Secașiu et al., 2019; Breda et al., 2013).

Following bibliographical research we can appreciate that the data in specialty literature strictly referring to the compared morphology of the two species is relatively lacking. Lister, A. (1996) offers one of the most detailed descriptions regarding the differences between the bones and teeth, but it makes no references to anatomical elements pertaining to the skull.

There is a series of works which manifest the interest of the authors regarding the morphology of the horns, being a known fact that these are present in all the males of the wild cervid species and they are game trophies, in some cases very valuable. The morphology of these organs is an indicator of the quality of the environment in which these species live (Yudha, 2019; Evans et al., 2005). Lastly, there are morphometric studies regarding the skulls of some cervid species (common deer, red deer, fallow deer) which deal with the differentiating criteria in relation with the area, age or sex of a certain individual (Markov, G., 2014; Onuk, B. et al., 2013).

Considering the reduced number of information referring to the comparative morphology of the two species of large cervids pertaining to the game fauna of Romania on the basis of which establishing the provenience of bones or bone fragments would be possible, we have decided to conduct this study.

The aim of this study is to identify a series of skull particularities, such as differences in conformation, size comparisons, or even the presence or absence of some anatomical elements on the basis of which the origin of the bone or bone fragment can be correctly deduced, as well as offering data for legal medicine which can provide answers regarding the origin of the bones that can be evidence in legal disputes.

MATERIALS AND METHODS

Our research was based on trophy skulls, ten belonging to red deer (*Cervus elaphus*) and eight to fallow deer (*Dama dama*).
The provenience, and their ages were very different. Some were provided by the General Association of Hunters and Sportive Fishers of Buzău County, while some originated from private collections. According to the evaluation data, all individuals were males of various ages, collected between 2005 and the present day.

RESULTS AND DISCUSSIONS

In both species, the following bones, listed caudo-rostrally, participate at the formation of the **dorsal side** of the skull: occipital bone, parietal bones, frontal bones and nasal bones. Some minor contributions can be attributed to the temporal, lacrimal, maxillary and incisive bones (Figure 1).

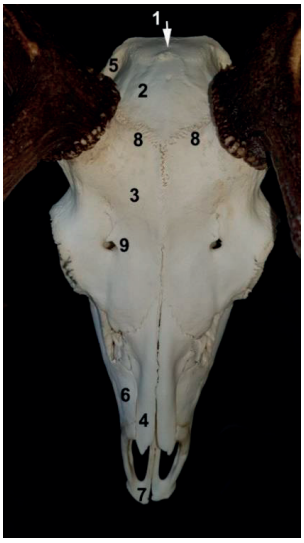

Figure 1. The dorsal side of the skull of the fallow deer (original): 1 - occipital; 2 - parietal; 3 - frontal; 4 - nazal; 5 -portion of the temporal bone; 6 - maxillary; 7 - incisive; 8 - fronto-parietal sutures; 9 - supraorbital foramen

The occipital bone participates at the formation of this side through its squamous portion. The dorsal side of this portion is separated by the nucal portion through a very wide crest, laterally continued through a nucal crest. A sagittal external crest cannot be identified, rather some reliefs (rough spots) of muscular insertion, disposed medially. Rostral to the occipital bone is the territory of the parietal bones, which are slightly concave rostro-caudally in the red deer. This territory also sees a series of muscular insertion fossae.
The symmetrical parieto-frontal sutures form an approximately 90 degree angle. The temporal lines are distanced from the median plane, with 5-6 cm between them at their closest, their rostral side reaching the aforementioned suture (Figure 2). The frontal bones delimit the largest area of the dorsal side, and include, in males, the thick horn processes, distanced from the orbit. The rostral extremity of the frontal bones is articulated with the caudal edge of the nasal bones. This rostral edge forms an acute angle with the lateral edge. In the fallow deer this angle is rounded.

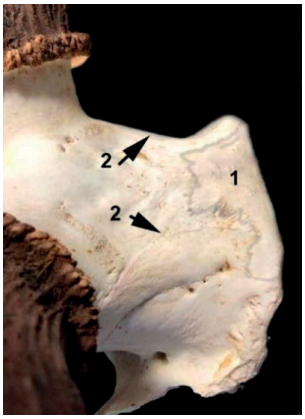

Figure 2. The dorsal side of the neurocranium in the red deer (original): 1 - the scvamosal part of the occipital bone which participates in forming the dorsal side; 2 - temporal lines

The nasal bones are rectilinear aboral-rostrally in the fallow deer and convex in the red deer (Figure 3 A). Transversally they are convex in both species. Both species present a large fronto-lacrimal-maxillo-nasal fissure. Each nasal bone has an apex oriented in the continuation of the lateral edge. Between the apexes of the two bones there is a slight arcade. In the red deer, the rostral processes of the nasal bones are better developed.
In the lateral view, at an initial analysis, two distinct elements can be observed between the skulls: the profile of the dorsal side of the neurocranium is concave in the red deer and

convex in the fallow deer, while the maxillary region has the superior edge convex in the red deer and rectilinear in the fallow deer. Moreover, the line of the diastema is slightly concave in the red deer and rectilinear in the fallow deer (Figure 3 B).

In the temporal region, the drawing of the sutures is almost identical in both species, but the nucal crest describes a line curved dorso-ventrally at the red deer and broken at the fallow deer. Also in this region it can be observed that in the red deer the temporal crest is very well represented, prominent at the level at which the zygomatic process of the squamous portion of the temporal bone detaches (Figures 3-7).

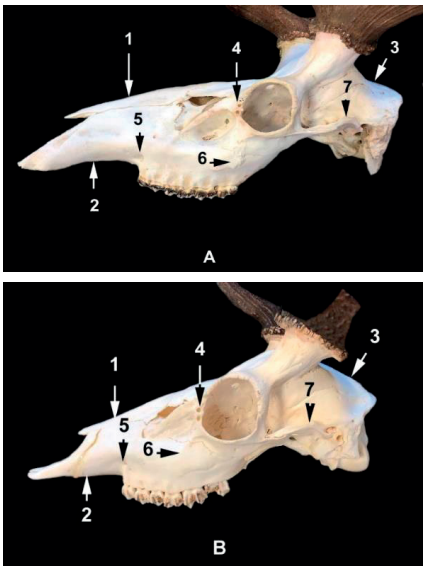

Figure 3 The lateral aspect of the skull for the red deer (A) and fallow deer (B) (original): 1 - the profile of the nasal bone; 2 - the line of the diastema; 3 - the profile of the dorsal side of the neurocranium; 4 - lacrimal holes; 5 - infraorbital hole; 6 - facial tubercle; 7 - temporal crest

The retroarticular process, visible on the lateral-ventral side, is notably different in both species. Its lateral and medial angles are equal in the fallow deer, while at the red deer the medial angle is rounded and better represented than the lateral angle (Figure 4).

The orbital region is characterised through a complete orbit, with prominent edges. In both species there are two lacrimal holes, placed on the contour of the orbit, caudally from the external lacrimal fossa. The latter is very deep at the red deer

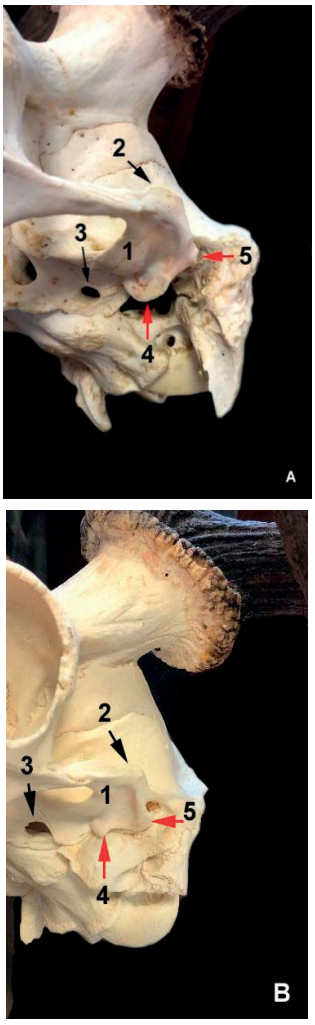

Figure 4. The base of the zygomatic process of the temporal bone in the red deer (A) and fallow deer (B) (original): 1 - condyle; 2 - temporal crest; 3 - oval hole; 4 - medial angle of the retromuscular process; 5 - lateral angle of the retroglenoidian process

The ethmoid hole is situated on the medial wall of the orbit, on the suture between the wing of the presphenoid and the orbitary portion of the frontal bone. In the orbitary hiatus the hole for the optic nerve can be identified, situated dorso-rostrally from the orbital-rotund foramen. We appreciate that the muscular tubercle which covers the latter orifice laterally is better represented in the red deer.

Making a rapport of the length between the alveolar process of the maxillary bone (measured between the oral edge of the first alveola and the aboral edge of the last alveola)

and the distance between the last molar and the rostral edge of the zygomatic process of the temporal bone, an average of 1.23 was obtained for the fallow deer and 0.87 for the red deer, in other words the molar arcade and implicitly the occlusal surface is larger in the fallow deer.
The positioning of the infraorbitary foramen is different. The length between the maxillo-incisive suture and the first alveola (Figure 5 a), in rapport to the distance between the contour of the infraorbitary foramen and the diastema (Figure 5 b) is on average 9.54 in the red deer and 3.07 in the fallow deer, which means the infraorbitary orifice for the former species is very close to the diastema (Figure 5).

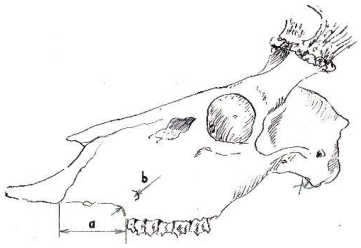

Figure 5. Topography of the infraorbitar hole (original scheme)

The facial tubercle, located under the external lacrimal fossa, is well developed in the red deer and rather dull in the fallow deer. A final appreciation regarding comparative aspects of the skull in a lateral view is represented by the rapport between the total length measured between the rostral extremity of the incisive and the most aboral portion of the occipital in a straight line (Figure 6 a), and the height of the skull at the level of the supraorbitary hole (Figure 6 b). This rapport is of 6.12 in the red deer and 4.07 in the fallow deer. Observations have proved that this value at the red deer is influenced by the development of the splachnocranium.

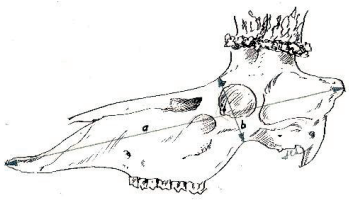

Figure 6. The ratio between the length and height in skull in *Cervidae* (original scheme)

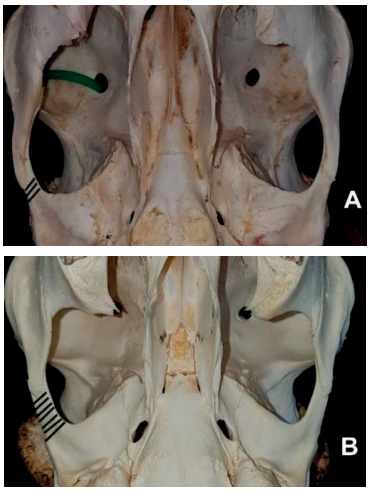

Figure 7. Differences regarding the development of the zygomatic arcade in the red deer (A) and fallow deer (B) (original)

On the ventral view, the junction of the zygomatic bone with the zygomatic process of the temporal bone is very close to the articular surface of the latter, while in the fallow deer this junction is approximately halfway to the zygomatic arcade (Figure 7).
The roof of the oral cavity has an rostral palatine foramen situated in the territory of the palatine process of the maxillary bone in the red deer, while in the fallow deer this orifice is placed on the maxillo-palatine suture (Figures 8, 9).

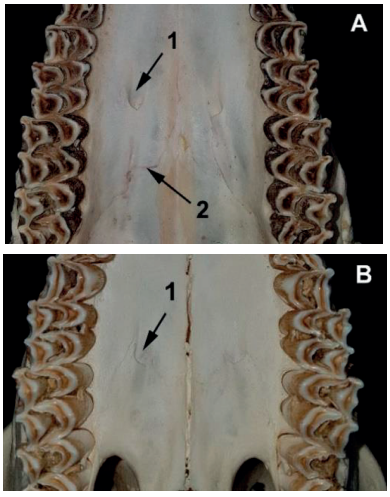

Figure 8. Differences regarding the topography of the rostral palatine foramen at the red deer (A) and fallow deer (B) (original): 1 - palatin rostral hole; 2 - maxilopalatin suture

Other differences in the region of the premolars and molars are insignificant, however it can be noted that the prealveolar portion of the maxillary bone is much longer in the red deer (Figure 9). It has been proven that the rapport between the length of the alveolar process of the maxillary bone and the length of its prealveolar portion, measured from the first alveola to the caudal side of the palatine fissure, is of 1.4 in the red deer and 2.2 in the fallow deer (Figures 9, 10). This proves that the splachnocranium of the red deer is more elongated than that of the fallow deer. In both species the spheno-basioccipital muscular tubercles are strong and well developed.

On the caudal view, the occipital foramen magnum is delimited by two condyles which are similar in shape for both species. The rapport between the width of the occipital foramen to the length of the skull was 16.33 in the red deer and 11.4 in the fallow deer, which proves that the orifice is more spacious for the fallow deer.

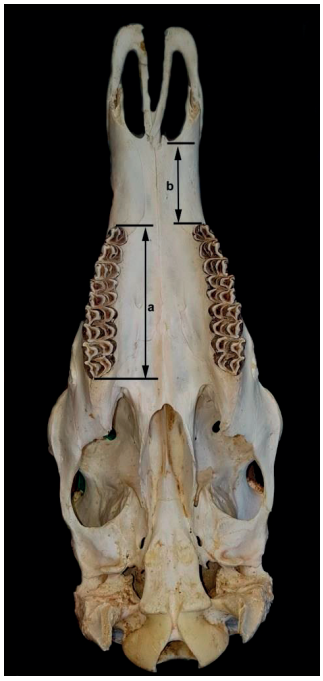

Figure 9. The ventral side of the skull in the red deer (original)

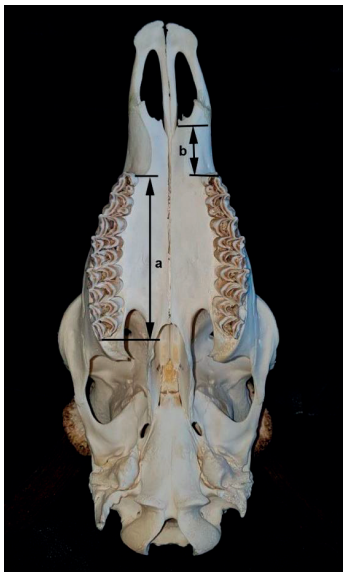

Figure 10. The ventral side of the skull in the fallow deer (original)

CONCLUSIONS

A first general conclusion is the fact that the different size between the two species cannot constitute a differentiating criteria of the skulls, especially when it comes to fragments.

The lateral exposure of the two types of skulls provides important differential aspects, namely the different profiles of the dorsal side of the neurocranium and splachnocranium, as well as the diastema.

The nucal lines and the temporal crests are distinct from a conformational point of view.

There are specific particularities regarding the topography of the infraorbitary foramen, the rostral palatine foramen and especially the retromuscular process. The measurements have allowed obtaining some clues through rapports between dimensions we have considered representative, totally different in the two species.

The final conclusion is that the large quantity. The data obtained is useful in establishing the origin of skull fragments when situations impose this, although the subject can very well be explored in the future.

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PHENOLICS CONTENT, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF SOME EXTRACTS OBTAINED FROM ROMANIAN SUMMER SAVORY AND LEBANON WILD THYME

Corina PREDESCU, Camelia PAPUC, Georgeta ŞTEFAN, Carmen PETCU

University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, 105 Splaiul Independenţei Street, 050097, District 5, Bucharest, Romania

Corresponding author email: durduncorina@yahoo.com

Abstract

The aim of the present study was to compare the antioxidant and antibacterial properties of summer savory (Satureja hortensis L.) from Muscel County flora (Romania) and wild thyme (Thymus serpyllum) from Lebanon. The aerial parts of plants were harvested in august, dried quickly and alcoholic extracts were prepared. Total phenolic content (TPC), total flavonoid content (TFC), DPPH radical scavenging activity, ferric reducing antioxidant power (FRAP) value, and antimicrobial properties were determined using the extracts. TPC showed that Lebanon thyme had higher concentration compared with Romanian thyme (13.78 ± 0.95 mg GAE/g DW and 12.14 ± 0.97 mg GAE/g DW, respectively). DPPH• was calculated as IC_{50} and the sample results were compared with gallic acid. FRAP results showed similar values 42.71 ± 4.24 µM Fe^{+2}/g DW (Romanian thyme) and 39.55 ± 4.21 µM Fe^{+2}/g DW (Lebanon thyme). The antibacterial activity of summer savory was found to have maximum effect against Staphylococcus aureus ATCC 9144. Bioactive compounds, measured as total phenolic content, were in higher concentration in both extracts which also relates to their antioxidant and antibacterial activities.

Key words*: Satureja hortensis, Thymus sepyllum, phenolics, flavonoids, antioxidant and antimicrobial activity.*

INTRODUCTION

In the past few years, the food industry producers have tried very hard to change direction toward a clean label. The replacement of synthetic preservatives with phenolic structures, such as butylated hydroxyanisole (BHA), with extracts obtained from aromatic plants rich in natural phenolics, has attracted the attention of the researchers, due to speculation about the possible toxic effects of synthetic antioxidants (Papuc et al., 2010).

Aromatic plants have been studied extensively because they are a rich source of natural antioxidants and antimicrobial substances which can be extracted relatively easily using different solvents. Summer savory and wild thyme, could achieve these demands due to their active compounds found in their extracts (Gedikoğlu et al., 2019). Recent trends for natural food additives made plant extracts with antioxidant and antimicrobial properties an important step to obtain a clean label and proved beneficial food products for the consumers (El-Guendouz et al., 2019; Gonelimali et al., 2018). It was reported that the antioxidant and antimicrobial effects of aromatic plants are closely related to the presence of phenolic compounds (Kulisic et al., 2005). *Satureja hortensis* L. (summer savory, thyme), member of *Lamiaceae* family, is a variety of an annual herbaceous crop species, flowering shrubs, found in many parts of the world, native from the western Mediterranean to southern Europe. Growing up to 30 cm tall, by 40 cm wide, it is a bushy evergreen subshrub with small, aromatic, grey-green leaves and clusters of purple or pink flowers in summer (Fierascu et al., 2018). Summer savory is known especially as aromatic herb and have an intense culinary use. In folk medicine, these herbs are used against headaches, toothaches, colds, asthma, and rheumatism (Gedikoğlu et al., 2019).

Thymus serpyllum is a perennial shrub, known as Breckland thyme, wild thyme, or creeping thyme; however, its specific name "*serpyllum*" is derived from the Greek word meaning "*to creep*", because of wild thyme's trailing habit. It is a species of flowering plant in the mint family *Lamiaceae*, native to regions of Europe, Asia and North Africa. It is a low, subshrub

growing to 2 cm tall with creeping stems up to 10 cm long. The oval evergreen leaves are 3–8 mm long. The strongly scented flowers are either lilac, pink-purple, magenta, or a rare white, all 4-6 mm long and produced in clusters. The hardy plant tolerates some pedestrian traffic and produces odours ranging from heavily herbal to lightly lemon, depending on the variety. It has high tolerance for low water and poor nutrient soils. The increase of pathogenic microorganism's multidrug resistant has led to extensive phytochemical and pharmacological studies of *T. serpyllum* as an important source of medicinal substances with antioxidant and antimicrobial properties (Jarić et al., 2015).

Based on *in vitro* tests, Uysal et al. (2015) reported that their chemical constituents, such as phenols and flavonoids, provide antimicrobial and antioxidant properties.

The ***objective*** of this research was to evaluate the antioxidant and antimicrobial activities of *Satureja hortensis* L. (summer savory), harvested from Muscel County, Romania and *Thymus serpyllum* (wild thyme) growing in Lebanon using a multiple-method approach in relation to their chemical composition, comparatively with synthetic antioxidants used in food industry.

MATERIALS AND METHODS

Reagents and chemicals. Spectrophotometric grade ethanol, 2,4,6 three(2-pyridyl)-S-triazine (TPTZ) reagent $AlCl_3$ anhydrous, sodium nitrite, sodium hydroxide, 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium bicarbonate anhydrous, gallic acid, catechin and Folin - Ciocalteu reagent were supplied by Sigma Aldrich (Germany). Iron (III) chloride hydrate and ferrous sulfate ($FeSO_4 \cdot 7H_2O$) were purchased from Fisher Scientific (UK). Tryptic soy agar, tryptic soy broth, and Muller-Hinton agar were supplied by Merck (Germany). Antimicrobial susceptibility test disks were purchased from Oxoid (UK).

Hydroalcoholic extract preparation. Fresh plant parts (flower, leaves and stems) of both *Satureja hortensis* L. (summer savory) from Muscel County, Romania and *Thymus serpyllum* (wild thyme) growing in Lebanon were collected. Samples were washed, dried and powdered and stored at room temperature, in darkness. For extraction, ten grams of each sample were weighed into a beaker and 100 ml of 60% aqueous ethanol (1:10 ratio of w/v) was added. After 30 min the sample was placed in water bath for 3 hours at 60°C. Next, the homogenates were filtered using Whatman no. 1 filter paper. The two filtrates were placed in 250 ml round-bottom flasks.

Total phenolic content. To determine the total polyphenol content, 0.5 ml of the sample extract and 7 ml of distilled water were mixed with 0.5 ml of Folin - Ciocalteu's reagent. After 5 min, 2 ml of 20% Na_2CO_3 solution was added, and the mixture was incubated for 60 min in the dark. The reaction mixture absorbance was measured at 760 nm, and the reaction mixture without the extract was used as a blank. Gallic acid was used as standard, and a 5 points standard curve was prepared (0-10 mg/dl). The TPC of the plant extract was expressed as gallic acid equivalents/g dry weight plant (mg GAE/g DW) (Singleton and Rosi, 1965).

Total flavonoid content. The total flavonoid content was determined according to the method of Zhishen et al., 1999. 1 ml of extract was placed in a ten ml flask that contained 5 ml distillate water. Then, 0.3 ml of 5% $NaNO_2$ solution were added. After 5 minutes 0.6 ml of 10% $AlCl_3$ were added and after another 5 minutes, 2 ml of 1 M NaOH solution were added and the volume was brought to ten ml with distilled water. The mixture was left 15 minutes at room temperature and the absorbance was read at 510 nm. The results were expressed as mg catechin equivalent/g dry weight plant (mg CAT/g DW).

DPPH radical scavenging activity. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging capacity was measured according to Blois (1958). Next, 50 µl plant extract (at different concentrations in methanol) was mixed with 5 ml of a 0.004% (w/v) DPPH▪ methanol solution. The reaction was allowed to stand at room temperature for 30 min, and absorbance was read against a blank at 517 nm. The inhibitions of the DPPH radical in percent were calculated as follows:

$$I\,(\%) = \left(\frac{A\ blank\ -\ A\ sample}{A\ blank}\right) \times\ 100$$

Where, A_{blank} is the absorbance of the control reaction (containing all reagents except the test sample), and A_{sample} is the absorbance of the extracts. Extract concentration providing 50% inhibition (IC_{50}) was calculated using the graph ‑ plotted inhibition percentage against the extract concentration. Gallic acid and butylated hydroxy anisole (BHA) were used as positive controls in concentration of 10 mg/100 ml. The IC_{50} value for each sample was determined graphically by plotting the percentage discoloration of DPPH• solution as a function of the sample concentration.

The ferric reducing antioxidant power (FRAP) FRAP assay is based on the ability of phenolics to reduce yellow ferric tripyridyl triazine complex (Fe(III)-TPTZ) to blue ferrous complex (Fe(II)-TPTZ) by the action of electron-donating antioxidants (Riahi et al., 2013). The FRAP reagent was freshly prepared by mixing acetate buffer (300 mM, pH 3.6), TPTZ solution (10 mM TPTZ in 40 mM HCl), and $FeCl_3 \cdot 6H_2O$ (20 mM) in a ratio of 10:1:1 (v/v/v). To carry out the assay, 1.8 mL of FRAP reagent, 180 µl distilled water, and 20 µl of plant extract were mixed. After 15 min at 37°C, the absorbance was measured at 595 nm, using the FRAP solution as a blank. The antioxidant capacity of plant extracts was determined from a standard curve plotted using the $FeSO_4 \cdot 7H_2O$ linear regression. Results were expressed as µmols Fe^{2+}/g DW. BHA and ascorbic acid were used as controls in concentration of 10 mg/100 ml.

Antimicrobial activity. Two Gram-positive and three Gram-negative bacteria were used as test organisms: *Staphylococcus aureus* ATCC 9144, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 25922, *Salmonella enteritidis* ATCC 13076, and *Salmonella typhimurium* ATCC 14028.

Disk diffusion assay (DDA) (Oke et al., 2009). All the bacterial species were first inoculated into tryptic soy agar and incubated overnight at 37°C. After checking for purity, the bacteria were suspended in a 0.9% NaCl solution. A spectrophotometer was used to adjust the final cell concentration (1.5×10^8 cfu/ml) by reading the DO at 600 nm. Then, 100 µl of the bacterial suspensions was spread on Mueller-Hinton agar. The 6-mm-diameter, sterile, empty disks impregnated with 20 µl of extracts were placed on the inoculated agar. Antibiotic standard disks were used as a control. The inoculated plates were incubated at 37°C for 24 h. As positive controls, ciprofloxacin and Ampicillin (30 µg/disk) were used for bacterial strains. Antibacterial activity was determined by measuring the *zone of inhibition* in mm without including the diameter of the disk (Valgas et al., 2007).

RESULTS AND DISCUSSIONS

Total phenolic and flavonoid content. Plants from *Lamiaceae* family are known to be rich in compounds possessing strong antioxidant activity. Thyme and wild thyme, are found in many parts of the world, especially in the Mediterranean region, are also regarded as medicinal herbs and condiments. Because of the highest concentration in active compounds, as phenols and flavonoids, thyme has positive effect on health of the consumers, when it is used as tea or added in food. In fact, the food products that contain natural antioxidants have a double benefit: better conservation during the viability period and the excess of antioxidants get to the consumers.

Table 1. Total phenolic compounds and total flavonoid content in hydroalcoholic extracts of Romanian and Lebanon wild thyme

Analysed plant	Total phenolic content (TPC) mg GAE/g DW	Total flavonoid content (TFC) mg CE/g DW
Lebanon wild thyme	13.78 ± 0.95	8.54 ± 0.84
Summer savory	12.14 ± 0.97	7.49 ± 0.79

Values are the average of duplicates ± standard deviation.

The concentration of phenols in plants is dependent on climate and geographical position (Liu et al., 2018). *Satureja hortensis* L. (summer savory) from Muscel County, Romania and *Thymus serpyllum* (wild thyme) growing in Lebanon contain important concentrations of phenols and flavonoids. These findings are similar with other results regarding *Satureja hortensis* L. and *Thymus serpyllum* phenol and flavonoid contents (Ballester−Costa et al., 2017; Plánder et al., 2012; Kulisic et al., 2005). Lebanon wild thyme extract have shown that contains a high content of phenolic 13.78 ± 0.95 mg GAE/g DW and flavonoid 8.54 ± 0.84 mg CE/g DW

compounds when compare to Romanian thyme which had 12.14 ± 0.97 mg GAE/g DW and 7.49 ± 0.79 mg CE/g DW (Table 1). It was calculated that for both types of thyme, about 60% of the phenols were flavonoids. It was also calculated that the Lebanese wild thyme had 14% more polyphenols than the Romanian one. Also, the concentration of flavonoids was lower for the Romanian thyme compared to the Lebanese one with 14%. Statistically significant difference and positive correlation between the concentration of phenol and flavonoid contents of Romanian and Lebanese thyme ($p < 0.05$, $R^2 = 0.9999$).

DPPH radical scavenging activity. DPPH (2,2 - diphenyl - 1 - picrylhydrazyl) free radical scavenging activity was used to investigate the antioxidant activity of two thyme extract by comparation with gallic acid. The effect of antioxidants on DPPH radical scavenging is due to their ability to donate hydrogen. DPPH▪ is a stable free radical which accepts a hydrogen radical from an antioxidant molecule (AH) to become a stable diamagnetic molecule, in accordance with the equation below:

$$DPPH▪ + AH \rightarrow DPPH▪▪H + A▪$$

The concentration of antioxidant needed to decrease the initial DPPH concentration by 50% (IC_{50}) is a parameter widely used to measure the antioxidant activity (Brighente et al., 2007).

The free radical scavenging activity is higher when IC_{50} value is lower. The amount of extract needed to decrease the initial radical DPPH concentration by 50% is used for the free radical scavenging activity and is established as IC_{50}. Results of the DPPH radical scavenging activity test are shown in Table 2. IC_{50} of Lebanon thyme extract (85.25 ± 7.31 µg/ml) was significantly ($p < 0.05$) higher than that found for Romanian thyme extract (102.94 ± 8.14 µg/ml) radical scavenging activity when compared to BHA.

Some researchers reported that the phenolic compounds with hydroxyl groups attached to their structures present in aromatic plants are responsible for the important antioxidant effect (Shahidi et al., 1992). When comparing the sample results with the standard gallic acid (4 ± 0.35 µg/ml), the extracts in the current study had a much lower free radical scavenging activity. A significantly higher correlation was established between total flavonoid content and DPPH radical scavenging activity ($p<0.05$). The results indicated that both Romanian and Lebanon thyme extract have effective DPPH radical scavenging activities. It was observed that Lebanon wild thyme extract had the most effective DPPH radical scavenging activity.

Table 2. DPPH radical scavenging activity of hydroalcoholic extracts of Romanian and Lebanon wild thyme

Sample	IC_{50} value of DPPH (µg/ml)
Lebanon wild thyme extract	15.25 ± 1.31
Summer savory extract	17.94 ± 2.14
Gallic acid 10 µg/100 ml	4.51 ± 0.35
BHA 10 µg/100 ml	14.84 ± 1.57

Values are the average of triplicates ± standard deviation.

The extract of Lebanon thyme, with significant DPPH • scavenging activity, also had a higher quantity of total phenolics. This extract, which have a high antioxidant activity, also had a great quantity of flavonoids, as summarized in Table 1.

The ferric reducing antioxidant power (FRAP)

Prior et al. (2005) found out that FRAP mechanism is based on electron transfer rather than hydrogen atom transfer. The basis of FRAP assay is the ability of antioxidant compounds to reduce Fe^{3+} to Fe^{2+}.

The FRAP reaction is taking place in acidic medium (pH value equal to 3.6) in order to maintain iron solubility. The reaction mechanism is based on decreasing of ionization potential at low pH that drives hydrogen atom transfer and increases the redox potential. In the presence of 2,4,6-trypyridyl-s-triazine the reduction of Fe^{3+} to Fe^{2+} occurs. The reduction reaction is accompanied by the formation of a blue complex with Fe^{2+} with a maximum absorption at 593 nm. The reducing power appears to be related to the degree of hydroxylation and extent of conjugation in antioxidant compound (Huang et al., 2005). The results of evaluation of Ferric reducing antioxidant power (FRAP) are shown in Table 3.

Antioxidative activity of examined plant extracts measured with FRAP method bring more information regarding benefic effects of them. Results of the evaluations presented as

µmols Fe^{2+}/g DW, showed increasing activity with phenol extract's concentration. Highest FRAP values were found for ascorbic acid (54.14 ± 5.61 µmols Fe^{2+}/g DW) and in sample of Lebanon thyme extract (42.71 ± 4.24 µmols Fe^{2+}/g DW) and Romanian thyme extract (39.55 ± 4.21 µmols Fe^{2+}/g DW). Also, high activity showed BHA (34.12 ± 3.42 µmols Fe^{2+}/g DW). Similar results were observed by Gedikoğlu et al. (2019) and Birasuren et al. (2013). As the result of the FRAP analysis herbs extracts and standards were ranked as follows: BHA < Romanian thyme extract < Lebanon wild thyme extract < ascorbic acid.

Statistical analysis of relationships between ferric reducing antioxidant power of ethanol herbs extracts and total polyphenol content showed high correlations (R^2 = 0.9999, $p < 0.05$). Also, a significantly higher correlation was established between FRAP and DPPH radical scavenging activity ($p < 0.05$).

Table 3. Ferric reducing antioxidant power (FRAP) value of hydroalcoholic extracts of Romanian and Lebanon thyme

Sample	FRAP (µmols Fe^{2+}/g DW)
Lebanon wild thyme extract	42.71 ± 4.24
Summer savory extract	39.55 ± 4.21
Ascorbic acid (10 µg/100 ml)	54.14 ± 5.61
BHA (10 µg/100 ml)	34.12 ± 3.42

Values are the average of duplicates ± standard deviation.

Khosh-Khui et al. (2012) find out that water deficiency might increase antioxidants levels depending on plant genotypes and this can explain why Lebanon wild thyme extract showed higher antioxidant activity.

Antimicrobial activity. Two Gram-positive bacteria (*Staphylococcus aureus* ATCC 9144, *Staphylococcus epidermidis* ATCC 12228) and three Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Salmonella enteritidis* ATCC 13076, and *Salmonella typhimurium* ATCC 14028) were used to test antimicrobial activity of Romanian thyme extract and Lebanon thyme extract. Table 4 present the antimicrobial activities of thyme extracts and BHA against various organisms. The two extracts possessed antimicrobial activity against all tested bacteria, but the highest activity was showed by Lebanon wild thyme extract. BHA showed the lowest antimicrobial activity. This result regarding the antimicrobial activity of synthetic antioxidant were similar with other researches (Gavarić et al., 2015).

Thyme extracts are known to possess some antimicrobial activities and are used in various food preparations as flavour enhancers (Nzeako et al., 2006). Even the antimicrobial pathways are not fully known and understood, it looks that its action is due to the compounds present in the thyme extract (phenols and flavonoids among the others) (Gavarić et al., 2015). The synergistic effects of different compounds present into extracts can contribute to the antimicrobial activity through different mechanisms. Different extract compounds can interfere with bacterial membrane and thereby increase the cell leakage or act indirectly antimicrobial by facilitating the influx of antimicrobial phenolic compounds (Burt, 2004). For this reason, it is recommended to use plant extracts than pure compounds (Burt, 2004).

Table 4. Antimicrobial activity of hydroalcoholic extracts of BHA and Romanian and Lebanon wild thyme

Sample / Microorganism	Lebanon wild thyme extract	Summer savory extract	BHA (10 µg/100 ml)
	Zone of inhibition in mm		
Staphylococcus aureus	27.5 ± 3.1	28.1 ± 2.1	13.6 ±0.9
Staphylococcus epidermidis	24.1 ± 1.5	23.3 ± 1.9	14.1±1.0
Escherichia coli	25.2 ± 1.4	25.6 ± 2.1	11.2 ±0.9
Salmonella enteritidis	18.4 ± 1.1	16.4 ± 1.5	14.2 ±1.2
Salmonella typhimurium	13.2±1.1	11.0 ± 1.1	6.±0.7

Values are the average of triplicates ± standard deviation.

CONCLUSIONS

Both hydroalcoholic extracts were found to contain a noticeable amount of phenolics and flavonoids. Phenolics and flavonoids, may be the compounds responsible their antioxidant and antimicrobial activities in these plants extracts. The thyme extracts contain compounds with antioxidant activity that act as hydrogen/electron donors. Romanian thyme extract and Lebanon wild thyme extracts showed strong DPPH radical scavenging activity and strong ferric reducing antioxidant power when compared with standard. The results of this study show that the extract possessed antimicrobial activity against the tested bacteria and can be used as an easily accessible source of natural antibiotic. In

addition, the extracts of these plants can be regarded as plant-derived antioxidant and antimicrobial mixture in different fields (foods, cosmetics, pharmaceuticals).

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PERIPARTUM METABOLITES AND HORMONAL IMBALANCE THAT CAN INFLUENCE COW FERTILITY - A REVIEW

Cezar Mihai BERCEA-STRUGARIU, Nicolae Tiberiu CONSTANTIN, Dragoș POPESCU, Dragoș BÎRȚOIU, Constantin VLAGIOIU

University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, 105 Splaiul Independenţei Street, District 5, 050097, Bucharest, Romania

Corresponding author email: cezarbercea@gmail.com

Abstract

During the last decades breeding dairy cows has severely affected the fertility of this species because this is a multi-factorial objective that can involve genetic, environmental and managerial factors. The main purpose of this review is to describe the modification of different metabolites and hormones that are involved in cow's fertility after calving. High milk yield during the fresh period predispose dairy cows to enter in negative energy balance (NEB). This happens because of fat tissue is mobilised faster than the liver is able to metabolize it. For a normal metabolize, the liver needs glucose. Non-esterified fatty acids (NEFA) offers an alternate source of energy but in the same time it can lead to liver accumulation of ketone bodies (acetone, acetoacetate and β-hydroxybutyrate). A small quantity of ketone production is normal for dairy cows but high amounts can lead to clinical and subclinical ketosis. Such diseases can predispose to a decrease milk production, a low fertility, and even culling.

Key words*: dairy cows, non-esterified fatty acids, β-hydroxybutyrate, milk production, negative energy balance.*

INTRODUCTION

After the transition period, which is defined as the last 3 weeks before calving and the first 3 weeks after calving, there is a high increase in dry matter intake (DMI) and milk yield (Barletta et al., 2017); also during this period of time, the health condition of the cow is particularly at risk because of cellular and humoral response of the immune system which is highly active as the cow is predisposed to uterine and mammary infections (Constantin and Bîrțoiu, 2014).

All dairy farmers want to accomplish the old challenge: "one calf per year from each cow". For this, biologists, nutritionists and geneticists are working on understanding the biological mechanism of dairy cows that lead to impaired fertility and, also on strategies to avoid this aspect (Walsh et al., 2011).

The main factor which influences the postpartum health of the cow is the high milk yield production. The milk production is correlated with the energy consumption that is linked with blood volume that pass through portal vain into the liver. Because progesterone and estradiol are first metabolized in the liver tissue, the increasing blood flow leads to a decrease concentration of these hormones. Because of this, heat detection is harder as heats tend to be silent and fertility is impaired. (Farraretto et al., 2014).

Reproduction efficiency represents the number one priority for all breeding systems in dairy cows. Because of this, during the last seven decades the genetical changes have focused on increasing the yield milk production. For example, at the beginning of last century dairy cows had milk productions of 2000 kg/year; one century later these productions have multiplied by four (Miglior et al., 2017). According to Miglior et al. (2017), between 1917-2017 milk production was the most wanted aspect by amelioration (more than 65%) where the milk quantity, protein and fat percents were followed. Body aspect was the second fact (around 25%), then the longevity, easy calving, and work performance. Fertility was considered last (less than 5%).

BODY CONDITION SCORE AND NEGATIVE ENERGY BALANCE

High milk production dairy cows need a high energy level to supplement the increasing milk production especially between 4th and 8th weeks

postpartum. This high energy consumption is hard to achieve because of limiting appetite and a low dry matter intake. Low dry matter intake will predispose cows to entering a negative energy balance (NEB) and a high body reserve mobilization. The consequences of NEB are represented by increasing metabolic diseases that occur mainly in the first month of lactation, by immune function reduction and by decreasing fertility (Carvalho et al., 2014).

The evaluation of body condition score (BCS) is made by inspection and palpation, and the transitory modifications are used to check the nutritional estate and the health level of high yield milk production cows.

BCS loss is correlated with low reproductive performances. The females with low BCS at calving or those that suffer high looses of BCS early after calving, will have high chances to not ovulate, will show low rates of estrus appearance, will have low conception rate at first insemination, and they will also have high embryo mortality and prolonged calving intervals (Walsh et al., 2011). For cows with moderate milk production a BCS between 2.5 and 3 (scale between 1 to 5) is recommended. Cows that achieve this at the beginning of the transition period will have reduced body reserve mobilization and low NEFA and β-hydroxybutyrate acid (BHBA) concentrations (Barletta et al., 2017). A low BCS (between 1.5 and 2.5) is correlated with low fecundity of oocytes. In contrast, obese cows (BCS over 3.5) also have low fertility because of decreased DMI before calving in direct association with high fat mobilization after severe NEB postpartum (Walsh et al., 2011). NEB also reduces the growth of dominant follicles and estradiol production because of low insulin, low IGF-1 concentration and LH pulses alteration (Barletta 2017).

High temperatures can exacerbate the effects of NEB. During high temperature stress, lactating cows have a low appetite and a low BCS during the puerperal period. Also, the concentrations of glucose, insulin growth factor 1 (IGF-1) and cholesterol are reduced, compared to NEFA and blood urea nitrogen concentrations that are increasing in dairy cows that suffer of high temperature stress (Walsh et al., 2011).

Kafi and Mirzaei (2010) observed a delayed ovulation in cows that looses more than 1 BCS point in first 49 days in milk. Also, the risk of delayed ovulation increased by 16.5 times for females that loosed 0.75 of BCS during the same period of time.

METABOLIC DISEASES IMPLICATED IN INFERTILITY

During the transition period, cows cross through calving stress, lactation debut and a high amount of energy and protein needs for milk production. Because these cows have inadequate energy stores they will enter a NEB characterized by physiological, metabolic and endocrine imbalances. Cows that are immunosuppressed have a high risk to develop metabolite diseases as acidosis, retained fetal membranes and abomasal displacement. Cows that suffer metabolic imbalance and low dry matter intake before calving are more at chance to develop metritis, laminitis and endometritis, all of these ultimately leading to low reproductive parameters (Wathes et al., 2013).

At the start of lactation the liver and adipose tissue increase the release of growth hormone (GH).

Because of NEB, insulin concentrations remain low leading to a low liver GH and IGF-1 receptors expression. These two processes will alter the hipotalamo-hypophyses axis because insulin and IGF-1 are not capable to accelerate the action of gonadoreline (GnRH) on ovarian cells, preventing ovulation and retarding the estrous cyclicity (Carvalho et al., 2014).

LIPIDIC METABOLISM THAT IS IMPLICATED IN REPRODUCTION

Lipids are represented by cholesterol, phospholipids and triglycerides. These, among theirs derivates, offer energy support, are part of all cells membranes and are important elements involved in a large variety of endocrine mechanisms. The adipose tissue represent the main source of lipids, even if they are also stored in other tissues like muscles and liver. The majority (more than 95%) of fat tissue is composed by triglycerides stored as lipid droplets. The adipose tissue secretes some adipokins like: leptin, resistin, tumoral necrosis factor alpha (TNF-α) and interleukin 6 (IL-6). These adipokins send

signals to hypothalamus and other tissues that are involved in energy homeostasis (Wathes et al., 2013).

The liver plays a major role in lipid metabolism. When the liver capacity for lipids export is over-passed, the triglycerides accumulation into hepatic cells leads to fatty liver syndrome. This is true for almost all dairy cows, with highest incidence during the second week of lactation. Beyond the hepatic cell disorder, triglycerides induce apoptosis of endoplasmic reticulum, stress and derangement of mitochondrial' membranes (Wathes et al., 2013). Mitochondria represents the critical point for energy storage and controls the energy balance metabolism of cells and is also the main intracellular oxygen consumer.

In vitro maturation of oocytes offer high amounts of mature oocytes that are capable to support embryo development (Prates et al., 2014). Also, *in vitro* studies showed that oocytes and embryos accumulate fatty acids into their cytoplasm. Marei et al. (2012) showed that bovine oocyte maturity in the presence of linoleic acid influences mitochondria distribution into cellular cytoplasm and increases the level of reactive oxygen species. Moreover, Van Hoeck et al. (2011) obtained reduced oocyte quality because of different NEFA exposure. Similar effects were obtained for bovine embryos cultivated in blood serum of heifers fed with rich diets in lipids and palm oil. All of these show that an abrupt NEB after parturition will be followed by increase concentrations of NEFA and oocyte quality alteration (Wathes et al., 2013).

In contrast, Sinclair (2010) obtained better results using alpha linoleic acid into oocytes growing medium and a better enhance of post fertilization of oocytes. The mechanisms associated with this positive response showed an increase concentration of prostaglandin E_2 at the level of *cumulus ooforus*-oocyte complex.

A cow can't breed short after calving because the genital tract takes time to regenerate and to be capable for a new pregnancy. Probably the main cause for high embryo mortality is represented by unsuitable uterine environment that is seen in dairy cows with repeat breeding syndrome. At this moment the uterus can be contaminated by different pathogens because the immune status is off during this period, cows developing endometritis. Because of this, a local insulin resistance effect is present. The healing process of endometrial tissue will be arduous because IGF-1, an important restorer element, is low.

INSULIN, LEPTIN AND IGF-1 IN RELATION TO COWS FERTILITY

Insulin is a peptide hormone IGF like and is liberated by the pancreas as a response to high level of glucose. Insulin induced by glucose stimulates glycogen formation, enhances glucose absorption by almost all tissues (except muscles and liver), inhibits lipolysis, gluconeogenesis and stimulates lipid storing in the liver (Veerkamp et al., 2003). Fat accumulation into non adipose tissues (like muscles and liver) is linked with peripheral insulin resistance. In humans fatty accumulation at organ level leads to metabolic syndrome and a combination of medical disorders as cardiac hypertension and diabetes type 2 (Sinclair, 2010).

Negative energy balance is defined by a increase mobilization of all body resources, by a decrease concentrations of insulin, IGF-1 and glucose, and by an increase concentrations of NEFA and BHBA. It seems that high GH and NEFA concentrations antagonize insulin action, leading to peripheral insulin resistance in fresh cows (Thatcher et al., 2011). Insulin concentration during dry-off period decrease dramatically (60 days before calving the insulin concentration is around 0.63 ng/ml, and 5 days before parturition is less than 0.26 ng/ml) (Baruselli et al., 2016).

Even if insulin has an important role in cells metabolism, an excess of it can interfere with reproductive and metabolic processes (Baruselli et al., 2016). The highest insulin limit in cows is considered to be over 37 mIU/ml.

It seems that IGF-1 is the most important mediator of GH from milk because it regulates milk secretion by mammary gland (Knop et Cernescu, 2009).

IGF-1 concentrations reflects low amount release of GH by the liver. It seems that IGF-1 has a longer anabolic action comparing to acute insulin action. For example, IGF-1 enhances protein synthesis and theca and granulosa cell proliferation, increases steroidogenesis, and releases luteinizing hormone (LH) (Veerkamp et al., 2003).

During NEB, IGF-1 - GH axis is down regulated because of GH receptors deficiency of liver, in direct link to reduced IGF-1 concentration and with increased GH concentration. All these, along with low insulin level, offer an endocrine environment that sustains the direct action of GH for lipolysis and for gluconeogenesis at the start of lactation (Knop et Cernescu, 2009).

Leptin is a cytokine like hormone released by fat tissue and acts on the central nervous system, controlling the LH pulses. Most probable, leptin acts together with insulin for ovulation relapse (Rodney et al., 2018).

NEFA AND BHBA EVALUATION FOR A BETTER REPRODUCTION

Dairy cows with high milk yield production cross a tremendous period of glucose, amino acids and fatty acids shortage that starts soon before calving.

At the start of lactation, all adipose resources are mobilized as NEFA for energy support. Through increased blood flow these acids are transported to the liver where they are metabolized into triglycerides and very low density lipoproteins by oxidation and re esterification (Barletta et al., 2017).

The liver metabolizes by oxidation around 15-20% of NEFA and produce ketone bodies (acetone, acetoacetate acid and BHBA) (McArt et al., 2013). Blood BHBA concentration can indicate the grade of fatty acids oxidation. It seems that more than 50% of dairy cows cross a short period of subclinical ketosis during the first month of lactation. This fact is an adaptative strategy for glucose maintenance (Knop and Cernescu, 2009) because BHBA acts as energy source for the brain and heart (McArt et al., 2013).

Circulating NEFA concentrations are inversely with DMI. For example, cows with BCS higher than 4 at calving will present increased concentrations of NEFA in the first 7 weeks of lactation.

A number of studies reported a negative relationship between NEFA and reproductive activity. Ospina et al. (2010a) showed that NEFA concentrations during transition period were associated with low pregnancy rate after 70 days in milk, and Ospina et al. (2010b) signaled the same result for the first 21 days in milk.

Females with severe NEB are more susceptible to develop different diseases. For example, subclinical hypocalcemia, increased level of NEFA, metritis, respiratory and digestive problems predispose the cow to a delayed estrous cycle over 50 days of lactation, negatively affects embryo quality, decreases the number of pregnant cows per service and increases embryo loss. High level of NEFA is corelated to a low pregnancy rate per service, and high concentration of NEFA and BHBA are associated with different clinical disorders (Barletta et al., 2017).

Ospina et al. (2010) proposed to measure NEFA and BHBA variations during the transition period for a better diagnosis of ketosis and NEB. Hence, antepartum cows with NEFA $\geq$ 0.27 mEq/L and with postpartum NEFA $\geq$ 0.72 mEq/L and BHB $\geq$ 10 mg/dL presented low chances to remain pregnant after 70 days of voluntary waiting period. The milk production was lower for cows that have antepartum NEFA $\geq$ 0.33 mEq/L. For cows in first lactation a decrease milk production was registered when NEFA and BHBA values were 0.72 mEq/L and 10 mg/dL, respectively (Ospina et al., 2010). For NEFA measurement, blood must be collected 14 to 3 days before calving because in the last 3 days before labour an increase in NEFA concentration is naturally present (McArt et al., 2013). With a level of 1.4 mmol/L of BHBA, Raboisson et al. (2014) certified that fresh cows are in a clinical ketosis state even though clinical signs appear over 3.0 mmol/L (McArt et al., 2013). Kafi and Mirzaei (2010) showed that cows with delay ovulation have high BHBA concentrations in the first 42 days in milk.

ESTROUS CYCLE RELAPSE

For dairy cows, normal puerperal period is defined as complete uterine involution, relapsing of follicular growth, ovulation of dominant follicle soon after calving, and continuous estrous cycles at 21 days intervals. A number of factors are involved in the delay of first ovulation after calving. Heifers need more days to ovulate after parturition (31.8 $\pm$ 8.3 days) comparing to multiparous (17.3 $\pm$ 6.3 days). Moreover, primiparous cows need higher amounts of energy for body growth and for

lactation, and this predisposes them to a faster NEB than multiparous cows. Other risk factors are postpartum disorders, seasonal calving, mastitis, lameness, and severe decrease of BCS. For diagnosed with mastitis and lameness, ovulation relapses with a delay of 7 to 17 days. Comparing to healthy cows, animals that suffer of endometritis have 4.5 times chances for a delay ovulation, and 4.4 times chances to have a longer luteal phase. Because of bacteria environment, endometrial cells secretion of prostaglandin $F_{2\alpha}$ is shifted to prostaglandin E2 (Walsh et al., 2011).

CONCLUSIONS

Even though in the last decades the dairy industry has been focusing on continuously improving milk production through genetic selection in detriment of fertility, this review shows that high milk yield production and NEB represent strong reasons for a change in this strategy on long-term. Also, farmers and veterinarians should focus on using cow side tests for NEFA and BHBA concentrations for a correct monitoring of dairy cows during the peripartum period.

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IUC D AND *PAP C* VIRULENCE-ASSOCIATED GENE PRESENT IN ROMANIAN AVIAN PATHOGEN *ESCHERICHIA COLI* ISOLATES

Maria Rodica GURĂU, Hasan Majid HAMEED, Dragoș COBZARIU, Doina DANEȘ

University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd, District 1, Bucharest, Romania

Corresponding author email: otelea_maria@yahoo.com

Abstract

Avian colibacillosis produced by Escherichia coli, a common infection facing poultry flocks, affects poultry industry by the broad spectrum of induced entities: systemic acute infection - colisepticemia, localised infection as omphalitis, cellulitis, enteritis, salpingitis/peritonitis, cronic disease - choligranulomatosis, the chronic respiratory disease and many more. The E. coli infection is causing large losses for the poultry industry, impairing the performances: decreased egg production, with drown of the carcasses at slaughterhouse and the increased mortality rate, are recorded in the affected flocks. E. coli strains inducing poultry diseases are a specific group of strains, called Avian Pathogen E. coli (APEC) strains: these strains randomly share more virulence-associated genes. 13 E. coli isolates from different Romanian poultry outbreaks/flocks have been investigated for the presence of Iuc D and Pap C genes. The Pap C gene is responsible for adhesin assembly and its frequency is associated with the cystitis strain like Santo E. proves in his article from 2006. Gene Iuc D of the plasmid encodes a membrane-bound enzyme synthesizing N6-hydroxylysine, the first product of the aerobactin biosynthesis pathway. The DNA extraction was made using QIAamp cador Pathogen Mini Kit (Qiagen). The amplification protocol was: a cycle of denaturation at 94°C for 30 s followed by 25 cycles of 94°C for 30 s, 58°C for 30 s and 68°C for 3 min, and a cycle of 72°C for 10 min. The Iuc D gene was present in 100% (13/13) isolates. Pap C gene was present in 23.07% (3/13) isolates. *Considering these preliminary results, it can say that Iuc D and Pap C genes are independently expressing their virulence and further research should be performed to establish the pathogenicity of each of the two genes together with other pathogenic genes.*

Key words*: PCR, gene. Iuc D, Pap C.*

INTRODUCTION

Escherichia coli is frequently found in poultry flocks and the strains of *E. coli* are classified in entero-toxigenic, entero-pathogenic and entero-haemorrhagic.

The *E. coli* infection - Avian Pathogen *Escherichia coli* (APEC) strains - is causing large losses for the poultry industry, impairing the performances: decreased egg production, withdrawn of the carcasses at slaughterhouse and the increased mortality rate, are recorded in the affected flocks (Barnes et al., 2003; Dou et al., 2016). The clinical signs in young are: septicaemia, enlarged spleen and liver. Airsacculitis, pericarditis are present in sub-acute form of the disease. It is already known that the virulence factors of the APEC strains are coded by genes located into the nucleus and plasmids of the bacterial cell (Akram et al., 2017; Dozois et al., 2003).

The APEC strains harbor more virulence-associated. The pathogenicity of an APEC strain relates with the multitude of virulence factors it encodes (Vandekerchove et al., 2005). There are different associations of virulence genes, rarely repetitive in APECs strains (Delicato et al., 2003; Chakraborty et al., 2015). APECs are a heterogeneous group of strains. The virulence factors that an APEC strain posses, could be assessed by PCR and thus can be established its role into the outbreak.

In our study 13 *E. coli* isolates from different Romanian poultry farms/outbreaks have been evaluated for the presence of *Iuc D* and *Pap C* genes. The genes were frequently identified in APEC strains from other countries (Rouquet et al., 2009; Li et al., 2008).

MATERIALS AND METHODS

13 *E. coli* isolates were investigated for the presence of *Iuc* D and *Pap* C genes.

The strains came from the Romanian counties Brasov, Calarasi, Dambovita, Giurgiu, Vrancea

and Iasi, belonging to the three historical regions, Transylvania, Muntenia and Moldova. The age of the poultry flocks where these strains has been isolated ranged (1 day, 10 days, 7 days, 23 weeks, 24 weeks, 25 weeks, 65 weeks and 87 weeks), the flocks being broiler or layers. The DNA extraction was performed with the QIAamp cador Pathogen Mini Kit (Qiagen, Dusseldorf, Germany). The lysis of the samples was carried at the room temperature with proteinase K and the VXL buffer to inactivate the nucleases. The purification of the DNA was done with the ACB buffer that strengthened the binding conditions, and then the solution was transferred to the column in order to be purified. The elution of nucleic acids was made by adding the AVE buffer into the column and by centrifugation it was eluted (Qiagen).

The PCR amplification protocol were: 94°C 3 minutes, 25 cycles with 94°C for 30 seconds, 58°C for 30 seconds and 68°C for 3 minutes. The final elongation: 72°C for 10 minutes.

The mix for the reaction was made in a volume of 25 µl with 2 µl DNA template, 1µl dNTPs 10 mM, RNase free water 18.7 µl, 0.4 µl of Taq platinum polymerase (5 U/µl) (Invitrogen®, Itapevi, São Paulo, Brazil), 2.5 µl of PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.0), $MgCl_2$ (1.5 mM) 2 µl and 0.1 µl of primers forward and reverse for each of the two tested genes (10 pmol) (Table 2). It was carried one reaction for the two genes because the annealing temperature of primers is the same for the *Iuc D* and *Pap C* genes.

The primers anneling temperature was before optimized as the two tested genes to have the same temperature.

The sequence primers for *Iuc D* and *Pap C* genes, are described in the Table 1. The amplicons were visualized by electrophoresis in 1.5% agarose, at 90 V, 1.5 A, for 35 min.

Table 1. Sequence of primers-forward and reverse – used for amplification of *Iuc D* and *Pap C* genes fragments and expected size

Primers name	Sequence	Size (bp)
iucD F	ACAAAAAGTTCTATCGCTTCC	714
iucD R	CCTGATCCAGATGATGCTC	
Pap C F	TGATATCACGCAGTCAGTAGC	501
Pap C R	CCGGCCATATTCACATAA	

Table 2. The reagents and the quantities of the reaction mix for the *Iuc* D and *Pap* C genes

Reaction mix	
Reagents	**µl/sample**
RNase free water	18.7 µl
10 X PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.0)	2.5 µl
$MgCl_2$ (50 mM)	2 µl
dNTP solution (10 mM - Promega, USA)	1 µl
P_A (100 pmol)	0.1 µl
P_B (100 pmol)	0.1 µl
Pc(100 pmol)	0.1 µl
Pd(100 pmol)	0.1 µl
Taq platinum polymerase (5 U/µl) (Invitrogen®, Itapevi, São Paulo, Brazil)	0.4 µl
Total	25 µl

RESULTS AND DISCUSSION

The predicted size for the PCR amplicons to be visualized in electrophoresis for the *Iuc D* is 714 bp, and for the *Pap C* is 501 bp (Ewers et al., 2005).

The *Iuc D* gene was present in all investigated strains (13/13) isolates. *Pap C* gene was present in 23.07% (3/13) isolates.

The strain no. 8 isolated from broiler, 7 days old, from Calarasi County was positive to *Pap C* gene, also the strain no. 12, broiler 7 day old from Giurgiu was positive to *Pap C* gene. The strain no. 13 from Iasi, positive to *Pap C* gene, was also from broiler, 11 day old. All these 3 samples (8, 12, 13), positive for *Pap C*, show in electrophoresis a product of 501 bp (Figure 1), as expected for the size of *Pap C*.

As for the *Iuc D* gene, the results shows that all the 13 strains were positive, as seen in electrophoresis, were the amplicon at 714 bp was present in all 13 samples (Figure 1).

The isolate no. 1 was from broiler 7 day old, from Vrancea, no. 2 from Dambovita, 23 weeks, layer, no. 3 strain *E. coli* was from Iasi, 25 weeks, layer also. The strain no. 4 was layer, 87 weeks, from Brasov, no. 5 strain from Calarasi, broiler 10 day old, no. 6 from Dambovita, 24 weeks, layer, and the strain no. 7 also from Dambovita, broiler 1 day old. The strain no. 9 from Brasov, 65 weeks, layer, no. 10 from Vrancea 11 days, broiler, no. 11 Iasi 11 days, broiler.

Table 3. The PCR-results of the tested isolates for the presence of *Iuc D* and *Pap C* virulence genes

Isolates	Gene *Iuc D*	Gene *Pap C*	County	Age of the originating bird
1	**X**	**-**	Vrancea	broiler 7 day
2	**X**	**-**	Dambovita	23 weeks, layer
3	**X**	**-**	Iasi	25 weeks, layer
4	**X**	**-**	Brasov	87 weeks, layer
5	**X**	**-**	Calarasi	10 day, broiler
6	**X**	**-**	Dambovita	24 weeks, layer
7	**X**	**-**	Dambovita	1 day, broiler
8	**X**	**X**	Calarasi	7 days, broiler
9	**X**	**-**	Brasov	65 weeks, layer
10	**X**	**-**	Vrancea	11 days, broiler
11	**X**	**-**	Iasi	11 days, broiler
12	**X**	**X**	Giurgiu	7 day, broiler
13	**X**	**X**	Iasi	11 day, broiler

X = mark the strains containing the gene.

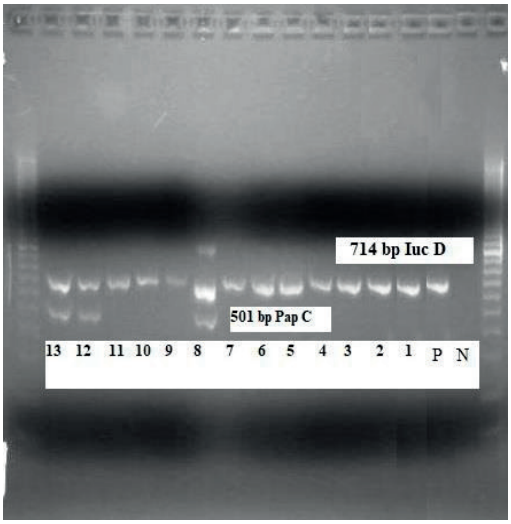

Figure 1. The PCR testing result of the *Iuc D* gene-714 bp; samples from 1 to 13 are positive, and for *Pap C* gene-501 bp, samples 8, 12, 13 are positive; P = positive control; N = negative control

The isolates no. 8, 12 and 13 presented both genes, *Pap C* and *Iuc D*. The pathogenicity of the APEC can be correlated to the association of the genes not only with the presence of them. *E. coli* strains have different pathogenic genes that are not expressed on all APEC strains (van der Westhuizen and Bragg, 2012; Gurau et al., 2018; Kemmett et al., 2013). As in our previous studies on APEC genes, in witch was not found all the virulence genes in all strains, in this fallow up study was found in all strains only one of the two genes *Iuc D* and *Pap C* was found only in 3 strains (Gurau et al., 2018).

If compare our results with the literature data, we can note that the prevalence of the strains containing the *Iuc D* gene was lower than in our study 78.79% (52/66) in Paixao et al. (2016) and 100% (13/13) in our study. In the same study the *Pap C* gene has almost the same prevalence 22.73% (15/66) as our study 23.07% (3/13).

Our results on the APEC strains show a randomised distribution of virulence genes. This is supporting the differences in terms of patogenicity expressed by the different APEC strains. Higher pathogenicity of the APEC determines primary infections while lower pathogenicity causes clinical signs and disease only if the poultry are stressed (Dho-Moulin & Fairbrother, 1999).

CONCLUSIONS

The *Iuc D* gene has a higher prevalence in this study being present in all isolated comparing with the *Pap C* gene which has a much lower prevalence than *Iuc D*. The virulence-associated genes *Iuc D* and *Pap C* were both identified in this study, supporting the higher pathogenicity of these strains, compared to the other strains, containing only the gene *Iuc D*. These results come to confirm other results from the literature, in which, different *E. coli* strains possess different pathogenic genes but not all virulence genes are expressed in all *E. coli* strains.

According to these preliminary results it could be assumed that *Iuc D* and *Pap C* genes are independently expressing their virulence.

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THERAPEUTIC APPROACHES IN SEVERE, COMPLICATED CANINE BABESIOSIS: A CASE REPORT

Camelia ION, Ioan Liviu MITREA, Mariana IONITA

University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, 105 Splaiul Independenţei Street, District 5, Bucharest, Romania

Corresponding author email: ani.malz@yahoo.com

Abstract

Canine babesiosis is a potential life-threatening tick-borne disease affecting dogs worldwide. Early diagnostic and specific therapy in due time are emergency issues, as the infection may progress rapidly to severe, acute, and complicated potentially fatal disease. Here we describe a clinical case of babesiosis in a 6-year-old male Bichon Frise dog which was referred to the Clinic of Faculty of Veterinary Medicine of Bucharest, in April 2019, with clinical signs (fever, 39.9°C, jaundice, haemoglobinurie, weakness) compatible for babesiosis. Blood samples were collected and subjected for hematological and biochemical investigations, and for parasitological testing. The blood smear analysis showed intraerytrocytic piroplasms compatible for large Babesia form. Subsequently of the clinical and paraclinical investigations the dog was diagnosed with severe babesiosis, characterized by severe anemia (PCV=15.8%; Hgb=5.8 g/dL), neutropenia, trombocitopenia, and acute renal injury; a guarded to poor prognosis was considered. Accordingly, the therapeutic protocol aimed firstly to stabilize the animal by blood-transfusion, administering compatible tested blood, at a dose rate of 22 ml/kg body weight, over the course of 4 hours, followed by the babesiicid therapy, using imidocarb dipropionate, at lower dose of 3 mg/kg b.w. (two intramuscular injections, 24 h apart). Additionally, supportive therapy was administered under permanent monitoring of the animal within the intensive care unit. Following the treatment, the dog's status improved rapidly and clinically recovered within 10 days. This case-report describes a successful complex therapeutic protocol based on multiple approaches (blood transfusion, specific treatment, intravenous fluids and oral supportive treatment) for critical, life-threatening cases of canine babesiosis.

Key words*: dog, babesiosis, severe, clinical case, Romania.*

INTRODUCTION

Canine babesiosis is a significant disease with specific tick vector transmission that affect dogs worldwide (Irwin, 2009). Currently it is emerging in many European co untries (Schnitter et al., 2012). It is caused by large and small intra-erythrocytic protozoan piroplasms of the genus *Babesia* (Apicomplexa: Piroplasmia) (Carret et al., 1999; Mitrea, 2011). Clinical infection is usually characterized by fever, apathy, and anaemia, but single and/or multiple organ disfunctions such as, kidney, liver, respiratory, nervous dysfunctions, associated with coagulation deficiencies and electrolyte imbalance, may occurr (Lobetti et al., 1996; Leisewitz et al., 2001; Zygner et al., 2014; Eichenberger et al., 2016). However, clinical presentation and disease progression are highly variable, characterized by mild, moderate up to severe anemia, depending of multiple factors, both parasite- (antigenic properties, specific virulence, strain, and species) and/or host- (age, immunity and other concurrent diseases) related factors (Birkenheuer et al., 1999; Jacobson, 2006; Irwin, 2009).

Additionally, chronic evolution of the disease can be often associated with complex pathology (hepatic, renal, cardiac, and neurological dysfunctions).

The late diagnostic and lack or late babesiicid treatment may lead to fulminating disease with high mortality (Ayoob, 2010; Leica et al., 2019). Therefore, early diagnostic and careful monitoring of disease's progression provides important data supporting adequat therapeutic protocols to be applied, according to the clinical presentation of infection (Lobetti et al., 1996; Leisewitz et al., 2001; Jacobson, 2006; Irwin, 2009).

In Romania, in the last decade, the epidemiology of canine babesiosis has been showing rapid changes, many reports

describing endemic foci, particularly in South-Eastern areas, with clinical presentation and pathological changes varying from mild, moderate up to severe, complicated diseases (Mitrea, 2011; Ionita et al., 2012; Leica et al., 2019). Additionally, increased abundance of tick populations associated with climate changes, socio-economic, and environmental related factors represent high potential risks for canine tick-borne diseases in Romania (Ionita et al., 2016; Ionita and Mitrea, 2017).

Therefore, there are strong evidences that clinicians should be aware that establishing early diagnostic is an emergency issue in canine babesiosis, as the clinical signs may progress rapidly to severe acute disease, evolving to complicated, life-threatening, fatal disease, especially on lack and/or delayed babesiicid therapy (Irwin, 2009; Ayoob, 2010; Solano-Gallego et al., 2011).

Here we describe a clinical case of severe, complicated canine babesiosis. The therapeutically approaches and clinical follow-up are discussed.

MATERIALS AND METHODS

Case presentation

A 6-year-old male Bichon Frise dog was referred to the Clinic of Faculty of Veterinary Medicine of Bucharest, in April 2019 with clinical signs compatible with babesiosis (fever, 39.9°C, jaundice, haemoglobinuria, weakness).

The dog was subjected to a routine physical and clinical examination and thereafter blood samples were collected and subjected to hematological, biochemical, and parasitological investigations.

Hemathological parameters were determined by using IDEXX VetAutoread™ Hematology Analyzer. Serum biochemistry parameters' analysis was performed with VetTest 8008 / Catalyst Dx analyzer (IDEXX U.S.).

For parasitological testing, thin blood smears, Giemsa stained, were microscopically examined for intraeritrocytic piroplasms.

Imagistic investigations were performed using ultrasound examination according to abdominal assessment with sonography for triage (AFAST) (Codreanu et al., 2017).

RESULTS AND DISCUSSIONS

At clinical examination the dog displayed fever (39.9°C), generalised jaundice, dyspnoea, severe vomiting, and haemoglobinuria.

Microscopical examination of the blood smear showed large intra-erytrocytic piroplasms (Figure 1), compatible for *Babesia* large piroplasms. Additional, severe non-regenerative, normocytic, normochromic anemia, as well as neutropenia, vacuoles in monocyte's cytoplasm, and severe thrombocytopenia were registered.

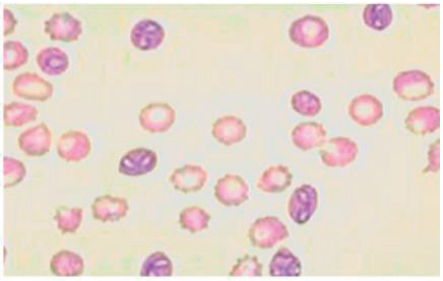

Figure 1. Dog blood smear, Giemsa stained, showing intraerythrocytic large piroplasms (x 1000)

Haematology parameters analysis revealed severe anaemia (packed red cell volume: PCV=15.8%; haemoglobin: HGB=5.8 g/dL), leukopenia and thrombocytopenia (Table 1).

Table 1. Dynamics of haematological parameters in a 6 year old Bichon Frise with babesiosis, during the follow-up period

Parameter tested*	*Reference limits*	Day 1	Day 2	Day 3.	Day 18
PCV	*37-55%*	**15.8↓**	**28.2↓**	**26.4↓**	**33.0↓**
HGB	*12-18 g/dL*	**5.8↓**	**8.4↓**	**9.7↓**	**10.7↓**
MCHC	*30-36.9 g/dL*	36.7	**29.8↓**	36.7	32.4
WBC	*6-16.9 K/µL*	**5.00↓**	13.00	14.50	18.10
GRANS	*3.3-12 K/µL*	3.40	7.00	7.40	14.60
% GRANS	*%*	68%	53.8	51.0	80.7
NEUT	*2.80-10.50 K/µL*	2.80	5.98	NT	NT
EOS	*0.5-1.50 K/µL*	0.6	1.02	NT	NT
PLT	*175-500 K/µL*	>15↓	>932↑	67↓	499

*PCV=packed red cell volume; Hgb=hemoglobin; MCHC: mean corpuscular hemoglobin concentration; WBC= white blood cells; GRANS=total granulocyte count; NEUT= neutrophils; EOS: eosinophils; PLT: platelet count;
D1=day of admission; D2-18=days after admission

Serum biochemistry panel's analysis showed acute renal injury (azotemia- BUN=126 mg/dL) and elevation of hepatic enzymes (TBIL >27.9 mg/dL; ALT 270 U/L) (Table 2).
In Tables 1, 2 dynamics of the hematological and biochemical parameters, from day of dog admission and during of the follow-up period (approx. three weeks), are displayed.

Table 2. Dynamics of serum biochemistry parameters in a 6 year old Bichon Frise dog with severe babesiosis, during the follow-up period

Parameter tested*	*Reference limits*	Day 1	Day 2	Day 3	Day 18
GLU	*74-143 mg/dL*	87	NT	NT	97
CREA	*0.5-1.8 mg/dL*	0.8	1.1	NT	1.5
BUN	*7-27 mg/dL*	**126↑**	**124↑**	**62↑**	**56↑**
ALT	*10-100 U/L*	36	NT	NT	**270↑**
ALKP	*23-212 U/L*	**+++↑**	NT	NT	**602↑**
TBIL 1:1	*0.0-0.9 mg/dL*	**>27.9↑**	NT	**>27.9↑**	0.9
LIPA	*200-1800 U/L*	**1884↑**	NT	NT	NT

*GLU=glucose; CREA=creatinine; BUN= blood urea nitrogen; ALT=alanine aminotransferase; ALKP= alkaline phosphatise; TBIL 1:1=total bilirubin; LIPA=lipase; D1=day of admission; D2, D3, D18=day after admission. +++↑: over the detection limits.

The abdominal ultrasound examination showed enlarged spleen and liver, a fine layer of abdominal fluid.

Diagnostic

By corroborating the clinical and paraclinical investigations' results the dog was diagnosed with severe babesiosis, caused by piroplasms compatible for large *Babesia* form, and characterized by severe anaemia, complicated with acute renal and hepatic injurries.

Treatment and follow-up

Due to the severe, life-threatening anemia (PCV of 15.8%), blood-transfusion was the first therapy approach. Fresh whole tested blood (D.E.A.1.1 negative) was administered, at a rate of 22 ml/kg body weight, over the course of 4 hours. No side effects following the blood transfusion were registered, therefore, after completion of the blood transfusion, a babesiicid therapy with imidocarb dipropionate (Imizol®, Merk, Animal Health, Intervet Inc.) was administered, as follows: firstly, one intramuscular injection of at a dose of 3 mg/kg, followed by a second injection in the next day (at 24 h). No side effects were registered after the babesiicid therapy.
Additionally, supportive therapy including intravenous fluids (continuos rate infusion with aminoacids, isotone cristaloids - ringer solution, at a rate of 5 ml/kg/h), antibiotics (ceftriaxone 20 mg/kg every 12 hours), renal and hepatic supplements (metoclopramide 0.5 mg/kg, at 12 hours, and cyancobalamine, 50 mcg/kg/day) were administered. The dog was close monitorized for vital functions (breathing rate, heart rate, capillary refill time, temperature, blood pressure and urinary output) within the intensive care unit.
A rapid improvement of the clinical signs was registered after the babesiicid therapy, and both hematological and biochemical parameters showed improved values in the next 3-10 days post-treatment (Tables 1, 2).
Over the course of 10 days in intensive care unit, the dog responded well to the treatment, its general status showing substantially improvement. Therefore, at 10 days post-admission, the dog was released, with recommendation to continue the supportive therapy (Doxycycline 10 mg/kg/day, supplements for supporting renal function, and liver supplements, for 30 days; B12 vitamin, 100 mcg/kg, once a week, for 4 weeks; probiotics), and tick prevention.
A clinical and parasitological follow-up was established a week post-releasing (D-18), when the blood smear examination was negative for intraerytrocytic piroplasms. Additionally, the relevant haematological and serum biochemistry parameters showed substantially improvement. The supportive therapeutic protocol continued, as recommended.

Discussions

The clinical case described in the present paper, clearly demonstrates that canine babesiosis represents an emergency in certain situations such as a late diagnostic, which may impact the prognosis, and may lead to severe disease progression with complex pathology, as previously described (Furlanello et al., 2005; Ayoob, 2010). In such cases, hemotransfusion may be used for controling anemia (Uilenberg et al.,1981).
From clinical and pathological point of view, monitoring disease progression, assessment of

the clinical signs, as well as the outcome of treatment all represent the base for optimizing the therapeutic protocol, as described in the present case.

Blood transfusion should be correlated with the clinical status of the patient, in cases of acute anemia with a haematocrit lower than 20.0% (Costea, 2016). The hematocrit value increased rapidly after the fresh whole blood administration (from 15.8% to 28.2% at 24 h), and continued to improve during the monitoring period (up to 33.0%, two weeks post-treatment; Table 1), along with improving of the dog clinical presentation.

Babesiicid therapy is based on imidocarb dipropionate injection, usually recommended at a dose rate of 6.6 mg/kg (Plumb, 2015). However, due to the critical clinical presentation of the dog, a therapy protocol with a lower dosage of imidocarb dipropionate (3.3 mg/kg, 24 h apart) was chose, in order to avoid potential side effects, associated with permanent monitoring.

Additionally, supporting and fluid therapy, in order to recover and maintain the optimal vital signs (cardiac frequency, pulse, diuresis), including restoring electrolyte levels and reducing dehydration, was administered (Ayoob et al., 2010). Continuos rate infusion and symptomatic treatment continued until clinical monitoring revealed vital signs stabilisation and the dog was able to receive oral feeding and therapy.

The additional therapy with doxycycline (10 mg/kg/day for 30 days) was recommended as there are reports on other drugs that can positively contribute to the babesiosis management (Solano-Gallego et al., 2016). For instance, doxycycline has been described as reducing the severity of clinical signs, along with reduction of morbidity and mortality for *B. canis* and *B. gibsoni* infections (Vercammen et al., 1996; Lin and Huang, 2010). Additionally, experimental studies on plant extracts (i.e. artemisinin and its derivates inhibited the *in vitro* growth of *B. gibsoni*) might be potential drugs for treatment of babesiosis (Goo et al., 2010; Iguchi et al., 2015).

In our case, the therapeutical approach, under a continuous monitoring, showed a rapid clinical improvement of the dog, despite of the severe clinical presentation and pronounced pathological changes. Nonetheless, the severity of organ dysfunctions, early diagnostic, and specific and supportive treatment may have a serious impact on the efficacy of therapy and diseases's progression.

CONCLUSIONS

This case-report describes a complex successful therapeutic protocol based on multiple approaches (blood transfusion, specific treatment, intravenous fluids and oral treatment) on severe cases, with life-threatening disease. Additionally, it is emphasized that early diagnostic and specific therapy administered in due time are critical points of successful management of babesiosis.

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DOG VERTEBRAL COLUMN SURGERY IN A T12 FRACTURE USING A RECONSTRUCTION METALLIC PLATE ADAPTED AND MODIFIED: A CASE STUDY

Laurenţiu Ionuţ ISPAS[1, 2], Ionuț-Cristian GÂRJOABĂ[1], Alexandru-Gabriel NEAGU[1], Niculae TUDOR[1], Constantin VLAGIOIU[1]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, 105, Splaiul Independentei Street, District 5, Bucharest, Romania
[2]Cabinet Veterinar Dr. Laurentiu Ispas, 2A George Cosbuc Street, Ploiesti, Romania

Corresponding author mail: drlaurentiu@gmail.com

Abstract

Vertebral column fracture surgery in dogs is used to correct severe cases of spinal deformity and spinal cord compression. In the last years more and more cases of vertebral column fractures or luxation in dogs by car accidents as our case are met with varying degrees of injury to the spinal cord. In our study we present a dog hit by a car, which presented clinical sign of vertebral column fracture with little ventro-lateral displacement and suspected of spinal cord compression at the level of T12-T13 with paralysis; this was confirmed by x-rays and Magnetic Resonance Imaging (MRI). The anesthesia used was an inhalation type. The surgery was made to stabilize and reposition the vertebral column through repositioning the two splitted parts of the vertebral column in the normal anatomic position using a dorso-lateral approach and a metallic plate with 3 screws adapted and modified especially for the patient and for the fracture difficulty's needs. We concluded that the recovering was fast and with complete motion on the back part of the body due to rapid diagnosis and intervention even if the surgery came was performed five days later after the car accident and also with the help of the adjuvant therapy (vitamin B therapy, pain-killers, antibiotics, Myodine).

Key words*: column dog fracture, metallic plate surgery.*

INTRODUCTION

Spinal fractures, especially those with displacement, have a poor prognosis, due to the compression effect exerted on the spinal cord. For this reason, a preoperative neurological evaluation and a better stabilization of the fracture site is necessary (Matis, 2007; Tobias and Spencer, 2012). For appropriate postoperative recovery, both neurostimulatory therapy and physiotherapy sessions are used.

Our study case is about a male dog a cross-breed Teckel (Duchhound) of the age of 1 year old, who present a severe fracture of the vertebral column at the level of T12 vertebra caudal joint facet with a ventral displacement causing a spinal cord compression.

This helps us to a very good fixation of the modified plate in the spaces between vertebral articular processes (cranial and caudal) and the vertebral spinous processes together with the screws ensuring a good stability of the spine which does not allow the plate to move from the fixed position while allowing an elasticity enlargement of the spine in the area relative to the simple rectangular thick metal plates.

Due to the way the metal plate is placed with this figure of 8 lying down the middle area between the screws, it is based on the cranial and caudal vertebral articular processes that are "well" molded which does not allow the plate to move either in the cranial direction or caudal and not in lateral or medial direction.

Over all the assembly is also fixed by the 3 screws placed obliquely at the base of the spinous processes and in slightly different directions to decompose the forces resulting from the forces of traction, torsion and pressure existing on the spine in different planes.

A torn column with displacement and paralysis described above were later shown to be very efficient, the operated dog recovered very quickly its motor and sensory functions along with a very good recovery of the muscle mass (Kube and Olby, 2008; William, 2018), (Douglas and Slatter, 2003). The dog operated walks and runs very well.

MATERIALS AND METHODS

Place of the research activity:
Private veterinary Clinic, Ploiesti, Prahova county, Romania and Faculty of Veterinary Medicine in Bucharest, Romania.

Period of the case study:
11 November, 2019 - 1 March, 2020

Case presentation
An 1-year-old intact male Teckel (Duchhound) cross-breed dog ("Rocky"), weighting 6.9 kg was presented at the veterinary clinic after a car accident in November 13, 2019 (Figure 1).

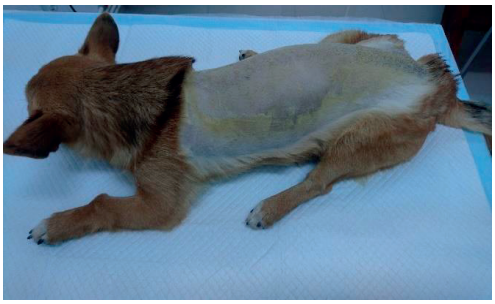


Figure 1. The patient before surgery

Clinical presentation: the dog presented paralysis in the back half of the body, inability to move on the hind legs, inability to stand, apathy, body temperature of 38.8º C.
The cause of traumas: road accident; trampled over the thorax area.
Position: the dog is in lateral decubitus and cannot stand on the paralyzed hind limbs.
Neurological reaction: absent at the pinching of the extremities; almost absent reaction to deep pain. Additional, deformation and inflammation of the spine in the thoracic area T12-T13 bent was registered.
The dog was subjected for x-ray and Magnetic Resonance Imaging (MRI) investigations.

RESULTS AND DISCUSSIONS

The *radiological examination* highlighted the T12 thoracic vertebral fracture of the caudal joint facet (Figure 2).

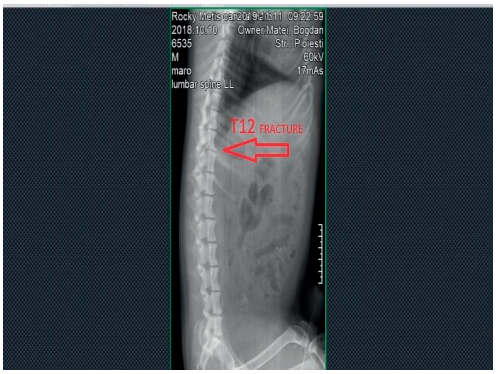


Figure 2. X-Ray of a the showing T12 vertebral fracture

MRI examination was performed at the Faculty of Veterinary Medicine of Bucharest on November 14, 2019 for the certainty of diagnosis of medullary compression.
It revealed the fracture of thoracic vertebrae T12 of the caudal articular facet, with the ventral displacement of the caudal portion of the interrupted spine and a compression on the spinal cord in the T12-T13 intervertebral space, without intervertebral disc herniation and intervertebral disc extrusion, and without major spinal cord injuries (Figure 3.1-3.4). MRI diagnostic: T12 fracture and the vertebral column displacement.

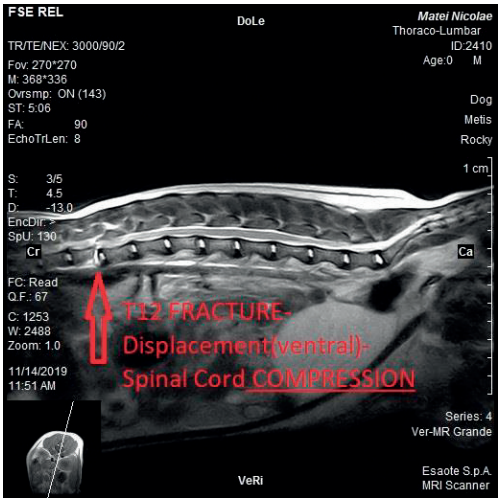


Figure 3. Magnetic Resonance Imaging of a dog with spine T12 fracture and spinal cord compression

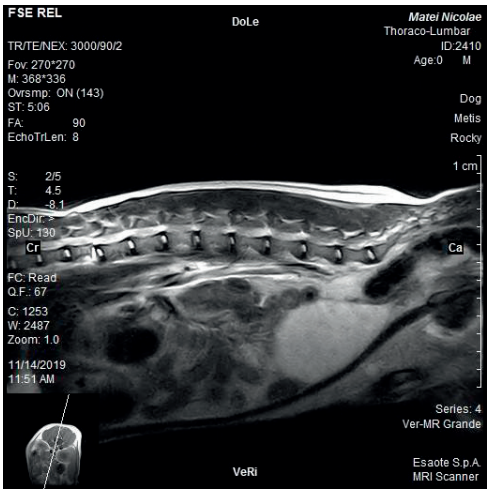


Figure 3.1. MRI in a dog with Spine T12 fracture

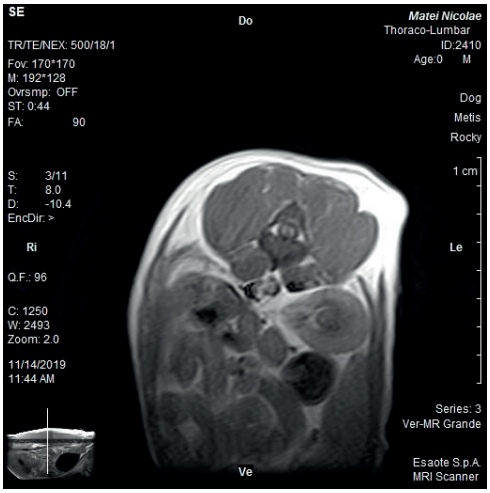


Figure 3.2. MRI in a dog with Spine T12 fracture

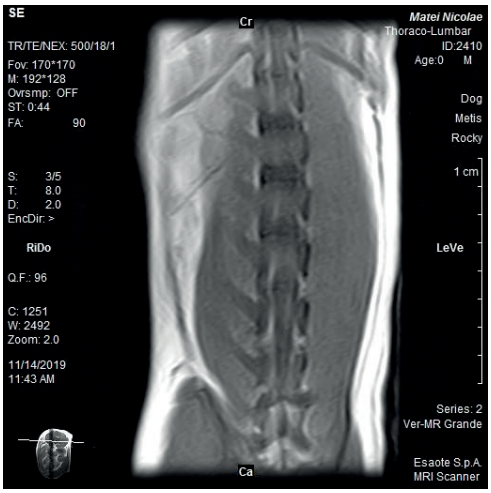


Figure 3.3. MRI in a dog - Spine-vertebral column

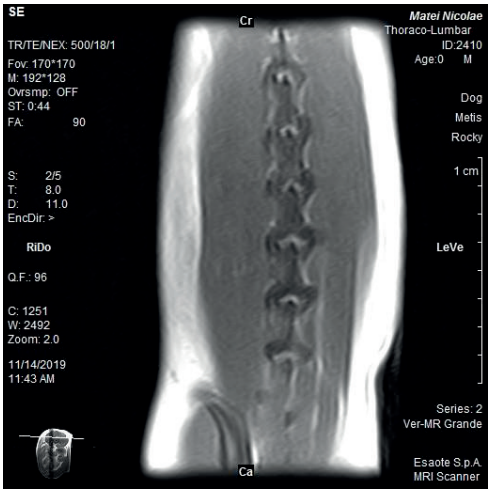


Figure 3.4. MRI in a dog with Spine T12 fracture

The dog was subjected for surgery, that was performed 5 days after the car accident, to decompress the spinal cord at the level of the T12-T13 intervertebral space and the reposition in anatomic way and stabilization of the fractured spine at the T12 level, which has a slight ventral displacement of the interrupted caudal portion as seen in the Figure 3.

Preoperative: 24 hours before surgery, analgesic, antibiotic, and anti-inflammatory therapy were administered along with performing blood testing for biochemical and hemathological parameters.

For anesthesia, an inhalation type with isoflurane gas was used.

Osteosynthesis materials used: we choose to use for a good stabilization of the fractured spine and reduce its displacement a reconstruction plate (length 40 mm and thickness 2.2 mm) with 3 holes and 3 screws (3.5 mm) adapted and modified especially for the dimensions of the "Rocky" patient vertebra to be fixed in 3 vertebrae: T12, T13 and L1 through the 3 screws each screw fixed on the spinous processes base of each vertebra.

This plate specially adapted and modified for the described surgery is derived from a metal plate of reconstruction type "Y" with a length of 80 mm and thickness of 2.2 mm-the model (Figure 4.1) and the dimensions measured of the part used in the surgery (Figures 4.2, 4.3).

It has been cut and adjusted with making rounded edges that do not damage the vertebral periosteum and serve our surgery perfectly.

Figure 4.1. Metalic stainless steel reconstruction plate Y model - picture and brand “Sky surgicals”

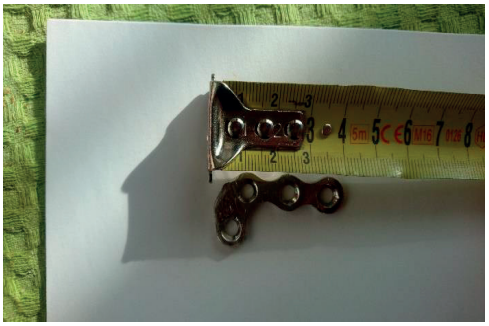


Figure 4.2. Length of the plate part 40 mm before preparing for use in the surgery

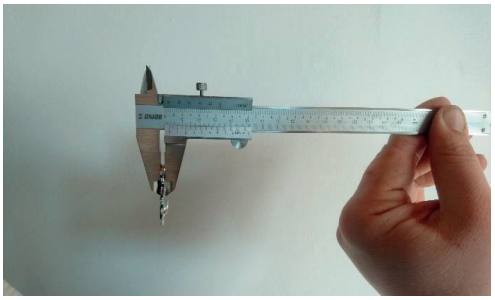
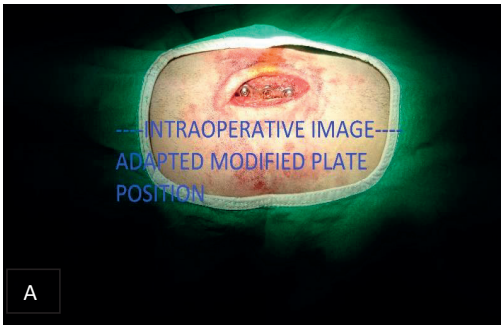


Figure 4.3. The thickness of the plate 2.2 mm

One of the important advantages using this part of the Y plate is that we performed 3 different such of vertebral surgeries for 3 different similar dogs (with a 4.5-9 kg body weight) with vertebral column fractures using only 1 Y plate divided and adapted as we need.

The application of this plate was performed in the anatomically existing spaces between the vertebral articular processes (cranial and caudal) and the base of the vertebral spinous processes so that the neck between the plate mesh sits to fit on the lateral surface of the vertebra to the articular processes.

Therefore, it will not allow the movement of the plate forwards or backwards, nor lateral or medially, also offering a strengthened by the fixing screws located at the base of the spinous processes, harmonizing very well with the movements of the spine while at the same time contributing to the reduced thickness of the plate (Figures 5-7).



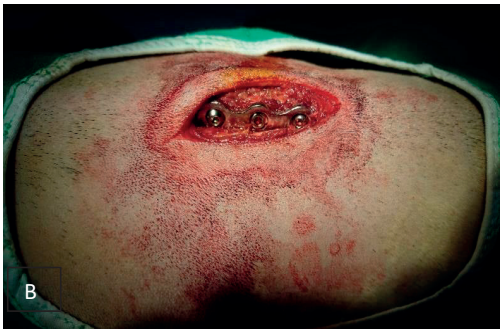


Figure 5 (A-B). Dog intra-surgery aspects showing fiting the plate on the regional anatomy

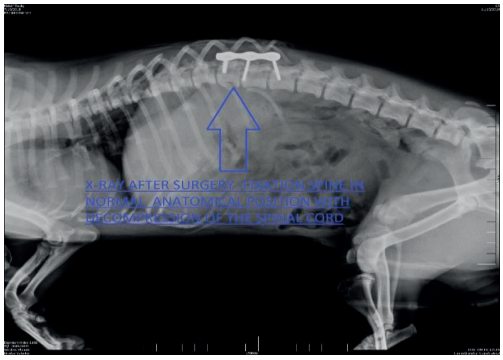


Figure 6. Postoperative radiography - plate and screws

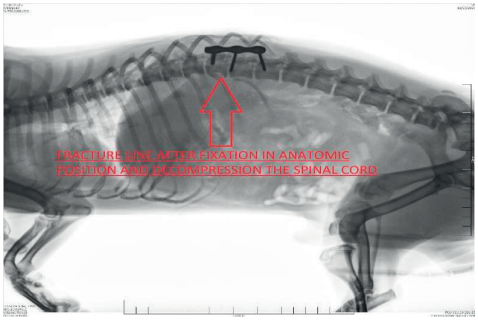
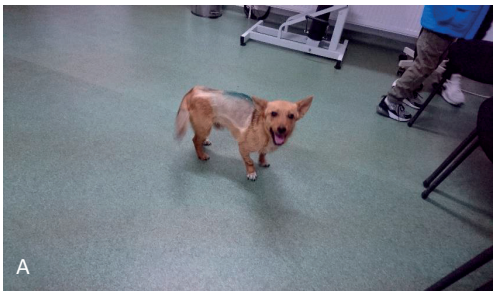


Figure 7. Postoperative X-ray (negative view) after fixation of the spine with decompression of the spinal cord

Post-surgery, analgesics, anti-inflammatories, antibiotics, neurostimulant medications were administered (as vitamin B12, B1-B6, Myodine) with very good results and fast recovery of the nervous system and muscle mass (Gârjoabă and Săvescu, 2019).
Sensory and motor neurological functions visibly greatly improved immediately after the surgery. The reaction to pain on the hind limbs was very good with the immediate withdrawal of the tested limb with very good response (Figure 8).

Figure 8. The dog immediately after surgery - sensitivity test

At 4 days post-surgery, the dog started to get up and walk easily helped and trained of the clinic team to exercise walking under careful supervise and monitoring (Figures 9, 10).



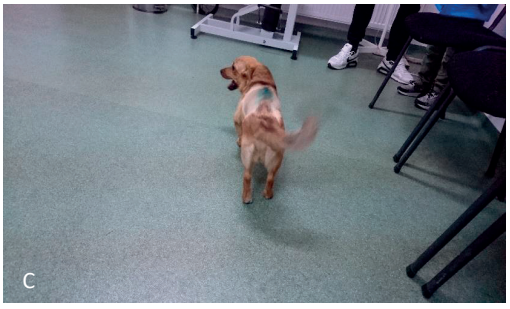


Figure 9 (A-C).The dog at four days after surgery: walking

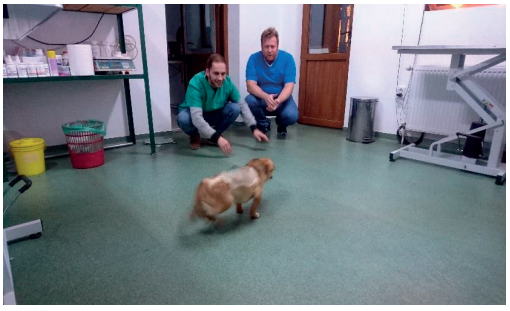


Figure 10. The dog during of recovery exercise training

At 7 days post-surgery, the dog displayed a normal clinical behavior with very small imbalances when running back or running (Figure 11, A-C).

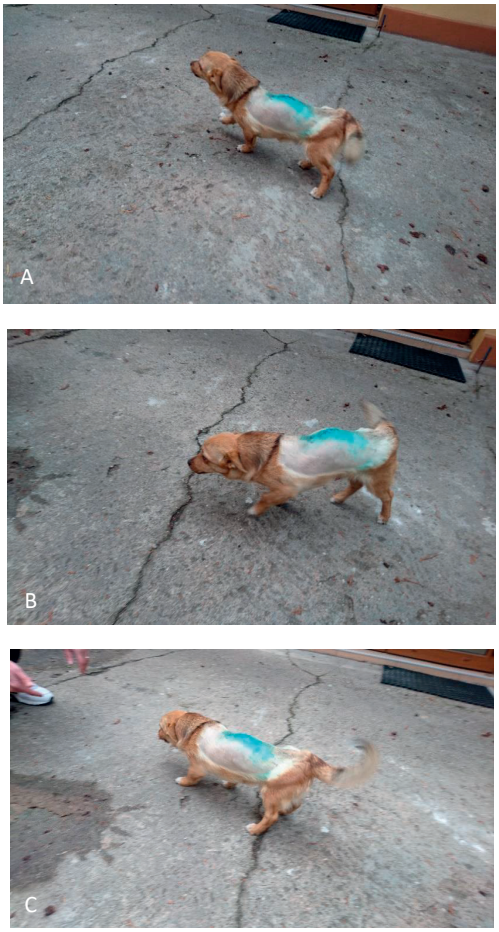


Fig. 11 (A-C). The dog seven days post-surgery, displaying normal status

At 3 weeks post-surgery the dog run, jump into bed as it can be seen in the following: https://youtu.be/XGM2K7EQETk
Similarly, at 3-4 months post-surgery, the dog run very well, being very agile and living normaly as before the car accident (as it can be seen in the following link https://youtu.be/5SqV8Psr2oc).

DISCUSSIONS

The success of the surgery of this case report was confirmed by the very fast restoration of the nervous system with the complete recovery the functions of the spinal cord and the functions of the vertebral column physiology, in only 7 days. Early surgical intervention is the best treatment option available in veterinary medicine for compressive or unstable lesions. Early decompression has been shown to enhance neurologic recovery in several animal studies (Kube and Olby, 2008; Merck, Manual of Veterinary Medicine 10th edition, 2014).
A good effect in the post-surgery treatment in this case was registered with the neurostimulant medications like vitamin B12, B1-B6, combined with pain killers and Myodine to improve fast the recovery of muscle mass lost in the days of paralysis and convalescence and recover the complete neuron's functions.
The position, place and location of this modified plate with screws in the described area creates a better long-term stability than a simple classic plate, because it fits very well on the shape of the lateral thoracic vertebral anatomy.
Use of a small amount of osteosynthesis metallic materials provides good results over time with a minimum biological impact comparatively with the double plate fixations on the both sides on the vertebral column (Welch, 2018; Shores and Brisson, 2017).
Another positive aspect is supported by a better acceptance from the animals (dogs in this case) of the internal on single plate versus the external fixators systems with many pins and screws in the body and other metallic systems outside the body which can be easily damaged of the dogs moving; also, external fixation systems can be easily hooked by any objects found around the dogs with high risks for compromising the surgery.
Additionally, this method doesn't cause deformation of the vertebral column and allow all the natural biomechanical movings as it is showed also in the video link performed after surgery.
We mention also other positive aspects such as, the shorter duration of the surgery with a reduced incision on only one side of the vertebral column; less chance of the body rejecting osteosynthesis material over time.
Moreover, no signs of the body reaction to the stainless steel plate implant during the time of the study 4 months were registered.
By this original method of placing the modified metallic Y plate, an economic efficiency of the

existing osteosynthesis materials was provided, without other special additional costs; it is known that the osteosynthesis materials used in spine surgery are usually very expensive. Therefore, this can be an economical option for clinics with limited possibilities and especially for social cases, unmanaged dogs, shelter dog, or zoo parks where funds are extremely limited. It could be also a very useful method for isolated areas clinics where supplies come usually 1 time every 2-3 months, for emergency situations (like, quarantine affected countries) to adapt one implant from another existent in the clinic and solve such cases of surgeries.

CONCLUSIONS

By this case we report the possibility of achieving a good stabilization of the fractured spine by this original method of placing the modified metallic Y plate. Metallic plate was adapted for fixing and stabilizing the spine, in a very short time, using another plate existing in the clinic stock. Other benefits consists in shorter surgery time, less anesthetics quantity used, less surgical materials used, and less costs because only one side incision method.

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INTRAVESICAL ADMINISTRATION OF CYTOSTATIC IN A DOG WITH URINARY BLADDER CARCINOMA - CASE STUDY

Catalin MICSA, Dorin ȚOGOE, Gina GÎRDAN, Maria RoxanaTURCU, Cristina PREDA, Andrei TANASE

University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Mărăști Blvd, District 1, 011464, Bucharest, Romania

Corresponding author email: mcscatalin@yahoo.com

Abstract

A 9-year-old spayed female mixed Pit Bull Terrier of 33.2 kg was referred with a complaint of stranguria, pollakiuria, hematuria, and abdominal pain of 5 weeks duration, not responding to treatment. The results of physical examination were unremarkable. A abdominal ultrasound revealed a large mass on the wall of the urinary bladder. The regional lymph nodes and the other abdominal organs present normal sonographical features. Thoracic radiography showed no evidence of metastatic disease. Blood samples have been taken for biochemistry work, CBC and urine samples. The dog underwent a cystoscopy, histopathological examination of the retrieved specimen reveal $T_2N_0M_0$ TCC (muscle-invasive transitional cell carcinoma). Due to the mass location on the wall of the bladder, surgery was not an option. Considering the result of the investigation, chemotherapy treatment has been applied: intravesical every 2-week cycle consisted of alternating epirubicin and 5-fluorouracil and intravenous holoxan. The treatment was well tolerated with no occurrence of any side effects. The abdominal ultrasound was repeated every 1 month and showed no progression of the disease.

Key words*: carcinoma, chemotherapy, cystoscopy.*

INTRODUCTION

Bladder cancer is one of the leading lethal cancers worldwide. Although urinary bladder cancer is reported to comprise only 2% of all reported cancers in dogs, bladder cancer affects tens of thousands of dogs every year worldwide.Transitional cell carcinoma (TCC), also referred to as urothelial carcinoma, is the most common form of urinary tumour in dogs, a malignant tumour that develops from the transitional epithelial cells that line the bladder. This tumour invades into the deeper layers of the bladder wall including the muscle layers. As the cancer enlarges in the bladder, it can cause obstruction to the flow of urine from the kidneys to the bladder or from the bladder to the outside of the body. Canine TCC also has the ability to spread to lymph nodes and to other organs in the body (lung, liver, others). TCC most frequently is found in the bladder, but can also develop in the kidneys, ureters, prostate, and urethra. Canine TCC is usually a high grade invasive cancer (Fulkerson, 2015). Problems associated with TCC include obstruction, distant metastases in > 50% of affected dogs, and clinical signs that are troubling both to the dogs and to their owners. Risk factors for TCC include exposure to older types of flea control products and lawn chemicals, obesity, female sex, and a very strong breed-associated risk.

This knowledge is allowing pet owners to take steps to reduce the risk of TCC in their dog.

The diagnosis of transitional cell carcinoma (TCC) can be made by histopathology of several tissue biopsies obtained with the help of cystoscopy or surgery. Studies showed that percutaneous aspirates and biopsies should be avoided due to the risk of tumour seeding. TCC is most commonly located in the trigone region of the bladder making impossible a complete surgical resection.

Although TCC is not usually curable in dogs, multiple drugs have activity against it, Regardless of clinical stage, systemic chemotherapy, having shown efficacy in bladder cancer, remains the standard approach for most of these dogs.

The appropriate treatment of patients with superficial bladder cancer requires the assessment of multiple variables, including

accurate clinical evaluation, understanding the natural history of the disease, pharmacology of the drugs currently used, and the expected efficacy of each drug (Badalament, 2017).
The treatment of superficial bladder cancer has three principal objectives: eradication of existing disease, to provide prophylaxis against tumour recurrence, and to avoid deep invasion into the muscle layers of the bladder. Almost every drug imaginable has been instilled into the bladder to treat superficial bladder cancer. However, five chemotherapeutic drugs (doxorubicin, mitomycin C, epirubicin, holoxan and fluorouracil) have widespread usage. Epirubicin is an anthracycline derivative of doxorubicin. Its antitumour effects are similar to doxorubicin, with a more favourable toxicity profile. In a study by Burk et al. (1989) involving 911 patients, no systemic toxicity was noted; chemical cystitis occurred in 15% and seemed to be related to drug oncentration.
Efficacy of intravesical chemotherapy can be measured in terms of tumour reduction, recurrence or progression (Lammet al., 1996).

MATERIALS AND METHODS

A 9-year-old spayed female mixed Pit Bull Terrier of 33.2 kg was referred with a complaint of stranguria, pollakiuria, hematuria, and abdominal pain of 5 weeks duration, the dog was not responding to treatment of classic cystitis. The results of physical examination were unremarkable and the lymphnodes were not enlarged or painful.
Then, urine samples have been taken for urine chemistry, sediment and cytology. The result came back with the following results: increased turbidity, increased RBC, increased WBC. The papillary tumours were characterized histologically by multiple papillae of spindle cells supported by thin, fibrovascular stroma and solid sheets of ovoid to round cells separated by similar stroma.
We also found presence of bacteria in sampled urine and most important neoplastic squamous cells.
A abdominal ultrasound revealed a large urethral mass extending to the trigone of the bladder, also affecting the inner bladder wall. The regional lymph nodes and the other abdominal organs were sonographically normal. Thoracic radiography showed no evidence of metastatic disease.
The owner elected for cystoscopy and histopathology of tissue biopsies (Figure 4). Blood biochemistry and CBC have been performed before anesthesia, the result being in normal limits.
The dog was premedicated for anesteshesia using Diazepam (0.2 mg/kg) and Butorphanol (0.2 mg/kg), Propofol (4 mg/kg) used for induction followed by endotracheal intubation using a 10 mm endotracheal tube and maintained with Isoflurane 2.5% and 100% oxygen. The dog was positioned in dorsal recumbency (Figure1).

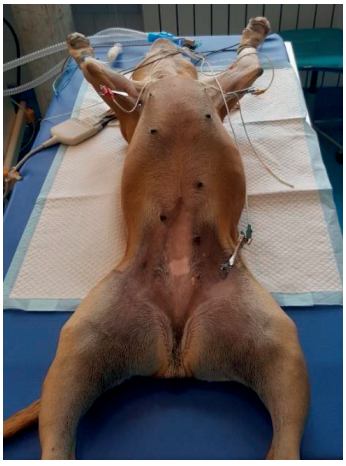

Figure 1. The dorsal recumbency postion for the procedure

For this procedure we used a Storz Hopkins forward-oblique telescope 30˚, 4 mm diameter and length 30 cm and a double action jaws, flexible biopsy forceps (Figures 2, 3).

Figure 2. Instruments for cystoscopy and biopsy samples

Figure 3. Monitor and modular camera control system

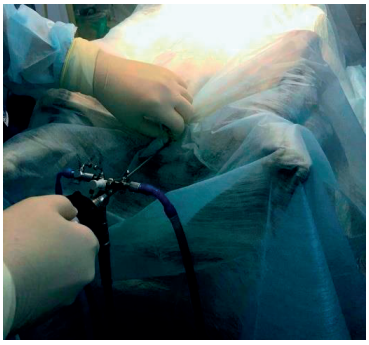

Figure 4. Undergoing the procedure

Inside the bladder, we tried to find areas with thickened or ruptured bladder wall or mass lesions within the urinary tract and take several biopsy samples for histopathological examination (Figures 5a, 5b, 5c).

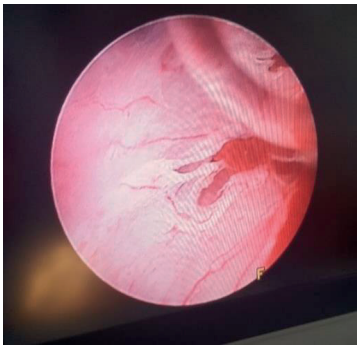

Figure 5a. Ruptured bladder wall

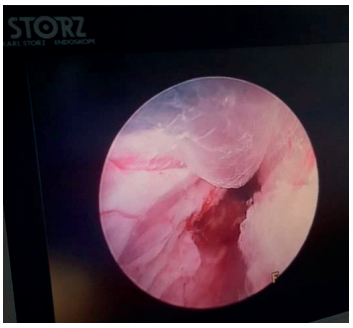

Figure 5b. Mass lesions inside the bladder wall

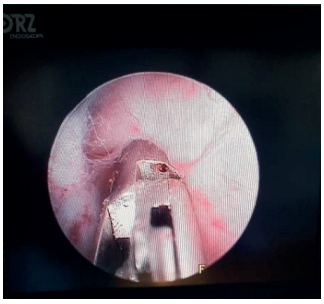

Figure 5c. Bladder biopsy sampling

Fluids have been provided during the procedure (Lactate Ringer) at 3 ml/kg/h rate. For the whole cystoscopy procedure, the heart rate, respiratory rate, concentration of CO_2 in expired gas, pulse, oxygen saturation and non-invasive blood pressure were measured. All the parameters were in normal limits during the procedure and the recovery period (Figure 6).

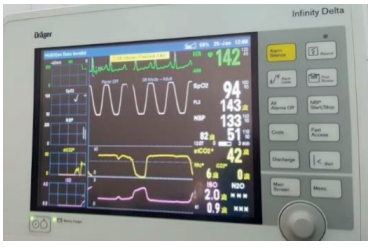

Figure 6. Vital sign monitor

RESULTS AND DISCUSSIONS

Pathological examination of retrieved specimen revealed to be Canine Transitional Cell Carcinoma. Due to the mass location on the bladder wall, surgery was not an option.
Consequently, treatment with intravesical chemotherapy has been applied: Epirubicin

alternated with 5-fluorouracil every 2 weeks and Holoxan i.v. Specific hepatic protective medication has been provided, such as liver enzymes and nutritional supplement for dogs suffering from cancer to greatly improve the animal's well-being during the treatments.

The chemotherapy was instillated using a semi-rigid urinary, sterile catheter, after eliminating and emptying the bladder and the dog had to walk and not urinate for 45-50 minutes. The dosage for Epirubicin was of 7 mg-3.5 mL, 5-fluorouracil was 4.5 mL both intravesical, and Holoxan intravenously 140 mg-3.5 mL.

The treatment was well tolerated with no occurrence of any side effects. We rechecked the blood chemistry, CBC and the abdominal ultrasound when the patient came for consultation after chemotherapy, as described in the tables below (Tables 1 and 2) and ultrasound pictures (Figures 7-10).

Table 1. Biochemistry results on different consults

Parameter	Reference interval	Day 1	Day 16	Day 60	Day 98	Day 125
GLU mg/dL	70-143	98	98	104	102	101
CREA mg/dL	0.5-1.8	1.2	1.1	1.0	1.9	1.5
BUN mg/dL	7- 27	13	11	12	16	15
TP g/dL	5.2-8.2	6.7	6.2	6.1	6.9	6.6
ALT U/L	10-100	37	61	79	70	75
ALKP U/L	23-212	74	64	82	65	77
CA mg/dL	7.9-12.0			10.1	10.9	11.1
ALB g/dL	2.2-3.9			3.2	2.8	3.3
Glob g/dL	2.5-4.5			2.9	4.1	4.4
GGT U/L	0-7			0	0	0
TBYL mg/dL	0.0-0.9			<0.1	0.2	0.3
AMYL U/L	500-1500			449	760	880
LIPA U/L	200-1800			436	524	613

Table 2. CBC results on different consults

Parameter	Reference interval	Day 1	Day 16	Day 60	Day 98	Day 125
HCT %	37.0-55.0	51.7	45	51.8	42.5	48.7
HGB g/dL	12.0-18.0	17.8	16.4	15.8	14.8	14.0
MCHC g/dL	30.0-37.5	34.4	36.4	30.5	34.8	35.2
WBC K/µL	5.50-16.90		5.90	5.29	4.50	3.91
GRANS K/µL	3.30-12.0	9.60	4.40		11.70	10.9
% GRANS		80.0	74.6		83.6	
L/M	1.1-6.3	2.4	1.5		2.3	1.8
% L/M		20	25		16	14
PLT	175-500	354	268		480	
RBC M/µL	5.50-8.50			7.22		
MCV fL	60.0-77.0			71.7		
MCH pg	18.5-30.0			21.9		
RDW %	14.7-17.0			15.3		
% RETIC				0.2		
RETIC K/µL	10.0-110.0			14.2		
% NEU				64.9		
% LYM				23.5		
% MONO				8.5		
% EOS				2.4		
% BASO				0.7		
NEU K/µL	2.00-12.0			2.86		
LYM K/µL	0.50-4.90			1.04		
MONO K/µL	0.30-2.00			0.38		
EOS K/µL	0.10-1.49			0.11		
BASO K/µL	0.00-0.10			0.03		
MPV fL				13.8		
PDW %				22.8		
PCT				0.61		

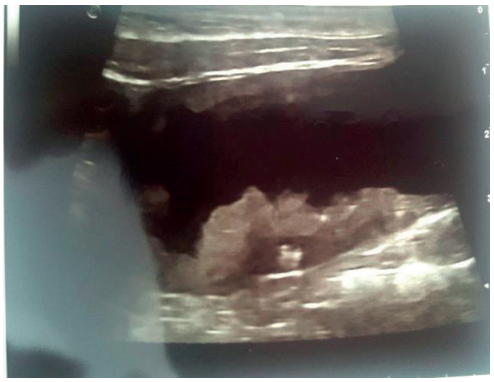

Figure 7. Longitudinal section with evidence of tumor formation in the middle region of the bladder and loss of parietal stratification

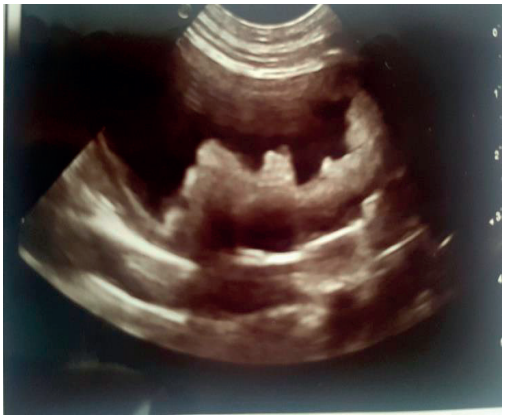

Figure 8. Longitudinal section of the middle and bottom region of the bladder and highlighting the tumor mass with irregular contour and broad-base in the middle region

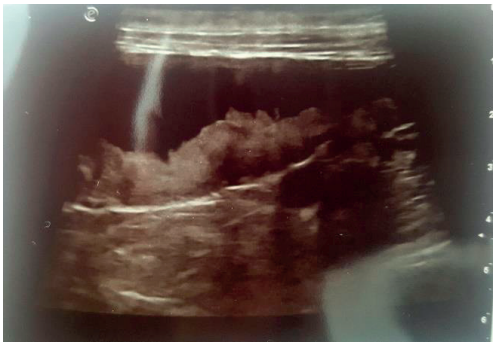

Figure 9. Longitudinal section of the middle region and neck of the bladder with highlighting the extension to the neck of the bladder

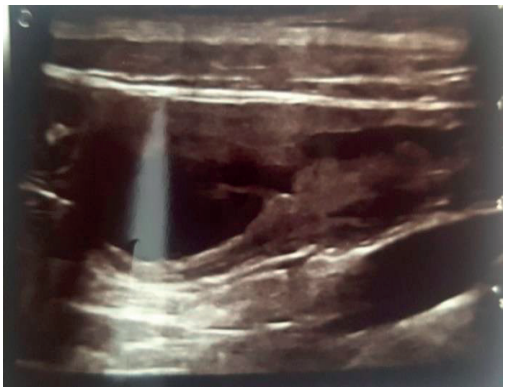

Figure 10. Longitudinal section with the highlighting of the tumor formation with regular contour and the tendency to reduce the size of the implantation base towards the bladder neck

The ultrasound examination was performed with the animal in the dorsal decubitus using a MyLab device and a 7.5 MHz microconvex probe.

After preparing the ventral abdominal region by trimming and applying the ultrasound gel, the bladder was examined both in the longitudinal section and in the cross-section, sequentially, starting from the neck of the bladder to the deep region.

As we can see in the pictures above, in the lumen of the bladder, a mass of increased size measuring18.3 mm thick and 27.4 mm length, was observed, occupying approximately 55% of the lumen with irregular appearance and large implantation base, with a strong infiltrative character in the deep parietal layers which causes the loss of the normal stratification of the wall in the region middle towards the neck of the bladder.

Subsequent examinations performed during the specific treatment revealed the tendency of localization of the tumour mass, with the obvious reduction up to size of 5.1 mm thick and 14.4 mm length with the reduction of occupied intralumenal space.

The aggressive infiltrative character of the formation, respectively the modification of the deep structure of the bladder wall was maintained throughout the ultrasonographic monitoring but with a clear reduction of the affected area.

CONCLUSIONS

The purpose of this study was to determine the antitumoural activity of 2 chemotheraphy agents administered intravesical, Epirubicin and 5-fluorouracil, in combination with Holoxan administered i.v. The study results provide evidence that this specific combination of drugs is more effective than either drug alone. It is not possible to know whether the Epirubicin and 5-fluorouracil enhanced the effects of the Holoxan or whether the Holoxan administered i.v. enhanced the effects of the Epirubicin, just that the combination resulted in a favourable remission rate.

Antibiotic treatment may cause reduction or temporary resolution of clinical signs. The urinary tract signs with TCC closely mimic those of a urinary tract infection.

Finding abnormal epithelial cells in urine and thickened bladder wall or mass lesions within the urinary tract also increases suspicion for TCC. Histopathological examination provides a definitive diagnosis of TCC and characterization of the different pathological types of TCC. Tissue biopsies from the bladder can be obtained by cystoscopy, and with this procedure the operator can visually inspect the urethra and bladder wall and obtain biopsies using this method.

In dogs with confirmed or suspected TCC, evaluation should include an assessment of overall health and tests to determine tumour stage, as this information will be used in planning treatment. This includes a complete blood count (CBC), serum biochemistry profile, urinalysis with or without urine culture, thoracic radiography, abdominal ultrasonography, and urinary tract imaging. Urine should be collected by free catch or catheterization; cystocentesis should be avoided as it could lead to tumour seeding.

When using ultrasonography to monitor changes in TCC masses, however, it is essential to have the same operator for performing each examination, to standardize the dog's position, probe position, and data collection, and to have a similar level of bladder distension for each ultrasound examination visit.

The main of TCC treatment in dogs continues to be systemic medical therapy which usually consists of chemotherapy. Although medical therapy is not usually curative, remission or stable disease (lack of progression) can be accomplished with several different drugs, and most treatments are well tolerated.

The best results often occur in dogs that sequentially receive multiple different treatment protocols over the course of their disease, like in our case.

Epirubicin appears to have the greatest level of antitumour activity against canine TCC, especially when combined with 5-fluorouracil.

Future studies with a larger population of dogs undergoing this approach and treatment should be performed to assess whether this strategy may be successful in dogs with Transitional Cell Carcinoma in the urinary bladder.

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PULMONARY STRONGYLIDOSIS OF SMALL RUMINANTS IN SERBIA

Ivan PAVLOVIC[1], Snezana IVANOVIC[1], Milan P. PETROVIC[2], Violeta CARO-PETROVIC[2], Dragana RUŽIĆ-MUSLIĆ[2], Narcisa MEDERLE[3]

[1]Scientific Veterinary Institute of Serbia, J.Janulisa 14, Belgrade, Serbia
[2]Institut for Animal Husbandry, Autoput 16, Belgrade-Zemun, Serbia
[3]Faculty of Veterinary Medicine, 119 Calea Aradului, Timisoara, Romania

Corresponding author email: academician Dr Ivan Pavlovic dripavlovic58@gmail,com

Abstract

In pasture breed condition helminth infection are common especially during late spring and autumn months. Research of goats and sheep parasites was made systematically last 10 years in Serbia. Most of the research related to gastrontestinal and something less about lung helminth infection. The research was carried out on several locations in Serbia in the period and included goat and sheep herds in the area of carried out in north, northeast, eastern, southern and south-eastern part of Serbia and at Belgrade area. We examined fecal samples using the Berman method. Slaughtered or dead animals we examined by necropsy and adult parasites separated from the lung section. Determination of adult and larval stage of parasites was based on the morphological characteristics. During our examination most abundant species was Dictyocaulus filaria, followed by Protostrongylus rufescens, Cystocaulus nigrescens and Muellerius capillaris.

Key words*: small ruminants, lung worm, Serbia.*

INTRODUCTION

The grazing diet allows the permanent contact of small ruminants with intermediate hosts and the eggs and larval forms of the parasite. From these reason parasitic infections are present worldvide in a large number of herds (Berrag and Urquhart 1996; Alemu *et al.,* 2006; Geurden and Vercruysse, 2007; Pavlovic *et al.,* 2009a, 2012, 2013; Alasaad *et al.,* 2010).

Lungworms of domestic ruminants are nematodes that belongs to the phylum Nemathelmenthes commonly named as round worms; classified under the super family Trichostrongyloidea and Metastrongyloidea (Tewodros, 2015). Of which, *Dictyocaulus* and *Protostrongylus* are causes of lungworm infection in ruminants (Taylor *et al.,* 2007; Palic, 2001; Pavlovic *et al.,* 2009b, 2010a). They induce verminous pneumonia which was a significant health problem of sheep and goats

Pulmonary strongilides of small ruminants are most commonly occur in pastures with lush vegetation (Pavlovic *et al.,* 2009b, 2010a). Pasture infectivity is related to rainfall which stimulates the activity of both the larvae and the mollusk. Moisture is essential for the survival and development of the larvae. The larvae is active at moderate temperature of 10-21ºC. Larvae survive best in cool, damp surroundings especially when the environment is stabilized by the presence of long herbage of free water.

In our paper we present the results obtained on the prevalence of pulmonary strongilides in sheep and goats in Serbia, based on research done in the last few years.

MATERIALS AND METHODS

Investigations performed in period 2012-2018 and included 430 herds of goats and sheep in the area of Belgrade, Stara Planina, north-eastern, central and southern part of Serbia and in Vojvodina.

In total, 4300 fecal samples were examined by the Berman method. At the same time, we have done the autopsy of 387 animals on the slaughter line or after death. After that routine necropsiesof the lungs were performed. The helminths within the particular lesion were found and their species were determined under the microscope.

Determination of parasites larvae and adult parasites was based on its morphological characteristics (Dunn, 1978; Euzeby, 1981).

RESULTS AND DISCUSSIONS

During our studies, infection with pulmonary strongilides was recorded in 327 (76.04%) herds. Based on the autopsy we revealed the presence of *Dictyocaulus filaria* (47.02%), *Protostrongylus rufescens* (74.01%), *Muellerius capillaris* (37.20%) and *Cystocaulus nigrescen* (19.12%). On the basis of autopsy and coprological examination average prevalence of *D. filaria* was 60.52%, *P. rufescens* 74.01%, *M. capilaris* 39.22% and *C. nigrescens* 19.31% (Figure 1).

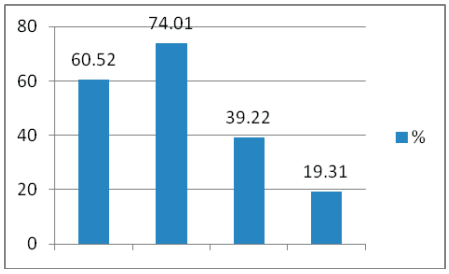

Figure 1. Average prevalence of lungworms infection

Dictyocaulus filaria was ocurerd at all small ruminants heard from all part of country and prevalence was from 12.24 to 60.52%. The highest prevalence is observed in the herds in Vojvodina, north and southeastern Serbia where predominate flat plain pastures. Due to microclimatic conditions, the infection usually occurs in the period April-May when the largest number of animals are grazing. This has been confirmed by research on grass on pastures for the presence of larvae of parasites (Regasa *et al.,*2010; Pavlovic *et al.,*2017).

These 3.5-9 cm long parasites live in the lumen of the bronchi and trachea. During autopsy, we found parasites in the bronchi where they caused chronic bronchitis and peribronchitis. The most common pathological changes we have encountered are presence of parasites and abundant purulent mucous membranes obstruct the lumen of the bronchi so that distal bronchial collapse, dark red atelectasis, or pale emphysematous fields (Figure 2).

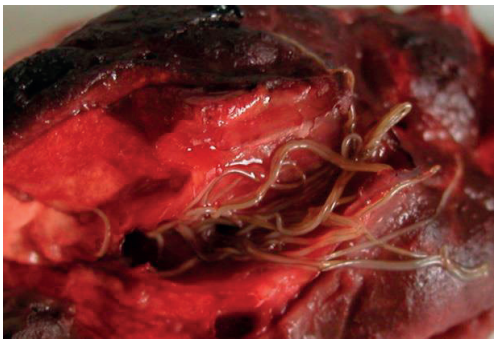

Figure 2. *Dictyocaulus filaria* in sheep lung

From the sufamily *Protostrongylinae* we registered genera *Protostrongylus, Cystocaulus* and *Muellerius* (Pavlovic *et al.,* 2010a; Ivanović and Pavlović, 2015). Parasites are predominant at herd grazed at hill side of mountains, especially at eastern, central and southern part of Serbia. *Protostrongylinae* are biohelminthes and need intermediate hosts for their development - snails and slugs (Diez-Baños *et al.,*1989; Manga and Morrondo, 1990; Morrondo-Pelayo *et al.,*1992). During ours examination in pasteures in various parts of Serbia we concluded that the most common intermediate hosts are *Abeda frumenta, Arion ater, A. subfuscus, Cepae vindobenensis, Helix aspersa, H. pomatia, Chondrula tridens, Fruticola fruticum, Derocercas reticulatum, Eucomphalia strigella* and *Helicella obvia.* Due to microclimatic conditions, the infection usually occurs in the March and April when the largest number of intermediate host are preset at pasture (Pavlovic *et al.,* 2010b).

During examination most abudant species was *Protostrongylus rufescens* occurred on 21.93 to 77.21%. These nematodes live in bronchioles and alveoli, are reddish in colour and relatively small - the male is 16-28 mm long and the female is 25-35 mm long (López *et al.,* 2011). The parasitic lesions established in the present study were located within the caudal lung lobes and were disseminated mainly within the dorsal subpleural parenchyma (Figure 3).

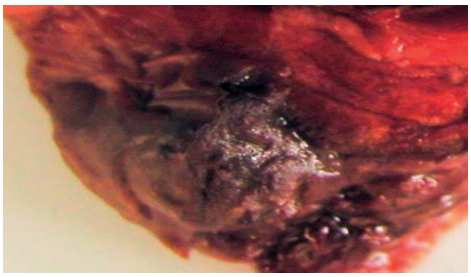

Figure 3. *Protostrongylus rufescens* in goat lung

From the genus *Cystocaulus* we occurs *C. nigrescens*. Adult parasites live in the pulmonary parenchyma (Mengestom, 2008). The parasites are gray-white in colour. Males are 18-24 mm long and female's 40-50 mm. Prevalence are ranges from 12.22 to 39.22%. On diaphragmatic lobes we found cone-shaped granulomas in lung tissue that varied in size, color, and degree of consolidation (Hubado, 2010; Domke et *al.,* 2013). They contain sexually active parasites with a mass of eggs and larvae. The nodules were as large as the head of a dark brown to black if *Cystocaulus* sp. was present (Figure 4).

Figure 4. *Cystocaulus nigrescens* in sheep lung

From the genus *Muellerius* occurs *Muellerius capillaris*. Parasites live in the bronchioles and alveoli. The body of the parasite is thin white. The prevalence ranges was from 4.23 to 19.31%. The lung lesions in goats infected with *M. capillaris* were nodular, firm, and gray located in the dorsal surface of the caudal lung lobes (Tenaw and Jemberu, 2018). The lesions in the lungs of sheep were more severe, regardless of the animals' age. In the most cases they were not formed as nodules and were dark grey to black areas affecting a large part of the lung surfaces (Figure 5)

Figure 5. *Muellerius capillaris* in sheep lung

From the presented data, it can be seen that the biodiversity of pulmonary strongylides of small ruminants is similar to that of the whole of Europe (Rose, 1973; Diez-Baños *et al.,*1989; Manga and Morrondo, 1990; Morrondo-Pelayo *et al.,*1992; Berrag *et al.*, 1996; Geurden and Vercruysse, 2007; Alasaad *et al.,* 2009; Stanchev *et al,* 2010; Panayotova-Pencheva and Alexandrov, 2010).

Our examination are the first systematically studies of pulmonary strongilides in Serbia, and are related to determining the prevalence of parasitic infections, species of lungworm of sheep and goats as well as their vectors.

CONCLUSIONS

Based on the results obtained, we can conclude that a large number of sheep and goats in Serbia are infected with pulmonary strongilides. *Protostrongylus rufescens* and *Dictyocaulus filaria* are the dominant species, while *Muellerius capillaris* and *Cystocaulus nigrescens* are present in a smaller percentage.

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THE USE OF TWO DIFFERENT ANESTHETIC PROTOCOLS FOR OVARIECTOMY IN *TRACHEMYS SCRIPTA ELEGANS*

Maria Roxana TURCU, Ruxandra PAVEL, Andra DEGAN, Gina GÎRDAN, Catalin MICSA, Ovidiu ROȘU, Lucian IONIȚĂ

University of Agronomic Sciences and Veterinary Medicine of Bucharest,
59 Mărăști Blvd, District 1, Bucharest, Romania

Corresponding author email: turcu.mariaroxana@yahoo.com

Abstract

This study was performed in order to evaluate two anesthetic protocols used for ovariectomy in turtles belonging to Trachemys scripta elegans species, between June and July 2019 at the Faculty of Veterinary Medicine of Bucharest. Patients aged one year old were divided in two study groups depending on the anesthetic protocol used. Group A was premedicated with Midazolam (2 mg/kg), Dexmedetomidine (0.1 mg/kg) and Ketamine (5 mg/kg) administered intramuscularly (IM) while group B had Midazolam (2 mg/kg), Buprenorphine (0.2 mg/kg) and Ketamine (2 mg/kg) IM. Induction was made with Isoflurane 5% in the induction chamber followed by Ketamine (10 mg/kg) given intravenously, in the supravertebral sinus (IV). All patients were intubated with an 18G IV catheter and maintained with Isoflurane 3% and 100% Oxygen. Patients in group B required two boluses of Ketamine (10 mg/kg/bolus) compared with patients in group A that didn't have any additional doses. Therefore the recovery was smoother and faster in patients from group A.

Key words*: anesthesia, ovariectomy, Trachemys scripta elegans.*

INTRODUCTION

The study analyzes and compares the effectiveness of two anesthetic protocols utilised in ovariectomy in *Trachemys scripta elegans* (red-eared slider turtle). The anesthesia of turtles have some dificulties, compared to the anesthesia of small animals. First of all, turtles are considered exotic animals, with anatomical and physiological particularities. Working with turtles for the first time can be very difficult. The doses need to be carefully calculated because they are different from the doses used in small animals.

The female presents a pair of ovaries that are saccular in shape and are covered with follicles. The ovaries are continued with the oviducts. The oviduct is continued with infundibulum, uterine tubes, isthium, uterus and vagina that open in the exterior in urodeum. The egg has three membranes inside and the eggshell, that is water resistant, allows gas exchange. All of these play a very important role in the development of the embryo (O'Malley, 2005).

Because of all this anatomical particularities, the turtles are prone to some conditions such as oviductal rupture, ectopic eggs in the coelum, chornic oviductal impaction or obstructive dystocia (Mans & Sladky, 2012). To avoid these conditions, as a prophylactic measure, the ovariectomy is recommended.

Turtles have anatomical and physiological particularities different from other small animals so the anesthetic protocols have to be modified according to those. One of these particularities is thermoreglation. Turtles are poikilothermic animals, which means that the body temperature is influenced by the outside temperature. Decrease in the body temperature affects the metabolic rate, which will increase the recovery period. There are also differences of the cardiovascular system. A unique feature of the cardiovascular system is the renal portal system. This cranial and the caudal portal vein form a ring of blood vessels around the kidney. The clinical importance of this particularity is that if the medication is administered in the bottom half of the body, the metabolism and excretion will be faster. Therefore, it is important to underline that the site for the intramuscular injection is in the forelimb muscles. As for the respiratory system, the turtles have a pair of saccular shaped lungs. The respiratory rate and the oxygen demand are

physiologically lower compared to the mammals. The hypoxia is well tolerated because turtles can change from an aerobic to an anaerobic metabolism (Sladky & Mans, 2012).

MATERIALS AND METHODS

The study took place at the Faculty of Veterinary Medicine Bucharest, between June and July 2019. A number of 10 turtles were used, all females, of age one year old and that were divided in two study groups, A and B, based on the premedication.

The first step in choosing anesthesia protocol was a thorough history and clinical examination. All the turtles were fed with turtle pellets and dried shrimps and kept in a glass aquarium filled with water. The physical examination revealed them to be bright, alert and responsive, with a heart rate and respiratory rate within normal limits (40 beats per minute and 5 breaths per 2 minutes).

Group A was premedicated with o combination of Midazolam, Dexmedetomidine and Ketamine, while group B received Midazolam, Buprenorphine and Ketamine. The main classes of substances that were used for the anesthesia were: benzodiazepines, α_2-adrenoreceptor agonist, opioids, dissociative agents and inhalant agents.

Midazolam is a benzodiazepine used for its sedation, muscle relaxation and anxiolytic effects. It is a water soluble compound, which means is not painful when administered intramuscularly. Midazolam is metabolised by the liver, with fast onset and a short action period. It doesn't have analgesic properties so it is used in different combinations with other classes of medication, like opioids and α_2-adrenoreceptor agonists. Flumazenil is competitive antagonist used to antagonize the effects of benzodiazepines (Costea, 2017).

Dexmedetomidine is the most potent and specific α_2-adrenoreceptor agonist available. This class of substances is known for its sedation, anxiolytic and analgesic properties. Because of its analgesic properties, dexmedetomidine reduces the need of opioids to almost 40% (Costea, 2017). Dexmedetomidine has some cardiovascular effects that include bradycardia, initial hypertension, followed by prolonged period of lower blood tension. As a result of the bradycardia, the cardiac output falls (Tranquilli et al., 2013). The respiratory depression is minimal. Other side effects of the dexmedetomidine administration include hyperglycaemia, decrease in the gastrointestinal motility and decrease in the intraocular and intracranial pressure (Clarke et al., 2013).

α_2 agonists actions can be antagonised by using Atipamezol. When used, Atipamezol antagonises both its sedation and analgesic effects.

Ketamine is a dissociative agent that produces a state called "dissociative anesthesia". This state is characterized by a dissociation of the thalamocortical and limbic system (Tranquilli et al., 2013). Ketamine acts as a non-competitive N-methyl-D-aspartate antagonist (Tranquilli et al., 2013). When administered alone, it has sympathomimetic effects, such as increase in the heart rate, cardiac output and blood pressure. (Costea, 2016).

Buprenorphine is a μ opioid receptor partial antagonist. It is more effective compared to morphine for chronic pain. The peak effect appears after 20 minutes of IV administration and the duration of the analgesic effects last 8 up to 12 hours. (Tranquilli et al., 2013)

RESULTS AND DISCUSSIONS

Before any medication was administrated, the turtles were measured so that the doses are according to the animals weight. The weight of the turtles varies from 1.2 to 1.7 kg (Figure 1).

The 10 turtles were randomized divided in 2 groups. Group A was premedicated with Midazolam (2 mg/kg), Dexmedetomidine (0.1 mg/kg) and Ketamine (5 mg/kg). Group B had Midazolam (2 mg/kg), Buprenorphine (0.2 mg/kg) and Ketamine (2 mg/kg). In both groups, the medication was given IM, in the forelimb muscle and then we kept them in a quiet place for 10 minutes so that the external sounds don't disturb them.

Figure 1. Weight measurement

For induction, all the turtles were kept for 10 minutes in the induction chamber where they received Isoflurane 5% and Oxygen 100%, followed by a bolus of Ketamine (10 mg/kg) IV, in the subcarpial sinus (Figure 2).

The next step after premedication and induction was intubation with a 18G IV cannula (Figure 3). Because so small endotracheal tubes were not available at the moment, we used an IV cannula, removing the insertion needle before intubation of the turtles.

Turtles are obligate nasal breathers (Kirchgessner & Mitchell, 2009). After opening the mouth, the glottis can easily be identified at the back of the tongue. The IV cannula is easily introduced inside the trachea. After the intubation, a breathing circuit is used to deliver 1 L of Oxygen 100% and Isoflurane 3% to the patient. The patient was maintained on Isoflurane 3% and 1 L of 100% Oxygen during the surgery (Figure 4).

Figure 2. Intravenous administration in the subcarpial sinus

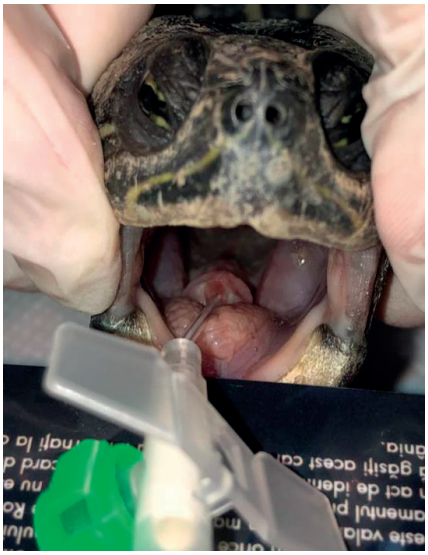

Figure 3. Intubation of the turtle using a 18G IV cannula

Figure 4. The turtle is connected to a breathing system to receive Isoflurane 3% and 100% Oxygen

Monitoring the pacient during anesthesia is a very important part of any surgery. The heart rate, respiratory rate and the end tidal CO_2 in the expired gas were recorded at regular intervals to facilitate early recognition of adverse trends. The normal vital signs of the turtles, before any medication was administrated were: heart rate 40 beats per minute and respiratory rate 5 breaths per 2 minutes.

This parameters change depending the substances that are used. For example, Table 1 presents the vital signs monitored during surgery: heart rate, respiratory rate and end tidal CO_2 in the expired gas of the turtles from group B after 5 minutes from receiving a Ketamine bolus (10 mg/kg, IV). Note the increase in the heart rate due to the positive inotropic action of the Ketamine.

Table 1. Vital signs of the turtles from group B after receiving a bolus of Ketamine (10 mg/kg, IV)

Parameter	T1	T 2	T 3	T 4	T 5
Heart rate (bpm)	75	73	65	70	63
Respiratory rate (rpm)	8	7	8	10	13
ET CO_2 (mmHg)	9	8	9	6	5

Table 2. Vital signs of the turtles from group A

Parameter	T 1	T 2	T 3	T 4	T 5
Heart rate (bpm)	35	30	41	38	36
Respiratory Rate (rpm)	8	7	8	9	8
ET CO_2 (mmHg)	9	9	8	8	8

Table 2 describes the same vital signs monitored during surgery of the turtles from group A. Note the decrease of the heart rate due to the use of α_2-adrenoreceptor agonist. In comparison to group B, the turtles from group A did not required additional boluses of Ketamine.

The mean duration of the surgery was 52.4 ± 7.4 minutes. During the surgery, an increase in the heart rate and the movement of the animal indicated that the plane of anesthesia was light so it required an extra bolus of Ketamine (10 mg/kg, IV). All the turtles from group B required one or two extra boluses of Ketamine (10 mg/kg, IV) during surgery. The time between each boluses was an average of 25 minutes.

After all the surgical procedures were completed, the turtles were extubated and kept under observation. During surgery and on the recovery period, the animals were kept on the heating pad. Cloacal temperatures were consistently the same as the ambient room temperature (28° ± 1°C) in all animals at each recorded time (at the beginning of the surgery and at the end of the surgery). All the turtles received Meloxicam (single dose, 0.5 mg/kg, IM) and Enrofloxacin (5 mg/kg/per day, 5 days). Every turtle from the two groups benefit from the administration of specific antagonists: group A receive Atipamezol (0.5 mg/kg, IM), while group B receive Flumazenil (0.05 mg/kg, SC). After 10 minutes from the administration of the antagonist, all the turtles in both groups presented an increase in the mandibular reflex. Also, the heart rate of the turtles from group A began to increase. After 20 minutes from the injection of the antagonists, the turtles from group A began to move (mean time of 15 ± 2.5 minutes), while the turtles from group B were alert and responsive (mean time of 21.8 ± 2.1 minutes).

A previous study by Greer, Jenne ans Digs evaluate the effects of a low and high dose combination of Medetomidine and Ketamine. The parameters that were evaluated were heart rate, palpebral reflex, limb and neck reflexion

and cloacal temperature and also the response to minor procedures. Using an α_2-adrenoreceptor agonist assures an adequate level of sedation and anesthesia and also allows endotracheal intubation (Greer et al., 2001).

Turtles from group B that didn't receive a α_2-adrenoreceptor agonist required one or two additional Ketamine boluses (10 mg/kg). Because of the additional bolus that the turtles in group B received, the recovery was prolonged, compared with the turtles in group A, in which the recovery was faster and smoother.

Figure 5 presents in comparasion the time that each turtle required to recover after the antagonist administration. Note that for group A, all the turtles required less than 20 minutes to recover (mean time of 15 ± 2.5 minutes), while the turtles in group B require more than 20 minutes (mean time of 21.8 ± 2.1 minutes).

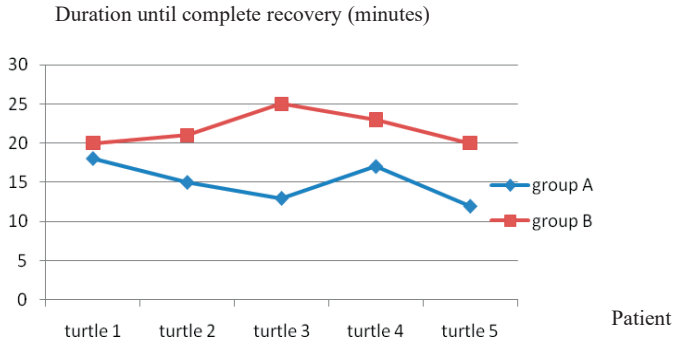

Figure 5. The recovery period (minutes) for the two groups

CONCLUSIONS

When using Midazolom - Dexmedetomidine - Ketamine in captive red eared slider turtles the recovery is smoother and the return to the normal physiologic parameters is faster.

To have a smooth and fast recovery, always consider using substances that have an antagonist to treat and prevent the emergency situations.

The monitoring of the vital parameters is very important during the anesthesia as it is in the recovery period.

For the anesthesia of the red eared slider turtle including an α_2-adrenoreceptor agonist increases the level of sedation and analgesia.

In combination with other drugs (ex. Benzodiazepines or Ketamine), the use of α_2-adrenoreceptor agonist produce a sufficient anesthesia for short to medium time duration procedures, without any addition boluses of substances.

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TESTING THE EFFECT OF *NIGELLA SATIVA* ESSENTIAL OIL SOLUTION ON CHICKEN BREAST pH AND TOTAL VOLATILE BASE NITROGEN DURING REFRIGERATION

Raluca-Aniela IRIMIA, Mara GEORGESCU, Liliana TUDOREANU, Manuella MILITARU

University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Mărăști Blvd, District 1, Bucharest, Romania

Corresponding author email: irimiaral@gmail.com

Abstract

Following consumers high demand for healthier food, the food industry is more than ever interested in replacing traditional chemical preservatives with natural preservatives such as plant extracts and oils. Therefore, we have tested the preservation potential of the 1% (T1%) and 2% (T2%) Nigella sativa oil solutions on chicken meat. Five chicken breasts (m. Pectoralis major) were purchased and each of them was divided in three aliquots, corresponding to Control, T1% and T2%. All chicken breast aliquots were refrigerated for 6 days. The pH and Total Volatile Base Nitrogen (TVB-N) was measured for Control and treatments on day 1, day 3 and day 6. After 6 days of refrigeration, the T2% treated meat had a pH = 6.062 ± 0.042 and a TVB-N = (26.100 ± 0.644) (*mg* NH_3*/100g meat), values which were significantly different compared to Control. The pH and TVB-N values measured for T1% treated meat were not significantly different from Control after 6 days of refrigeration. The T2% was found to be the most efficient treatment for preserving the meat during a refrigeration period of 6 days.*

Key words*: Nigella sativa, preservation, chicken breast, refrigeration, pH, TVB-N.*

INTRODUCTION

The use of essential oils for the meat products preservation is a healthy alternative to chemical preservatives, complying to consumers requirements (Hassanien, 2006; Marzieh et al., 2013; Georgescu, 2019). The latest scientific reports help producers, to identify the best alternative for improving the safety and quality of meat products by using natural oils and plant extracts instead of synthetic chemical derivatives (Osman et al., 2017; İlk et al., 2018).

One of the oils deeply studied, is that of *Nigella sativa* (black cumin). Following chemical analysis the *N. sativa* oil contains over 85% biological active compounds such as p-cymene, α-thujene, longifolene, α-pinene, β-pinene, thymoquinone and carvacrol (Karimi, 2019; Georgescu, 2018). Moreover the *N. sativa* extract demonstrated antimicrobial, anti-fungal and anti-oxidant properties which are highly useful characteristics for the current stringent needs of the food industry (Ramadan, 2016; Iwona et al., 2017). In addition, the *N. sativa* oil exhibited stronger radical dropping activity against 2,2-diphenyl-1-picryldiazyl radical compared to synthetic antioxidants (Ramadan, 2016; Kiralan et al., 2014).

Studies of the *N. sativa* oil effects were carried out on a multitude of foodstuffs (Osman et al., 2017) by using the essential oil in various forms, a great majority of the experiments being conducted for microbiological determinations. Thus, further studies in the field should also involve monitoring of the pH and Total Volatile Base Nitrogen in order to provide a broader view regarding the food safety and quality of the products treated with *N. sativa* essential oil.

MATERIALS AND METHODS

Five samples of chicken pectoral muscle (*m. Pectoralis major*) of approximately equal weight (200-250 g each) were purchased from a single producer and from different stores in order to randomize the process of sampling. The samples were transported in cooling bags with no refrigeration (approximately 15 minutes from the store to the laboratory, for each transport). Each chicken breast was divided into 3 aliquots of equal weight (Figure 1) and used for the "Control" (non-treated

meat), the 1% *N. sativa* oil solution treatment and the 2% *N. sativa* oil solution treatment (Figure 1). The Control and the two treatments contain each of them 5 aliquots of meat.

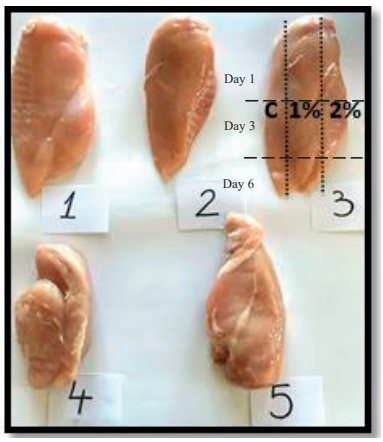

Figure 1. Breast samples collected - all aliquots were of equal weight. All parts (from the aliquots) were of equal weight

- Treatment solutions preparation

The treatment solutions were made by adding Black Caraway oil - cold pressed, 100% pure oil ("Ulei de negrilică", Carmita Classic SRL, Alba Iulia, Romania) to Deuterium Depleted Water (DDW). The solutions were: T1% - 1 ml of Black Caraway oil in 100 ml of solution and T2% - 2 ml of Black Caraway oil in 100 ml of solution.

- Meat samples preparation

Each of the five chicken meat breasts were sliced into three even aliquots. Thus, we had 5x3 meat samples which were used for the T1%, T2% and Control, as shown in Figure 1, for chicken breast number 3. The treatments T1% and T2% contained meat aliquots as described above. The treated aliquots were individually soaked into 20 ml of T1% and T2% solution respectively, and stored in polyethylene bags, for the whole duration of the experiment. Samples from the Control, T1% and T2% were analysed at 3 hours after the treatment (Day 1), after 3 days of refrigeration (Day 3) and after 6 days of refrigeration (Day 6), 45 samples in total. The refrigeration temperature was 2^0C.

- Measurement of pH and Total Volatile Base Nitrogen (TVB-N)

The TVB-N and pH (Figure 2) measurements were carried out according to SR ISO 2917:2007, SR 9065-7:2007, SR 9065-7:2007/C91:2009 and an AOAC method.

Prior to measurements, each collected part was grinded individually using a Braun MQ5020 grinder.

Figure 2. The pH determination

- Statistical data analysis

One-way ANOVA for independent samples was chosen to identify significant differences for pH and TVB-N respectively, between treatments in Day 1, Day 3 and Day 6. Our experiment has only n = 5 (very small sample size). Technically ANOVA can work if there is one value more than parameters to be estimated by the model (for k = 3 samples the minimum total sample size is n = k +1 = 4). However the rule of thumb for ANOVA is to have n=30 in order to generate a power of 80% of the analysis. Generally the accepted minimum of values per statistical sample is n = 7. When the sample size is very low (n = 5) the power of the analysis might be as low as 50%. Therefore, using 5 values per sample, for an ANOVA analysis there is a probability of approximately 50% to pick up on an effect that is present. Also we are aware of this situation and have decided to proceed with the experiment in order to acquire preliminary information we can use to develop a future larger scale experiment.

The data were tested for normality using the Shapiro-Wilk test. The Levene's test was used to test for equality of variances. When data were not normally distributed, we used the Kruskal-Wallis test.

The level of significance is 0.05.

When the data were normally distributed and the samples have unequal variances the choice for data analysis was Welch-ANOVA.
Dunnet test (with control) is the post-hoc test used for a significant ANOVA.
Following a significant Kruskal-Wallis test we used the post-hoc Conover test.
A two way ANOVA is not an option due to the fact that comparing the levels of pH and TVB-N between Day 1 and 6 has no practical meaning. Obviously, these values will be highly different.
The MedCalc Software version 18.10.2 and JMP 15 trial version were used for statistical data analysis.

RESULTS AND DISCUSSIONS

The pH and TVB-N values for all treatments during the 6 days refrigeration period are presented in Table 1.

Table 1. pH and TVB-N of chicken breast treated with solutions of *N. sativa* oil during 6 days of refrigeration at 2^0C

Treatments	DAY 1		DAY 2		DAY 3	
	pH Mean ± SD	TVB-N (mg NH_3/100g meat) Mean± SD	pH Mean ± SD	TVB-N (mg NH_3/100g meat) Mean± SD	pH Mean ± SD	TVB-N (mg NH_3/100g meat) Mean± SD
CONTROL	5.898 ± 0.131	24.980 ± 0.414	6.072 ± 0.051	26.000 ± 0.200	6.226 ± 0.055	27.200 ± 0.547
T1%	5.880 ± 0.129	24.860 ± 0.439	6.028 ± 0.071	25.620 ± 0.228	6.122 ± 0.055	26.620 ± 0.589
T2%	5.848 ± 0.135	24.700 ± 0.424	5.856 ± 0.225	25.300 ± 0.158	6.062 ± 0.042	26.100 ± 0.644

The statistical analyses of the treatment's results are presented in Table 2 to Table 5.
The Control pH values (Table 1) for the first day are similar to the data reported by Saláková (5.66 and 6.08) (Saláková et al., 2009) and to the data reported by Baston (5.82-6) (Baston et al., 2002), the mean value in our experiment being 5.898.
After three days of refrigeration the pH values of the samples treated with *N. sativa* oil solutions were not significantly different (Table 2) compared to the pH values measured for Control.
However the TVB-N values of treated chicken breasts were significantly different from the Control after three days of refrigeration (Table 3).
After six days of refrigeration only the samples of the T2% treatment were significantly different from Control (Table 5).

Table 2. Statistical analysis for pH and TVB-N values of chicken breast treated with *N. sativa* oil solution

Data	DAY 1 pH	DAY 1 TVB-N	DAY 3 pH	DAY 3 TVB-N	DAY 6 pH	DAY 6 TVB-N
Factor	Treatment	Treatment	Treatment	Treatment	Treatment	Treatment
Sample size	15	15	15	15	15	15
Levene statistic Significance level	0.00746 P = 0.993	0.0134 P = 0.987	3.805 P = 0.052	0.635 P = 0.547	0.724 P = 0.505	0.145 P = 0.867
Shapiro-Wilk test for Normal distribution	W = 0.8789 Reject normality (P = 0.0457)	W = 0.8780 Reject normality (P = 0.0443)	W = 0.8647 Reject normality (P = 0.0282)	W = 0.9120 Reject normality (P = 0.1451)	W = 0.8765 Reject normality (P = 0.0420)	W = 0.9076 Reject normality (P = 0.1243)
Kruskal-Wallis test Test statistic Significance level	1.2800 P = 0.527	1.6350 P = 0.435	5.3150 P = 0.069	Not applicable	9.1250 P = 0.0103	Not applicable
ANOVA Single factor F-ratio Significance level	Not applicable	Not applicable	Not applicable	12.878 P = 0.001	Not applicable	4.277 P = 0.040

Table 3. Dunnett's test (Comparisons with Control) for TVB-N values of chicken breast treated with *N. sativa* oil solutions after 3 days of refrigeration (Day 3) Control Group = CONTROL; α=0.05; |**d**|=2.50241. Positive values show pairs of means that are significantly different

LSD Threshold Matrix		
Level	Abs(Dif) - LSD	p-Value
CONTROL	-0.31	1.0000
T1%	0.067	0.0188
T2%	0.387	0.0002

Table 4. Post-hoc analysis (Conover test) for pH values for chicken breast treated with *N. sativa* oil solutions after 6 days of refrigeration (DAY 6)

Factor	n	Average Rank	Different (P < 0.05) from factor nr.
(1) T1%	5	7.50	(3)
(2) T2%	5	4.00	(3)
(3) CONTROL	5	12.50	(1)(2)

Table 5. Dunnett's test (comparisons with Control) for TVB-N values of chicken breast treated with *N. sativa* oil solution after 6 days of refrigeration (Day 6). Control Group=CONTROL; a=0.05; |d|=2.50241 Positive values show pairs of means that are significantly different

LSD Threshold Matrix		
Level	Abs(Dif) - LSD	p-Value
CONTROL	-0.94	1.0000
T1%	-0.36	0.2501
T2%	0.158	0.0234

These results suggest that the T2% is more efficient for longer refrigeration periods, however the power of the tests is around 50%, therefore, there is a probability of approximately 50% to pick up on an effect that is present. In these circumstances, it is recommended to develop an experiment with at least 20 samples per level (statistical group).
In Day 1 the values of TVB-N for the Control have a mean of 24.98 NH_3/100 g. These results are different from the ones reported by Baston (mean value 20.5 NH_3/100 g) (Baston et al., 2002). One of the main reasons for this difference could be that the meat samples were

not purchased directly from the producer, in the first day after slaughtering. However, our mean value are similar to the mean values found by Baston (22.2-24.9 mg NH_3/100 g) for refrigerated meat samples after 3 - 5 days of refrigeration (Baston et al., 2002).

The pH and TVB-N values for the treated chicken meat were not significantly different compared to Control, after 3 hours from the treatment in Day 1 (Table 2).

On the other hand, current studies that are correlating *N. sativa* oil effects with the possibility of shelf-life extension, are made particularly for fish products and according to Commision Regulation no. 2074/2005, the value of 25 mg NH_3/100 g should be considered adequate for human consumption, as reported by Georgescu (Georgescu, 2020). This value is similar to the value accepted for poultry. Therefore, as reported by Raeisi, the 2% and 4% *N. sativa* plant extract has an delaying effect on TVB-N formation on *Oncorhynchus mykiss* refrigerated fillets (Raeisi et al., 2015). It is noted that the treatment method in this situation, is different by the one used in our experiment, but the results are indicating the same conclusion over a similar period of refrigeration time (Raeisi et al., 2015). Also, Ozpolat noted that *N. sativa* oil has indeed an delaying effect regarding the TVB-N formation in fresh *Barbus grypus* fillets, during storage at 2 ± 1°C (Ozpolat et al., 2017).

CONCLUSIONS

The 2% concentration solution was more efficient compared to the 1% solution regarding the chicken breast preservation during a 6 days refrigeration period.

After 6 days the measurements for the T1% were not significantly different from Control (non-treated meat).

The 2% solution of *N. sativa* pure oil, could be used by the food industry as an alternative to chemical preservatives, however, further investigations are needed. One of the important area to be investigated could be to identify the possible organoleptic changes of the meat during cooking after treatments with *N. sativa* pure oil solutions.

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PARTICULARITIES OF NECROPSY IN CASES OF BIRDS KEPT IN CAPTIVITY

Iulia-Alexandra PARASCHIV, Raluca-Ioana RIZAC, Teodoru SOARE, Emilia CIOBOTARU-PÎRVU, Manuella MILITARU

University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd, District 1, Bucharest, Romania

Corresponding author email: iuliaaparaschiv@yahoo.ro

Abstract

Diagnosis in captivity bird pathology requires, in most cases, gross postmortem examination and is a valuable tool regarding potential zoonosis, improving husbandry conditions or establishing the cause of disease, which, sometimes cannot be completed in live birds by the clinician. The present paper is a study of 35 captivity bird cases, belonging to different species, submitted to postmortem diagnosis at the Department of Pathologic Anatomy, from the Faculty of Veterinary Medicine of Bucharest. Results showed that different instruments and multiple approaches for examination and evisceration are required depending on the size of the bird, from ophthalmologic scissors and blades to instruments used in mammal necropsy. Regarding necropsy technique, feather examination showed stress bars for two cases and eight cases of self-mutilation, marking the importance of husbandry conditions. In addition, changes in the air sacs, internal organ topography and modified organ size were revealed in seven cases. Autolysis was present in 10 cases, due to improper or long conservation, compromising diagnosis or histopathologic examination. In conclusion, necropsies of birds kept in captivity require special care and knowledge, both in preparation and during the examination.

Key words*: captive birds, avian pathology, necropsy.*

INTRODUCTION

Gross examination and postmortem diagnostic testing are important parts of avian medicine, requiring specific approach of the examination of organs and sample collection (Cooper, 2004; Rae, 2006; Bulliot et al., 2009).

Nowadays, exotic and captive bird necropsies are required not only to satisfy curiosity of the owner or the attending veterinarian, but also to provide useful information for the rest of the birds in the flock and for further knowledge of bird diseases and response in treatments (Rae, 2003; Dorrestein, 2008).

Due to great variability of anatomical characteristics, necropsies of birds require knowledge and are time-consuming for the veterinary specialists (Wobeser, 1997; Mayer and Martin, 2005; Samour, 2016).

Instruments and procedures vary among bird species and different techniques used today were developed with the experience of ornithologists and other specialists monitoring birds in the wild (van Riper III and van Riper, 1980; Cooper, 2004).

MATERIALS AND METHODS

The present study was carried during October 2014 until October 2019, at the department of Anatomical Pathology, from the Faculty of Veterinary Medicine of Bucharest. It comprised a total of 35 cases of birds kept in captivity, some of them as pets and some as rescued birds unable to be released. These cases were submitted to post-mortem diagnostic procedures by private owners. Most frequent cases examined in the present study were psittacine species, a total of 22 birds, including 12 budgerigars, three Nymph parrots, two Rosellas, two Small Alexander, one Monk parrot, one Aratinga and one Ara parrot. The remaining cases included five domestic pigeons, two canaries, two crows, one owl, one swan, one seagull and one penguin.

The diagnostic procedure included clinical history of the birds, necropsy and collection of samples for further investigation such as histopathology or microbiology.

Gross examination was performed with different instruments, depending on the size of

the bird carcass. Three categories of instruments were used: for large birds (over 1000 g) - instruments used in necropsies of medium sized mammals, for medium size birds (500-1000 g) - instruments used in small size mammals and small size birds (30-499 g) - instruments used in small size mammals and oftalmic instruments (scissors, blades).

RESULTS AND DISCUSSIONS

Preparations for necropsy

The present paper showed that most cases of birds submitted to necropsy lack of information regarding history, clinical signs and treatments administered due to poor comunication of the owners with veterinarians. Only 5 cases were accompanied by a clinical sheet from the clinician, out of the total 35 cases. For 20 cases, owners recalled important information such as time of disease, changes in the environment and if any treatment was applied to the bird. A number of 10 cases were submitted to diagnosis without any information from the owner, when large number of birds were kept and no veterinarian clinical sheet. Other literature sources mention that often, history of the cases submitted to necropsy also lack of information regarding treatments, type of death-natural of euthanasia, challenging the results by artifacts of genuine lesions (Rae, 2006; Bello et al., 2012; Samour, 2016).

In adititon, knowledge of the normal anatomical features before starting the procedure is an important step (Bulliot et al., 2009). Absence of different structures in the digestive tract is a common finding in some bird species (Rae, 2003; Samour, 2016). An anatomic particularity is the fact that ceca is absent in some Psittaciformes (Rae 2003); in our study, it was the case of the 12 budgerigars submitted to necropsy. Another organ is the gallbladder, which is normally absent in most Columbidae and many psittacines (Rae, 2003). In our study, 28 out of 35 did not have this organ.

External examination

The first step in gross examination of an exotic bird case is general assesment of weight, length and the exterior as seen in Figure 1. For 18 cases of birds kept in captivity submitted in the present study, the body weight was under the normal ranges regarding their species, showing poor husbandry conditions and predisposition to other pathologies further discovered during necropsic examination.

Figure 1. Measurement of body length-from the beak to the tail in the case of penguin

Feathers and external orifices are the first tissues to be examined (Graham, 1992; Dorrestein, 1997; Rae, 2006). In the present study, examination of feathers revealed stress bars for two cases belonging to the group of Psittaciformes. Examination of the plumage is important to differentiate normal molting from pathologic causes, including parasitosis, trauma or modified behaviour (Rae, 2006; Samour, 2016). Another eight cases of psittacines and paseriformes presented self mutilation in the pectoral and wing areas. These cases presented feather rods or partial destruction of feathers due to excessive grooming. Two cases presented also, scratching skin lesions. History of the cases recorded multiple birds kept in small cages with little sunlight.

In the cases of parrots and canaries submitted to necropsy, the legs and the facial areas were also carefully examined. One case of budgerigar presented infestation with *Knemidocoptes pilae.* Morphologic diagnosis was leg hyperkeratosis, confirmed by skin scraping (van Riper III and van Riper, 1980).

Internal examination

Examination of internal organs of avian species is done by positioning the carcass on ventral recumbency and dislocation of hip joints (Latimer and Rakich, 1994; Samour, 2016). For three cases of small birds wings and feet

were nailed on a board in order to facilitate further examination as seen in Figure 2.

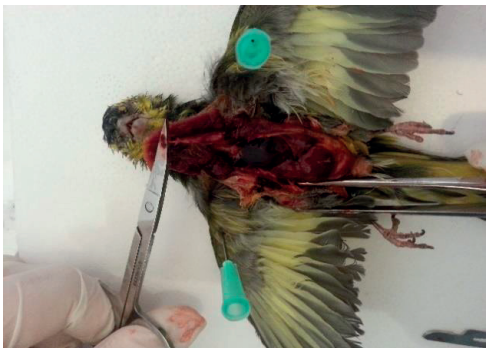

Figure 2. Ventral position of a canary *(Serinus canaria)* with pinned wings and internal examination of the carcass after sectioning the sternum: congestive organs and reddish liquid can be observed in the coelomic cavity

In most cases, feathers were plucked from neck and sternum and the rest were soaked in water. After skin incision and removal, muscular tissue was examined. Pectoral muscle atrophy was observed in 12 cases of birds, while excessive subcutaneous fat tissue was observed in two cases. The skeletal system is examined for traumatic, nutritional or infectious diseases (Samour, 2016). Sternal bone modified shape was recorded in three juvenile birds, two cases of pigeons and one case of the monk parrot, associated with other defects (small body weight, low bone mineralization, hip dysplasia) suggesting metabolic pathologies (Rae, 2003).

Next, thyroid glands were examined, before cutting clavicular bones. These bilateral organs are located in front of the thoracic inlet, closely to carotid arteries (Rae, 2003). In most cases, due to the small dimensions for gross examination, thyroids were sampled for histopathology.

In the present study, the following step was carefully removing the sternum and air sacs examination. Five cases presented gross changes of the abdominal air sacs. The aquatic birds submitted to diagnosis showed lesions associated with the cause of disease. The swan died of drowning after entanglement of the neck in wires. This cases was a rare situation of disease among the group species frequent diseases (Wobeser, 1997). The penguin and the seagull died of generalized granulomatous disease, including fibrino-granulomatous airsaculitis. After visual inspection, a small cut was done to take microbiologic samples with least contamination. The other three cases, two pigeons and one budgerigar presented discrete aerosaculitis of one or multiple airsacs, observed as whitish deposits and associated pneumonia or lung congestion. The history of the birds affected by granulomatous disease and aerosaculits included lethargy, weight loss and respiratory distress, but no specific clinical signs were recorded.

A true diaphragm is absent in avian species, but a pulmonary fold is situated ventral to the lungs, creating a pseudoseparation of celom and the peritoneal area (Work, 2000; Samour 2016).

The following steps in avian necropsic examination were the evisceration of liver and the heart. If the liver is dissected first, less manipulation is applied, except the cranial edge where the heart overposes. In the case of heart examination and evisceration first, some blood will cover the liver capsule and contamination can appear (Lowenstine, 1996; Dorrenstein, 1997). In the present study, the order of evisceration was chosen in every case, after visualization of possible lesions of these organs and, also in the cases with advanced autolysis, liver was eviscerated first, due to friability.

Liver examination revealed seven cases of circulatory lesions (anemia, active and passive hyperemia), four cases of inflammation and necrosis and 10 cases with autolytic changes.

The heart and pericardial sac revealed two cases with macroscopic lesions of heart dilation and pericardial effusion. Another 10 cases presented autolytic changes of imbibitions and post-mortem blood clotting or blood lysis.

The digestive tract is eviscerated from oesophagus or proventriculus up to the cloaca. In small sized birds, canaries, budgerigars and youngsters, better evaluation was obtained by evisceration from proventriculus to the large intestine.

The spleen requires identification and separation from the digestive tract, either before evisceration or following this step. It is situated between the proventriculus and the gizzard (Lowenstine, 1996; Samour 2016). The shape varied considerably, the examined canaries, pigeons and the penguin presented elongated shape, while the psittacine species,

the crows and the swan presented round shape, all within anatomic limits (Rae, 2006; Samour, 2016). In five cases of small birds, due to autolysis spleen could not be identified. The lesions identified in this organ were granulomatous splenitis, necrosis and congestion.

Next, examination of alimentary tract is similar to mammalian procedures, both of the exterior examination and opening technique (Rae, 2006). For small sized birds and juveniles, exceptions were applied, only opening areas of the intestines for better preservation in case of further microscopic examination or even evisceration en bloc and formalin preservation for further microscopic investigations (Rae, 2006).

Two psittacine species presented proventricular dilatation. One case, of a budgerigar presented a parasitic infestation with ascarids that migrated from duodenum to ventriculus and proventriculus, producing dilatation by an iritation mechanism. Scientific papers mention *Ascaridia* spp. as one of the most frequent parasitic diseases in cases of caged birds kept in crowded environments with multiple species and in individuals with low immunity (Lima et al., 2016). The other case, a young Ara parrot, two years old, presented a history of several months of weight loss and regurgitation (Figure 3).

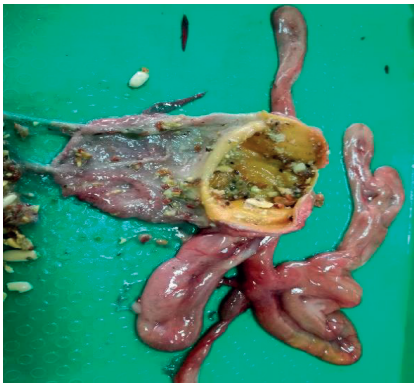

Figure 3. Proventricular dilatation, thinning of the proventriculus wall, catarrhal proventriculitis in an Ara parrot *(Ara macao)*

The second case, was likely affected by the pathology of Macaw Wasting Disease, but also scientific papers mention the possibility that dilatation can occur in juveniles and dissapears over the procces of growing as a result of hand feeding (Raghav et al., 2010; Staeheli et al. 2010). History of this case was limited and, although histopathologic examination did not confirm any viral inclusions, immunohistochemistry and virusologic tests were not performed due to financial reasons.

Also, with 10 exceptions, the rest of 25 avian cases submitted to diagnosis presented digestive tract disorders or post mortem changes due to gas formation and enlargement of the intestines. Catarrhal enteritis was observed in four cases, belonging to multiple species. Diagnosis was established based on necropsic examination in the cases of recently dead birds and confirmed by histopathologic examination. Post-mortem changes of the digestive tract were manifested as intestinal distension with gas formation along with sulfmethemoglobin imbibition and putrefaction, when more then 48 hours passed after death until submission to necropsy and improper conservation was performed.

Lungs are examined at the carcass by visual inspection and then eviscerated following anatomic particularities, with blunt dissection (Rae, 2003; Samour, 2016). In the present study, 30 cases presented gross changes of the lungs. Most birds suffered of local and diffuse lung congestion. This is most likely the effect of respiratory stress in both chronic and acute cases of diseases that lead to the death of the birds. Other, less frequent lesions were lung anemia, necrosis and autolytic changes. Three cases of birds previously identified with granulomatous disease and fibrinous airsaculitis presented similar lesions on the lungs (Figure 4).

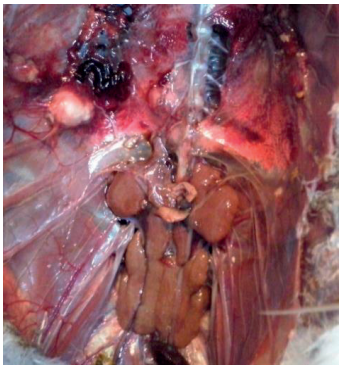

Figure 4. Carcass of a seagull *(Larus argentatus)* after removal of the digestive tract: lungs with granulomatous pneumonia and multifocal haemorrhages and kidneys with distrophic aspect

The organs examined on the carcass were the kidneys, the adrenal glands, the gonads and oral cavity and neck area (trachea, oesophagus and crop). One case of a budgerigar presented a testicular tumor, changing the topography of most organs from coelomic cavity (Figure 5).

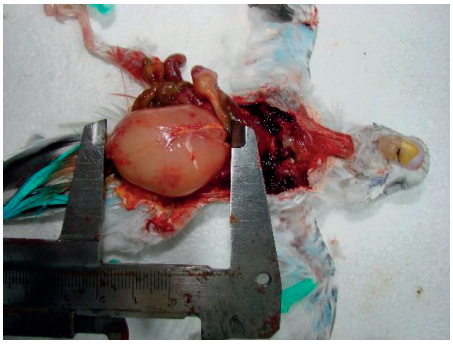

Figure 5. Necropsy of a bubgerigar *(Melopsittacus undulatus)* showing a 3 cm diameter, spheric tumour, located in the coelomic cavity, modifying internal organs topography

Last examination of the necropsy is the nervous system. Peripheric nerves were examined at the bird's, especially ischitic nerve and, for some cases the brachial plexus (Work, 2000). The brain, instead, was only examined in the cases of sudden death with little or no macroscopic lesions in organs and the cases with history of head trauma, tilt or other neurologic signs. This examination was performed in 5 cases of parrots submitted to the present study. Visual inspection was done on spaces created in the bone head, in two the small bird cases for better preservation in formalin and further histologic diagnosis. Scientific research mentions similar techniques of obtaining samples from brain regarding the size of the bird and focuses on further investigations for viral inclusions especially in Psittaciformes (Latimer and Rakich, 1994).

Advanced autolysis was a post mortem change observed in 8 cases and lead to impossibility of establishing the diagnosis and the cause of death. History of these birds included unknown moment of death in the enclosure, improper preservation at room temperature and several days (2-4 days) before being brought for examination.

Another aspect of interest is the method of preservation. Although it is best to refrigerate the bird for examination, 9 cases were freezed before being submitted to necropsy. For 6 of these cases, histopathology was performed, although results were partially compromised due to artifacts.

Necropsic examination was able to establish main lesions that caused the death in 25 cases, as seen in Figure 6, completed by further investigations for 19 of them. This group included two Psittacine birds with sudden death syndrome as the final diagnosis, although no lesions were identified on gross and microscopic examination and microbiologic and toxicologic results turned negative. The rest of 10 cases were affected by severe autolytic changes and potential lesions were masked.

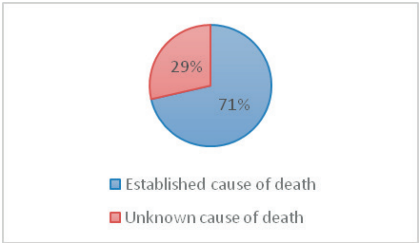

Figure 6. Percentage result of necrospic examination relevance in caged birds cases

CONCLUSIONS

Exotic bird necropsy is an important tool for diagnostics, but also for improving knowledge about these species. Preparations of the procedure, regarding species, history and instruments are key steps in obtaining best results of the examination. The order of examination and evisceration of organs and tissues is preset, in order to preserve tissues and organs, especially in small sized birds.

The present study showed the value of necropsic examination in obtaining a diagnosis in 71% of the dead birds kept in captivity.

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RESEARCHES REGARDING THE CONCENTRATIONS OF HEAVY METALS IN GAME MEAT (DEER AND WILD BOAR)

Florina RAICU, Constantin VLAGIOIU, Niculae TUDOR

University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Mărăști Blvd, District 1, Bucharest, Romania

Corresponding author email: stratoneflorina@yahoo.com

Abstract

The purpose of this article is to contribute to the subsequent establishment by the European Commission of maximum admitted limits for heavy metals in game meat. Heavy metals residues (Lead, Cadmium) are present in game meat because of the environmental excessive pollution and because of the hunting methods. Game meat samples (deer and wild boar meat) from Harghita county were analysed by: atomic absorption spectrophotometry. The results were compared with other categories of meat for which the maximum admitted limits are specified in the Regulation EC no. 1881/2006. The results were below the maximum admitted limits for other types of meat for which there is a specific European legislation, as following: cadmium concentration was highly below the highest admitted limit for meat categories. However for some samples, lead concentrations were comparable to the maximum admitted limit. Consequently, repeated analyses were performed to validate the results. The high quantities of lead residues could be explained by the hunting methods, but also by the samples collection close to the wounds produced by firearms. The hunting methods and the place for sampling too close to the wound caused by firearms (including the bullets) are responsible for finding high concentrations of residual lead in the analysed samples.

Key words*: lead, cadmium, game meat, atomic absorption, Regulation EC*

INTRODUCTION

Hunting is practiced by humans since the Palaeolithic era in order to obtain their food. At the same time, it is a conscious activity that exploits a renewable natural resource.

The game meat is an ever-expanding food alternative, due to its high nutritional properties, particular taste and interest in the livestock industry and the consumer's exploitation of hunting resources at the industrial level.

Therefore, the control and expertise of the quality of game meat is an important link in ensuring food security at national and international level, taking into account the density of the food supply, the traditional specificity and the effective functioning of the market (Stewart et al., 2011).

The game meat started to become very popular in Romania since the mid-2000s, when it appeared in hypermarkets or restaurants with specific hunting characteristics in some tourist areas. Seen at first perhaps with distrust, the hunting meat caught the attention of nutritionists very quickly, who appreciated its qualities and the important role it played in the diets of those who want to have a healthy lifestyle.

In addition, game meat is rich in vitamins A and D, zinc and iron, Omega 3, Omega 6, vitamins B1, B2, B3, B5, B7, B9 and B12 (which we must take from the diet, because our body does not process them naturally), calcium, potassium, magnesium etc. Very important is the fact that the game meat is high in proteins and low in fats. (Müller-Graf et al., 2017)

Table 1. Chemical composition of game meat (Popa G., Stănescu V., 1981)

Species	The constituent elements (%)			
	Water	*Proteins*	*Lipids*	*Mineral salts*
Wild boar	72.55	20.08	6.63	1.10
Deer	75.76	20.55	1.92	1.13

Some important things about game meat:

- The hunting meat comes from animals and birds raised close to their natural habitat (it could not happen otherwise, because hunting leads to completely different living conditions than farmed animals).

- The hunting meat is not treated with antibiotics or growth hormones.
- Wild game meat comes from strictly legally controlled hunting grounds - which is why animals feed on natural grass and fodder (Müller-Graf et al., 2017).

Concerning game meat, there is a continuous concern for the identification and quantitative and toxicological evaluation especially of heavy metals, the nutritional toxicity of these mineral elements (especially Pb, Cd, Hg) being directly influenced by their varying concentrations in water, air and habitat soil. In animals, the accumulation of heavy metals depends on the concentration in their food, the duration of exposure or the age of the animal.

Regarding the environmental excessive pollution in the last years, this is the main reason for the increasing the presence of chemical residues, including heavy metals, in game meat (Haldimann et al., 2002).

The hunting meat is recognized for its more intense coloration (Figures 1 and 2). Its variations depending on the type of the musculature, the diet, the age of the animal or the method of conservation.

Figure 1. Wild boar meat - macroscopic aspect

Figure 2. Deer meat - macroscopic aspect

Heavy metal toxicity presents serious consequences in the human body. The most affected systems and organs are: the central nervous system, leading to mental disorders, the blood components, which may damage the liver, lungs, kidneys and other vital organs, leading to serious systemic diseases (ATSDR, 1999).

Lead poisoning can be chronic or acute disorder. Acute exposure of lead can cause headache, loss of appetite, abdominal pain, fatigue, sleeplessness, hallucinations, vertigo, renal dysfunction, hypertension and arthritis while chronic exposure can result in birth defects, mental retardation, autism, psychosis, allergies, paralysis, weight loss, dyslexia, hyperactivity, muscular weakness, kidney damage, brain damage, coma and may even cause death (ATSDR, 2007; Lanphear et al., 2005).

In evaluation of its toxicity in humans, it was found that bone to blood mobilization increases during pregnancy, lactation, physiological stress, chronic disease, along with advanced age (Gulson et al., 2003).

In wild animals, **cadmium** concentrates in liver and kidneys. The literature indicates that in highly polluted areas, there is a Pb and Cd content of the mammalian organs (liver and kidney) of 20 and 16, respectively 30 and 100 mg/kg. In some low polluted areas, the values are between 1-5 mg/kg for Cd, respectively 4-10 mg/kg for lead (EFSA, 2010).

MATERIALS AND METHODS

For this article, there were analysed lead and cadmium concentrations from the following types of game meat: deer and wild boar (muscular tissue), coming from Harghita county, Romania.

In collected samples, lead and cadmium values were established by using the Graphite Furnace Atomic Absorption Spectrophotometry technique (Figures 3 and 4).

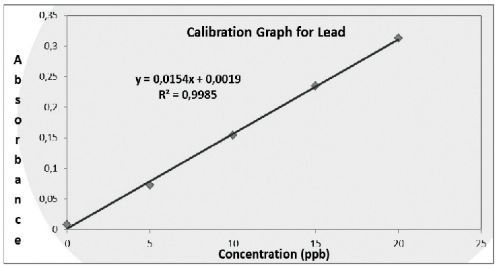

Figure 3. Calibration curve of lead performed by graphite furnace atomic absorption spectrometry

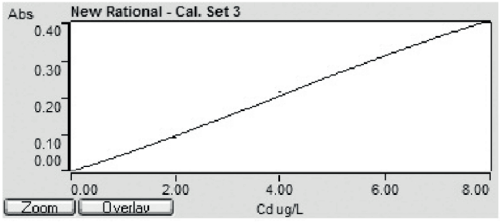

Figure 4. Calibration curve of cadmium by GFAAS

Processing the analyses

The stock solution of Cd of 1 µg/ml (1 ppm) is prepared from the concentrated solution (1000 mg/l), using nitric acid (0.1 mol/l).

The stock solution of Lead of 10 µg/ml (10 ppm) is prepared from concentrated solution (1000 mg/l), with nitric acid (0.1 mol/l).

From the stock solution of Cd (1 µg/ml), the calibration solution of 0.005 µg/ml is prepared using 0.5 ml of stock solution and HNO_3 0.1 mol/l.

From the stock solution (10 µg/ml) of Pb, the calibration solution with a concentration of 0.05 µg/ml is prepared using 0.5 ml of stock solution and 0.1 mol/l HNO_3.

Fresh calibration solutions will be prepared each day when making determinations on the Graphite Furnace Atomic Absorption Spectrophotometer.

Dry mineralization

The drying and calcination of the samples take place at an initial temperature of no more than 100 degrees Celsius.

Then, the temperature increases with a maximum speed of 50 degrees Celsius per hour up to 450 degrees Celsius and is allowed to the sample to stand overnight at this temperature.

If the sample is not completely burned, the ash is moistened with 1 ml to 3 ml deionized water or hydrogen peroxide, put the crucible back in the oven at no more than 200 degrees Celsius and gradually increase the temperature to 450 ± 10 degrees Celsius for 1-2 hours or more.

Repeat this operation until the sample is completely burned, which means that the entire ash is white/gray.

If there are black spots in the crucible that do not disappear after repeated burns, there is the possibility that they may actually be pieces of lead bullets, resulting at the time of the impact during the hunt. In this case, it is highly recommended to repeat the analysis (Trinogga et al., 2013).

Mineral processing

Add 5 ml HCl, conc. 6 mol/l so that all the ash comes in contact with the acid. The acid is evaporated on the sand bath. The residue is dissolved in 10 ml of 0.1 mol/l nitric acid. All ash must come in contact with the acid.

Allow the sample to stand from 1 hour to 2 hours. After this time, the solution is ready to be analysed.

In parallel, calibration solutions (cadmium of 0.005 µg/ml and lead 0.05 µg/ml), the matrix modifier (if applicable) are manually prepared.

The preparation of the calibration solutions for making the curve is automatically performed, the equipment is performing alone the dilutions prescribed in the calibration solution preparation program, indicating the concentration of the calibration solution used. To perform the calibration curve, the readings will be repeated until a correlation coefficient of minimum 0.990 is obtained.

For the analyses performed in this study, it was used the following matrix modifier: mixture of ammonium acid phosphate 1% and magnesium nitrate 0.1%.

All analyses were done in the Veterinary Sanitary and Food Safety Laboratory, Ploiesti, Prahova county. The interpretation of the results was done according to the current European legislation, more exactly to the Regulation EC no. 1881/2006.

This Regulation gives reference information regarding the maximum admitted limit of different contaminants in food products for bird meat, mutton, pork, lamb and beef.

RESULTS AND DISCUSSIONS

The obtained results have been related to the number of figures provided in the present regulations: Regulation (EC) 1881/2006 with subsequent amendments and completions.

For each reported result, the percentage of recovery obtained on the set of samples processed under the same conditions will be specified.

In the following table and charts there are presented details regarding average values of lead and cadmium concentrations found in muscular tissue samples of the game species.

Table 2. Distribution of deer and wild boar muscle samples (Lead, Cd) according to their concentrations

Sample	Sex		Age	Collection place	No. of samples	Assessed parameters	
						Lead (mg/kg)	Cd (mg/kg)
Deer	11 F	13 M	3.5-9 years	Harghita county	24	0.02 (min. conc., 10 samples) - 0.08 (max. conc., 6 samples)	0.001 (min. conc., 7 samples) - 0.03 (max. conc., 3 samples)
Wild boar	10 F	13 M	4-11 years	Harghita county	23	0.01 (min. conc., 5 samples) - 0.09 (max. conc. - 5 samples)	0.009 (min. conc., 8 samples) - 0.02 (max. conc., 3 samples)
Admitted limit according to EC Regulation no.1881/2006						0.100	0.050

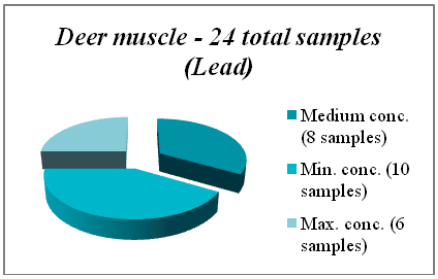

Chart 1. Distribution of deer muscle samples (Lead) according to their concentrations

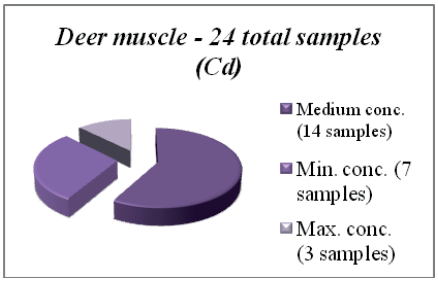

Chart 2. Distribution of deer muscle samples (Cd) according to their concentrations

As it can be seen in this table, all the analysed samples had lead and cadmium within the maximum allowed limits.

The values are within the ranges: wild boar 0.01-0.09 (lead), 0.009-0.02 (cadmium), deer 0.02-0.08(lead), 0.001-0.03 (cadmium).

The high values from these intervals are probably due to the bioaccumulation of heavy metals in the body. If the collection of the muscle tissue samples had taken place in the area near a wound obtained by shooting with lead bullets, the values of the lead would have far exceeded the maximum allowed limit.

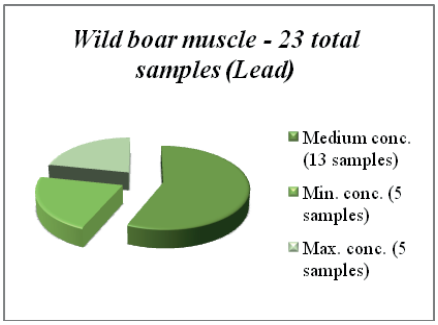

Chart 3. Distribution of wild boar muscle samples (Lead) according to their concentrations

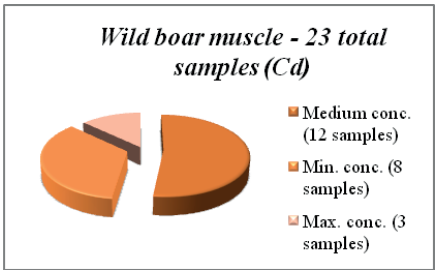

Chart 4. Distribution of wild boar muscle samples (Cd) according to their concentrations

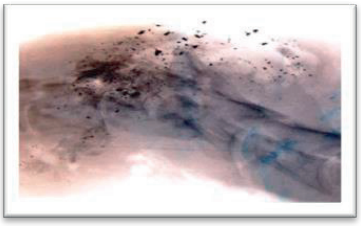

Figure 5. Lead bullet fragments - the dark spots

Figure 6. Lead bullet fragments removed from tissue

From this point of view, the sample collection was in conformity, the results expressing the

value of the analyte given by the residual accumulation of the heavy metals.
According to the obtained results, the samples of wild game muscular tissue from wild boar and deer collected from the Harghita county fall within the maximum limits allowed by the European legislation on the field of muscular tissue.

CONCLUSIONS

- Lead and cadmium may occur in muscle tissue samples as a result of the residual bioaccumulation of these heavy metals. Regarding the lead, meat can also be contaminated with pieces of lead bullets, as a result of their dissemination at the time of the hunting impact.
- Lead and cadmium residues are present in normal range in the muscular tissue of the deer and wild bear in samples from Harghita county. There were not recorded overvalues for these analytes.
- The lack of a European regulation putting up the maximum permissible limits of lead and cadmium for the muscular tissue of these animal species is due to the deficient monitoring of the heavy metal residues in the meat and the food of these animals (Taggart et al., 2011).
- The capturing procedures are the main reason for getting lead at a value that exceeds very much the maximum permissible limit of this analyte regarding the muscle tissue.
- None of the analysed samples had results above the maximum permissible limit of lead or cadmium for the muscle tissue category.
- In case of exceeded results, it is necessary to repeat the analyses including a close examination of the muscular tissue at macroscopic level, before starting working protocol. It is necessary to make the difference between contamination with pieces of lead bullets and residual accumulation of heavy metals.
- For the analysis of each sample, the entire amount of sample was chopped and homogenized so that the harvesting for processing is done in the most objective way possible.
- Do not wash the sample before analysing it because the tissue fluid will be lost and it contains an important amount of analytes. Otherwise, the result is a wrong one (the dilution process occurs).
- The hunting meat is a complex matrix from the analytical process' point of view. Therefore, choosing the matrix modifier is very important to achieve an objective result. The final choice is a rigorous selection after several attempts.

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Figure: 5,6 - www.huntingwithnonlead.org.

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COMPARATIVE STUDY OF OSTEOMYELITIS REPRODUCED ON RABBITS USING HUMAN STRAINS OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) AND METHICILLIN-RESISTANT *STAPHYLOCOCCUS EPIDERMIDIS* (MRSE)

Diana-Larisa ANCUȚA[1, 2], Teodoru SOARE[2], Diana SOARE[3], Maria CRIVINEANU[2], Cristin COMAN[1]

[1]"Cantacuzino" National Medico-Military Institute for Research and Development, Preclinical Testing Unit, 103 Splaiul Independentei, Bucharest, Romania
[2]University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, 105 Splaiul Independentei, Bucharest, Romania
[3]Histovet Laboratory, 30 A Doctor Paleologu Street, Bucharest, Romania

Corresponding author email: comancristin@yahoo.com

Abstract

One of the current concerns regarding orthopedic surgery is represented by the associated infections. Studies show that in 80% of human cases, the primary bacterial agent that causes osteomyelitis is Staphylococcus aureus. On the other hand, the epidemiological data claims that coagulase-negative staphylococcus, especially Staphylococcus epidermidis, have emerged as the predominant pathogens of the associated infection due to their ability to develop biofilm. The goal of the study was to induce osteomyelitis in rabbits using bacterial strains isolated from human patients and to optimize the concentration of the two species of staphylococcus capable of reproducing bone infection. The evaluation of the disease installation and the clinical evolution was completed by hematological and histological examinations. Comparing the results, it can be concluded that the MRSA strain is more pathogenic compared to MRSE. In both cases, the rabbit has been shown to be a good experimental model for the reproduction of osteomyelitis that can be used for the development of new treatments.

Key words*: experimental model, rabbit, osteomyelitis, MRSA, MRSE.*

INTRODUCTION

Osteomyelitis (gr. Osteon = bone, myeloma= bone marrow and itis = inflammation) (Beck-Broichsitter et al., 2015) is the inflammation of the bone marrow, which can be localized or diffuse, which is affecting the surrounding cortex, periosteum, and the surrounding soft tissue (Lew, 2004), even though it can be produced by a wide range of pathogens, the main source of infection is represented by the Gram-positive opportunistic bacteria of the genus *Staphylococcus* (Kavanagh et al., 2018), in particular *Staphylococcus aureus* and *Staphylococcus epidermidis*.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is the leading cause of overlapping infections that produce skin or organ damage (Guardabassi et al., 2019; Thapaliya et al., 2017), with varying degrees of severity (Abd El-Baky et al., 2019). *Staphylococcus epidermidis*, a saprophytic agent of the skin and mucous membranes, has become an opportunistic pathogen, resistant to â-lactam antibiotics and multiple antibiotics, becoming Methicillin - resistant *Staphylococcus epidermidis* (MRSE) (Widerström et al., 2016; Rolo, 2012), which is progressively involved in medical device related infections, through the formation of biofilm (Hofmans et al., 2018) and which increases the costs of care and also the mortality rate among the affected patients (Lakhundi et al., 2018; Wang et al., 2020).

The bacterial biofilm and the exopolysaccharide matrix produced by bacteria during the adhesion of different surfaces (Kývanç, 2018) offers them resistance to antibiotics, phagocytes and bacteriophages. Moreover, the bacterial infections in which the infectious agents have a high capacity for adhesion and biofilm formation are different from infections caused by planktonic bacteria, due to their etiology, sensitivity to various antimicrobial agents and the ability to adhere to non-

animated substrates, such as those used in medicine as prosthetic devices (Raksha et al., 2020).
The ability of the both bacterial strains to form biofilm as well as their resistance to antibiotics was an important criterion in establishing the objectives of this study. Using the rabbit as a model for osteomyelitis, the disease was reproduced by inoculating MRSA and MRSE, bacteria that were isolated from human patients. The bacterial suspensions that were tested at different concentrations, the clinical signs that emerged and also the complementary examinations that were performed have provided important data on the pathogenicity of both MRSA and MRSE, data that can be used to initiate new therapeutic schemes.

MATERIALS AND METHODS

Ethics statement
Animal studies have been approved by the ethics committee of the Cantacuzino Medical-Military Research and Development Institute (CI) and authorized by the competent authority, being carried out in accordance with the provisions of EU Directive 63/2010 respecting the rules of care, the use and protection of animals that are used for scientific purposes. The studies were conducted in Baneasa Station, the Preclinical Testing Unit, a unit authorized as a user by the competent authority. For the induction of the disease, sample collection and clinical supervision were taken all measures to reduce the suffering of animals.

The animals
For each study were used adult rabbits, "White New Zealand" strain, male and female, with an average weight of 3,000 grams. The animals were housed in the experimentation animal facility, in individual cages, with cycles of light and dark of 12 h each.
The rabbits were acclimated for five days under the same conditions of experimentation, at a temperature of 20-24°C and a relative humidity of 45-65%, during this time they received at their discretion food and water. The separation in groups was done by marking the rabbits on the ear using a permanent marker. All the groups have 4 rabbits.

Bacterial strains
MRSA - was obtained from human patients and received at CI from ROMVAC Company, Voluntari, Ilfov, Romania. MRSA was processed in the Microbiology Laboratory of CI by seeding it on Muller Hinton agar plates and by performing repeated passages, incubated at 37°C for 24 h. From the last passage, serial dilutions were made and the concentrations of 5×10^5 CFU/ml and 5×10^6 CFU/ml were the only ones sent for testing.
MRSE - was isolated from the knee prosthesis of a human patient and was received by CI from the Instituto Ortopedico Galleazzo, Milan, Italy. MRSE was processed under the same conditions as MRSA, and the tested concentrations were: 5×10^5 CFU/ml, 5×10^8 CFU/ml and 5×10^{10} CFU/ml.
As the metabolic activity of the bacteria is much more intense when they are organized in biofilms, we have created a favourable environment for its reproduction by using cotton meshes that represented the substrate for adhesion and colonization of the staphylococcal strains.

Surgical procedure
The animals were deeply anesthetized by neuroleptic analgesia using a mixture of Ketamine (50 mg/kg) and Acepromazine (1 mg/kg).
For all the animal groups, the election limb was the left hindlimb, the right hindlimb was taken as a control. The fur in the mid-proximal area of the tibia was trimmed and the skin was disinfected with Iodine 2%. After the incision of the skin layers and the exposure of the cortex, two bone defects were performed under continuous saline jet, using the same technique as in the previous studies (Ancuța et al., 2019). Using a drill with a diameter of 1.5 mm, two cavities were made up to the medullary canal, at a distance of 10 mm between them and approximately 10-15 mm distance from the tibio-femuro-patellar joint.
Each bacterial species, used in the study, were tested on groups of animals inoculated at different concentrations (Table 1).

Table 1. MRSA and MRSE concentrations/animal group

Bacterial species	Group no.	Number of animals/group	The used bacterial concentration
Methicillin-resistant *Staphylococcus aureus*	I	4	5 x 10^5 CFU/ml
	II	4	5 x 10^6 CFU/ml
Methicillin-resistant *Staphylococcus epidermidis*	III	4	5 x 10^5 CFU/ml
	IV	4	5 x 10^8 CFU/ml
	V	4	5 x 10^{10} CFU/ml

The created bone defects were augmented with the cotton meshes previously immersed in the bacterial suspension over which was inoculated an amount of 0.05 ml of bacterial culture/ defect.

The surgery was completed by suturing the wound with non-absorbable thread and applying compressive dressings. After surgery, in the next 3 days the rabbits received analgesic treatment (Ketoprofen - 3 mg/kg SC).

The monitoring period of all animal groups was 30 days, with an intervention point on day 14, when half of the total number of rabbits/group was euthanized by overdose of anesthetic to follow up the osteomyelitis onset and its passage from the acute phase to the chronic one, the rest of the animals were sacrificed at the end of the study. During the surveillance interval, the animals were examined clinically, hematologically, throw the necropsy examination and histologically.

Animal monitoring

Clinical examination: daily, follow - up local and general signs; thermometry, daily for the first 7 days, after that at day 14 and at the end of the study and weekly body weight monitoring.

Hematological examination: performed at CI, the blood samples were collected on day 0 and day 14, and their processing was performed using the ProCyte Dx analyzer (IDEXX Laboratories) for all groups.

The histological examination, performed for all tibias that were inoculated, from all the groups, followed the specific protocol: fixation in 10% formalin solution, 24-72 hours decalcification in a quick decalcification solution, dehydration in ethanol solution and paraffin embedding. The paraffin blocks were cut with the microtome to a thickness of 5 ìm and stained with eosin hematoxylin.

Statistics

Statistical analysis was performed using Microsoft Excel, the current version.

RESULTS AND DISCUSSIONS

Results

Survival rate was100% in groups I, III, IV, V and 75% in group II.

The clinical examination, performed daily to assess the general and local condition, was based on the measurement of body temperature and weight of the animals and care of the surgical wounds, intervening with pain management medication when signs of pain were observed.

Body temperature, measured intrarectally with the digital thermometer: For 2 days in the first 7, were recorded values above the physiological limits in groups II and V, which in the following days it returned to normal parameters (Figure 1).

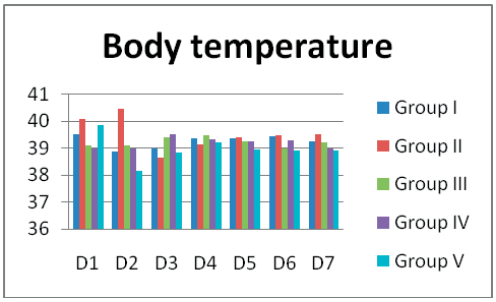

Figure 1. Evolution of average body temperature for each rabbit groups and bacterial concentrations used in the study

The body weight was evaluated at the beginning of the experiment for all subjects, then at day 14 and day 28. In the case of the animals that were inoculated with MRSA, a decrease in weight was observed in the first 14 days, especially in the group where the tested concentration was 5 x 10^6 CFU/ml, which, until the end of the study, these animals recorded increases in body weight. The animals infected with MRSE showed a favourable evolution, in groups III and IV and in the case of group V a slight decrease of the weight was observed in the middle of the monitoring interval, loss that was recovered until day 28 (Figure 2).

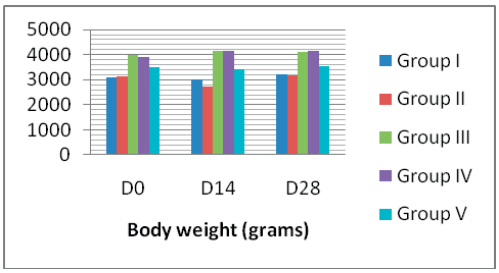

Figure. 2. Evolution of average body weight/groups

Haematological examination: the analysis of the average values for the parameters of interest, provided information about the organism's effort to neutralize the bacteria, without any relevance for the experiments performed (Table 2).

Tabel 2. Hematological average values on day 0 and day 14

The analyzed parameters	WBCx10^9/L		HETEROx10^9/L		LYMx10^9/L	
Reference Values	4.54 - 10.22		0.96 - 3.34		1.49 - 5.21	
	D0	D14	D0	D14	D0	D14
Group I	9.2	10.1	1.62	3.13	6.57	5.24
Group II	9.51	8.82	2.20	2.72	6.15	3.82
Group III	7.2	5.87	1.71	1.70	4.7	3.36
Group IV	5.18	5.91	1.8	2.22	4.36	2.89
Group V	8.57	7.33	2.16	2.72	5.66	3.44

The clinical signs assessment was performed daily, the groups I and II presented specific indicators of *Staphylococcus aureus* infection: fever in the first 2 days in most animals; in group II, the values of body temperature exceeded 40°C, a rabbit passing from the state of hyperthermia into hypothermia followed by death, 3 days after inoculation. Altered general state, lack of appetite, diarrhea and local signs of abscess starting from day 7 were observed. At 2 weeks after disease induction, all animals had open abscesses, thickened and necrotic wound edges. By the end of the study, both local and general signs had improved, in both groups that were inoculated with MRSA, but local lesions characteristic of the infection persisted even after.

The animals inoculated with MRSE have showed a favourable clinical evolution, regardless of the bacterial concentration used, the predominant local signs being inflammation, the indurations and the appearance of the abscesses encapsulated 7 days after the beginning of the experiment. In groups III and IV, abscess resorption has been observed, as well as progression to granuloma in animals from group V at the place of inoculation until the end of the study.

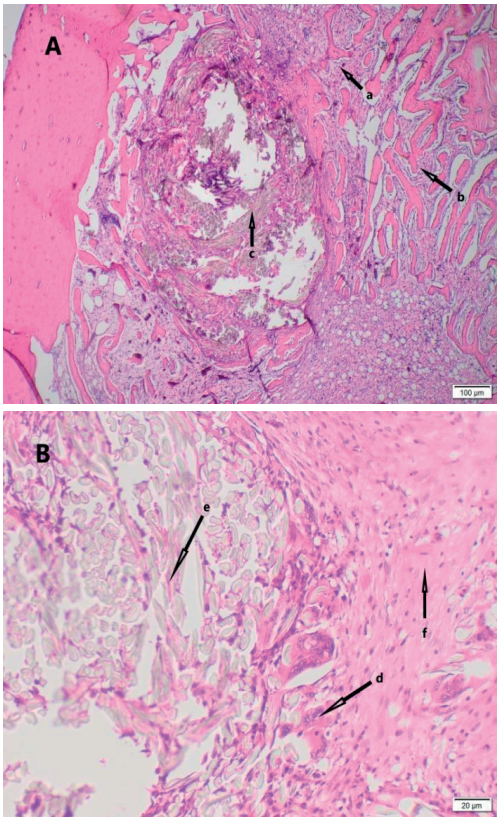

Figure 3. Cross section through tibia inoculated with MRSA 5 x 10^5 CFU/ml: A) Trabecular bone and hematopoietic bone marrow (a, b), textile implant and abundant cell population (c) (Hematoxylin-Eosin stain, 2x); B) Detail of the marginal area of the textile implant (Hematoxylin-Eosin stain, 10x) - presence of giant foreign body cells (d), surrounded by fibrous connective tissue (f), textile implant (e)

Histological examination: the predominant results from the histological analysis, in the groups inoculated with MRSA were (Figure 3):
- Thickened periosteum, with fibrous connective tissue, heterophils and

macrophages. In some rabbits, were observed areas of necrosis, which included even the compact on the implantation area.
- Compact bone with the presence of primary callus and granulation tissue (partial consolidation).
- The medullary area with the presence of abundant cellularity which is composed of giant cells of foreign body type that adhere to the textile implant and bacteria that are found intracytoplasmatically in macrophages.

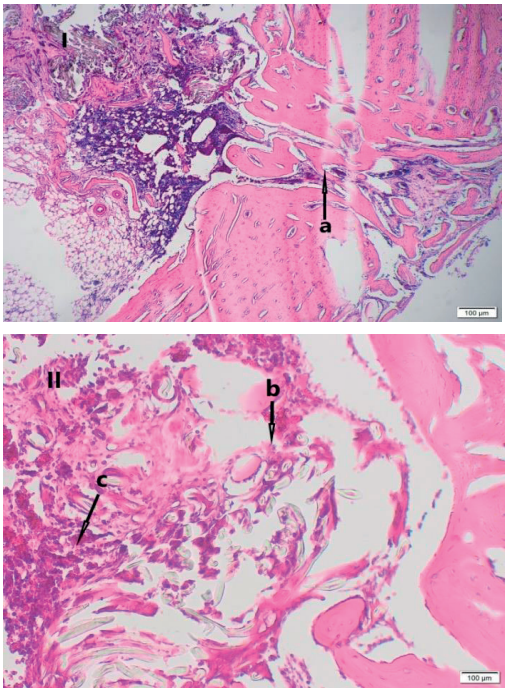

Figure 4. Sections through tibias inoculated with MRSE: I (5 x 10^5 CFU/mL) - Cross section through compact bone: lamellar fragments with a tendency of fusion that consolidates the bone defect (a), fibrous tissue and textile implant fragments in the medullary canal. II (5 x 10^5 CFU/ mL) - medullary area foreign body inflammatory reaction (b), sequestered hematopoietic tissue (c)

In the rabbits where the MRSE strain was tested, the histological changes were different, depending on the concentration used:
Group III (Figure 4):
- thickened periosteum, with fibrous connective tissue.
- Compact bone with regeneration elements - secondary callus.
- Fibrous tissue organized and oriented to the bone defect.
- The medullary with fibrous tissue, foreign body reaction (macrophages and multinucleated giant cells), hematopoietic tissue islands and discrete bacterial colonies (in some cases).

Group IV (Figure 5):
- compact bone with regeneration elements
- secondary callus, fibrous connective tissue
- Foreign body reaction in the marrow, preserved hematopoietic tissue.

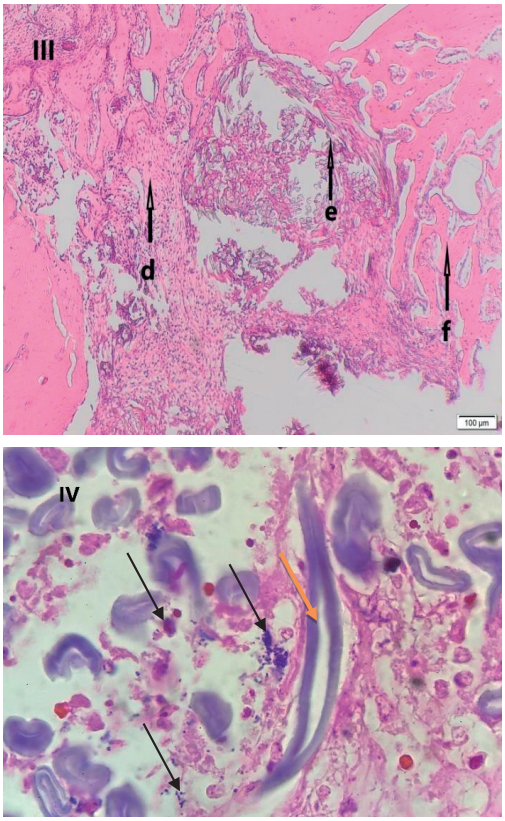

Figure 5. MRSE-inoculated tibia sections: III (5 x 10^8 CFU/mL) - detail of the compact bone with defect, fibrosis (d), textile implant (e) and incompletely fused bone lamellae (f). IV (5 x 10^{10} CFU/mL) - GRAM-positive coccoid bacteria (black arrow), textile implant (yellow arrow), GRAM stain (100x)

Group V (Figure 6):
- thickened periosteum, with the presence of inflammatory cells (macrophages, heterophils).
- Compact bone with regeneration elements - secondary callus, fibrous connective tissue.
- Medullary with debris from the textile implant and foreign body reaction, hematopoietic tissue islands present, heterophils, macrophages,

intracytoplasmatic bacteria and fibrous reaction.

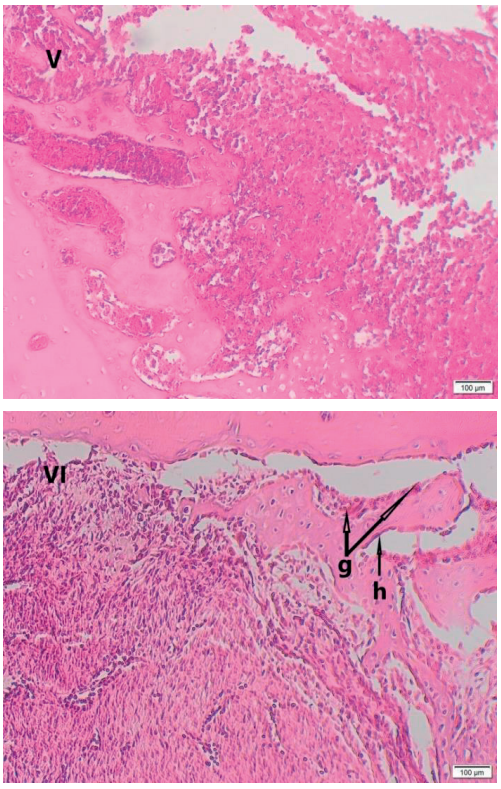

Figure 6. MRSE-inoculated tibiae sections: V (5 x 10^{10} UFC/mL) - Periosteal detail: necrosis area with numerous heterophils, macrophages and osteolysis. VI (5 x 10^{10} UFC/mL) - Detail of periosteal area with abundant fibrosis; compact bone tissue lamellae delimited by osteoclasts (g) and osteoblasts (h)

Discussions

Although, lately, surgical hygiene techniques and preventive antibiotic therapy have been significantly improved, bacterial infections remain a topical issue, especially in the case of orthopaedic surgeries involving the use of medical devices (Tran et al., 2019). Bacteria such as MRSA and MRSE irreversibly adhere to the surface of a device and develop to form a polysaccharide biofilm that protects them from the action of antibiotics or the defence mechanisms of the infected organism (Stewart, 2001).

In the current paper, we have focused on the ability of the two antibiotic-resistant bacteria, of significant importance in orthopaedic infections, MRSA and MRSE, to reproduce osteomyelitis and evaluated their pathogenicity by inoculating different concentrations in a rabbit tibia model.

Based on the premise that osteomyelitis is reproduced in a group of animals if the viability rate is over 75%, it can be said that all concentrations of MRSA and MRSE have been able to reproduce the disease but the correlation with the performed examinations, offers different data.

The longest period of time for the development of bone infection was four weeks (Giavaresi et al., 2008), during which the clinical signs were monitored. The groups inoculated with MRSA expressed a more spectacular symptoms than those in which MRSE was tested. The fact that in group II, a subject died shortly after the beginning of the experiment, presenting at the autopsy examination specific lesions of septicaemia as well as the prolonged febrile condition in the rest of the animals, the more pronounced local signs and the significant decrease in weight in group II in comparison with group I, were indicators of the aggressiveness and the action of the MRSA strain at the bone level, aspects mentioned also in other studies (Nijhof et al., 2000).

The rabbits tested with MRSE showed an attenuated symptoms, only the group that received 5x10^{10} CFU/ml expressed signs of bone infection, an interesting aspect for our study, especially when you take in consideration the rising curve of the body weight and temperature.

The histopathological diagnosis in the case of osteomyelitis has a considerable diagnostic value (Tiemann, 2014), groups I and II presenting specific lesions of active-chronic bone infection, represented by fibrosis, the presence of the multinucleated giant cells of foreign body, heterophils and macrophages (Fig. 4), groups III-IV showed signs of regeneration with the presence of bone spicules in the defect area accompanied by the activity of osteoblasts and the presence of numerous osteoclasts that ensure the remodelling of the newly formed bone (Figure 5, III), and in the last group there were bone remodelling and the activity of osteoblasts, the bone matrix being evidently present in some areas accompanied by different degrees of cartilaginous metaplasia with partial mineralization (Figure 6), probably

due to the negative influence made by the introduction of the inducing agent, that was prior immersed in the bacterial suspension with very high concentration.

If, in the case of the animals inoculated with MRSA, the symptoms and the results of the paraclinical examinations were more than sufficient to establish the diagnosis of osteomyelitis, in the case of the animals inoculated with MRSE with regard to groups III-V, the onset of the disease was insidious, requiring the use of increasing bacterial concentrations, as also mentioned by Park et al. in rats studies (Park, K.H. et al., 2017).

Various studies and models for osteomyelitis in different species have been described in the literature. Reizner et al. have described an extensive set of animal models suitable for the reproduction of osteomyelitis with staphylococcal strains (Reizner et al., 2014). Thus, the chosen animals should have immune and musculoskeletal characteristics similar to humans, and the bacteria should be clinically representative and capable of biofilm formation (Brunotte et al., 2019). In order to reproduce an environment favourable to the adherence and colonization of infectious agents, we used cotton meshes (Bottagisio, 2019), which proved to be effective for the purpose of our study, the reproduction of osteomyelitis being possible in all groups, especially those inoculated with MRSA and MRSE - group V. Comparing groups I and III that have been tested with the same bacterial concentration but different strains it can be concluded that *Staphylococcus aureus* is much more aggressive on the bone area than *Staphylococcus epidermidis*.

One of the our studies weaknesses is the number of animals that has been used, which should be "sufficient but not excessive" for the proposed goal (Penny S. Reynolds, 2019), and in our case, the results could not be quantified as a result of this aspect. Also, the lack of radiological examinations or the analysis of the tibias using the MicroComputer Tomography, is a limitations of our studies.

CONCLUSIONS

Both MRSA and MRSE strains have reproduced osteomyelitis in rabbits, but in relation to body weight and temperature, local signs and histopathological examination results, the optimal concentrations for the induction of the disease are: 5 x 10^5 CFU/ml MRSA and 5 x 10^{10} CFU/ml MRSE and further demonstrates the much higher pathogenicity of MRSA.

Comparing groups I and III that have been tested with the same bacterial concentration but different strains it can be concluded that *Staphylococcus aureus* is much more aggressive in bone than *Staphylococcus epidermidis*.

The work may serve as a basis for further studies because the rabbit is a good experimental model for osteomyelitis reproduction that can be used to optimize therapeutic doses or to develop new treatments.

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MEDICINAL PLANTS USED IN TRADITIONAL VETERINARY MEDICINE TO TREAT RUMINANTS IN THE CURVATURE SUBCARPATHIANS AREA, ROMANIA

Cristina CĂȘARU[1], Anca BULGARU[2], Doina DANEȘ[1]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd, District 1, Bucharest, Romania
[2]SC Pasteur Filipesti Branch SRL Bucharest Working Point, 333 Giulesti Str., Bucharest, Romania

Corresponding author email: casarucristina@yahoo.com

Abstract

Medicinal plants have been used since the earliest times to treat different disorders of humans and animals. The aim of this study was to identify the plant, mineral and animal remedies used in the treatment of cattle diseases traditionally raised, in the submontane area of the Curvature Carpathians. Relevant information has been collected from 237 interviewed subjects on the treatments used to control diarrhoea, mastitis, external mammary gland injuries, mammary papillomatosis, indigestion, acute meteorism and respiratory disorders, foot injuries/ infections, external and internal parasite infections. There have been identified 56 plants, 8 mineral substances and 6 substances of animal origin used in the treatment of the aforementioned disorders. According to the answers of the interviewees, the most frequently used plants belong to the Asteraceae family (32.35%), followed by Rosaceae (17.64%), Aliaceae, Betulacea and Fabaceae (8.82%), and the most frequently used species were Robinia pseudoacacia, Artemisia absinthium and Sempervivum tectorum.

Key words*: ethno-veterinary, traditional knowledge, cattle disease, Romania.*

INTRODUCTION

In the modern era, traditional veterinary medicine is practiced throughout the entire world, usually in rural areas, where the economical situation is precarious and veterinary services are difficult to access. Traditional medicine is based mainly on rural wisdom, its practice being cheap, secure, tested throughout the centuries, from generation to generation (Confessor et al., 2009; Phondani et al., 2010).

In the last decade, numerous studies published in developed or developing countries prove that the practices of ethno-veterinary medicine are now returning to a new level through the scientific approach. A major factor in the return of this therapeutic approach is the progress of organic farming. If in 1999 the agricultural area occupied 11 million hectares, in 2017 it reached 70 million hectares, with a turnover of 97 billion US dollars (Chakraborty & Pal, 2012; Willer & Lernoud, 2019).

The increasing emergence of resistance to antimicrobial and antiparasitic molecules is another major factor boosting the research of phyto-therapeutic principles (Mayer et al., 2014).

In Europe, there are few ethno-veterinary studies, the most numerous being in Asia and Africa, where the use of medicinal plants has a continuous tradition.

In Romania, such studies are emerging, although the potential of our country is very high and the medicinal plants have been used since the earliest times. Romania's flora registers over 4000 species of plants, of which 800 have therapeutic properties, properties confirmed by scientific studies for over 50% of them (Segneanu et al., 2019).

Dioscorides (a Greek physician, pharmacologist, botanist in roman army) gives the earliest description of medicinal plants used in different treatments on Romania's territory in his book "De Materia Medica". He emphasised that the medicinal plants are widely used in Dacia. Of the 600 species of plants he describes in his work, 40 originate from the Dacian territory: this is the oldest evidence of phytotherapy practice in our country (Fierascu et al., 2017; Segneanu et al., 2019).

Knowledge of veterinary practices has been preserved and passed on to nowadays in mountainous and sub-mountainous areas, these human communities being more conservative. One such territory is the submontane area of the Curvature Carpathians, the area where this study was conducted.

The aim of the study was to identify the medicinal plants used by farmers from the area of the Curvature Sub-Carpathians, in the treatment of cattle. The therapy based on natural resources (plants, animals, minerals), often associated with ancient religious practices and rituals, still remains an alternative in the treatment of various conditions. This knowledge is oral transmitted, from generation to generation, but will gradually disappear due to socio-economic and cultural environment evolution: the reduction of the number of micro-farms or the development of technology.

MATERIALS AND METHODS

The studied area

The research has been carried out in 25 villages belonging to 8 administrative communes (Bisoca, Gura Teghii, Mânzălești, Lopătari, Bătrâni, Poseşti, Jitia and Vintileasca) located in the submontane area of the Curvature Carpathians, in the counties of Buzău, Prahova and Vrancea (Figure 1)

Figure 1: Map of the study sites in Sub Carpathian area, Romania. Image adapted from Google Maps (https://www.google.com/maps)

Data collection and analysis

The research was carried out between April and October 2019. The interviewees was selected randomly, being recorded only the relevant answers on the therapies based on natural resources, used in the treatment of ruminants. There have been recorded 237 interviews.

The questions referred to the following conditions: diarrhea, indigestion, acute meteorism, mastitis, superficial mammary wounds, breast papillomatosis, pododermatitis, respiratory disorders, ectoparasites and endoparasites. The requested information was about the type of these natural resources (of vegetable, animal or mineral origin), data on the formulation and posology (administration and how long).

Conservation and taxonomic identification of plants

The plants were harvested and conditioned in a herbarium and subsequently identified at the University of Medicine and Pharmacy of Bucharest "Carol Davila", Faculty of Pharmacy, discipline of Pharmacognosy. Where harvesting was not possible, the identification of the plants was done with the help of the botanical atlas “The Illustrated Flora of Romania. Pteridophyta et Spermatophyta” (Ciocârlan, 2000) and specialized websites.

Classification of plants and statistical analysis of the data obtained

According to the classification made by Lans et al (2007), the plants were grouped into four categories, depending on the therapeutic properties, supported or not by the scientific data, categories designated by the letters A, B, C and D, as follows:

- A: high level of confidence, the plant is considered effective if the ethno botanical and pharmacological data from the literature are consistent;
- B: medium level, if there is pharmacological and phytochemical information that certifies the use of the plant in the treatment of different diseases;
- C: low level, if the plant (or plants of the same genus) is used for the same type of diseases in other areas, but there is no pharmacological information to confirm their effectiveness;
- D: minimum level if no information has been identified that can confirm the effectiveness of the plants, and they could be inactive.

It was also noted the frequence of each plant species (U) and the frequency of occurrences expressed as a percentage (F), based on the total number of the considered interviews. The

frequency is as higher as the number of citations of the plant is higher (Parthiban et al., 2016).

RESULTS AND DISCUSSIONS

Ethnobotanical knowledge

Most often, concrete information was obtained from people aged over 60 years (most of them being breeders who practice or have practiced subsistence farming).

For people aged under 40, the presence of the veterinarian is required to control the most of conditions, diarrhea being the only condition that is sometimes treated with the phyto-therapeutic remedies.

There were identified 56 plants, out of 34 families (Table 1), the largest representation being of the *Asteraceae* family (32.35%), succeded by *Rosaceae* (17.64%), *Aliaceae*, *Betulaceae* and *Fabaceae* (8.82%).

The frequency of the other nominated families was below 5.89%.

A study carried on ethno-veterinary medicine in Europe identified 590 plant species from 102 different families, used in animal treatment (Mayer et al., 2014). Relevant families were *Asteraceae, Fabaceae* and *Laminaceae*, while Bartha et al. (2015) in Covasna, a county located inner of the Curvature Carpathians, reported a number of 26 plants, 2 remedies of animal origin and 17 of other origin.

Parts of plants used and the formulation

As other ethno-veterinary studies, it was noted that the most commonly used are the aerial parts of plants, especially leaves (Figure 2), being easier to harvest and available throughout a wider period of the year, unlike fruits and flowers (El-Mahdi, 2019; Parthiban et al., 2016; Verma, 2014).

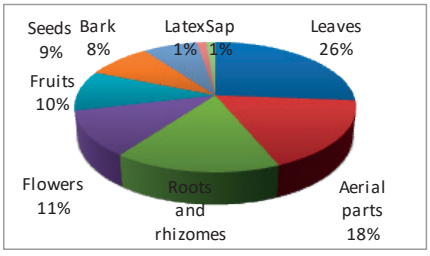

Figure 2. Percentage of parts of plants utilized

The plants are used in fresh form, given in ration, in the form of infusion, decoction, alcoholic macerate, poultices, ointments or oil infusion. Similar modes of administration are found worldwide.

Alcoholic extracts are prepared using the traditional drink, obtained from the alcoholic fermentation of plums, ţuica, with an alcoholic strength of 37-42°. For the cold season, animal breeders cultivate the plants, condition them properly and use them if necessary (Miara et al., 2019; Verma, 2014).

Treated pathologies and pharmacological records

Information was collected on 135 herbal remedies, of mineral or animal origin, used to treat 15 cattle diseases: the diarrhoea is the first one, followed by mastitis, internal and external parasitoses, acute meteorism, foot infections, respiratory diseases, indigestion, superficial mammary wounds and papillomatosis. For the treatment of haemorrhages, anemia, eye disease or skin wounds, only two remedies have been reported (Table 2). Most often, these remedies are delivered in alcohol (*ţuica*), in sunflower oil or in vinegar, and various empirical recipes are proposed.

Among the conditions listed above, diarrhea is the most commonly treated with herbal medicines, using either fresh herbs administered in the ration or as an infusion, decoction, alcoholic maceration. The antidiarrheal effect of the used plants is due to their antimicrobial, astringent, antispastic and antihelmintic action.

Gentiana lutea is the most widely used plant, especially in adult bovine diarrhea (cited 45 times). The root and the rizome of the plant are used, less often the leaves and the flower. It is given as an alcoholic extract, macerated or decocted. The European Medicines Agency conducted a study recognizing the effectiveness of *Gentiana lutea* especially in digestive disorders. Numerous studies have also demonstrated its antimicrobial action (Šavikin et al., 2009; Scarlattilaan, 2018).

Šavikin et al. (2009) have tested the action of the plant's compounds, gentiopicrin, mangiferin and isogentisin, which separately do not play an important antibacterial role, but their synergistic action in the methanolic extract of leaves and flowers proves to be effective against Gram-positive, Gram-negative bacteria and *Candida albicans*).

Table 1. Plants used in ethno-veterinary medicine in Subcarpathians'Curvature

Binomial name/ family	Parts used	Medicinal value (condition, formulation, posology)	U*	F (%)**	Classi-fication
Achillea millefolium *Asteraceae*	flower	diarrhea/infusion/oral ocular disease/infusion/topical	4	1.68	A***
Aesculus hippocastanum *Sapindaceae*	seed	diarrhea, indigestion/decoction, maceration in alcohol, baked seeds/oral	50	21.09	A
Allium ascalonicum *Alliaceae*	bulb	interdigital dermatitis/ raw/topical	6	2.53	A
Allium cepa *Alliaceae*	bulb	antihelmintic/juice/oral indigestion/ 4-5 bulbs (pasta) with 0,5 l oil, 0,25 l *țuică* and 2 ground paper tablespoon/ oral teat papillomatosis/juice/topical	22	9.28	A
Allium sativum *Alliaceae*	bulb	diarrhea/ pasta with eggs and vinegar/oral mastitis and antihelmintic/with alcohol/oral ruminal meteorism/pasta with petroleum/oral teat papillomatosis/juice/topical	18	7.59	A
Alnus glutinosa *Betulaceae*	bark	diarheea/decoction/oral	1	0.4	A
Armoracia rusticana *Brassicaceae*	root	respiratory diseases/raw/oral	11	4.64	A
Artemisia absinthium *Asteraceae*	aerial parts	diarheea/infusion/oral indigestion/infusion or raw with salt and vinegar/oral respiratory diseases , antihelmintic/decoction/oral external parasites/ decoction with *Canabis sativa* leaves/washes foot disease, skin injuries/decoction/topical	114	48.1	A
Atropa belladonna *Solanaceae*	leaves, root fruit	mastitis and immunomodulator/leaves or root raw/oral external parasites/row/oral	10	4.21	B***
Asarum europaeum *Aristolochiaceae*	leaves	mastitis/raw/oral	4	1.68	B
Betula pendula *Betulaceae*	bark sap	diarrhea/decoction/oral antianemic/raw/oral	4	1.68	B
Calendula officinalis *Asteraceae*	flowers	diarrhea/infusion/oral mamary injuries/ cream/ topical	17	7.17	A
Cannabis sativa *Cannabaceae*	leaves	external parasites/decoction/washes	5	2.1	A
Chamomilla recutita *Asteraceae*	aerial parts	foot disease, skin injuries/ infusion/ washes digestiv/ with oil/oral	7	2.95	A
Chelidonium majus *Papaveraceae*	aerial parts latex	external parasites/decoction/washes skin injuries/cream with Callendula flower/topical teat papillomatosis/latex/topical	37	15.61	A
Cornus mas *Cornaceae*	fruits	diarrhea/decoction or alcoholic macerate/oral	6	2.53	C***
Corylus avellana *Betulaceae*	leaves fruits	mastitis/raw/oral mamary injuries/ cream/topical	3	1.26	B
Curcubita maxima/pepo *Cucurbitaceae*	seed	antihelmintic/raw/oral	8	3.37	A
Equisetum arvense *Equisetaceae*	aerial parts	antihemoragic/decoction/compresses external parasites/decoction/washes	5	2.10	A
Fragaria vesca *Rosaceae*	leaves	diarrhea/infusion/oral	4	1.68	A
Gentiana lutea *Gentianaceae*	root, rhizome, leaves, flowers	diarrhea/decoction or alcoholic macerate/oral	45	18.98	A
Heleborum niger *Ranunculaceae*	root leaves	antihelmintic, immunomodulator, respiratory disease/ transcutaneous implantation on the ear mastitis/50g dry leaves/oral	32	13.5	A
Helianthus annuus *Asteraceae*	seed oil	indigestion, acute meteorism/with eggs, milk or alcohol/oral antihelmintic/with vinegar/oral	67	28.27	B
Hylotelephium telephium/spectabile *Crassulaceae*	aerial parts rhizomes	mastitis/raw or alcoholic macerat/oral	54	22.78	B
Hypericum perforatum *Hypericaceae*	aerial parts	diarrhea/infusion/oral mamary injuries/ oil macerate/topical	15	6.32	A
Inula britannica *Asteraceae*	flowers	diarrhea/infusion/oral	45	18.98	A
Inula helenium	root	diarrhea/infusion/oral	18	7.59	A

Fam Asteraceae	rhizomes leaves flowers	mastitis, antihelmintic/decoction/oral respiratory diseases/raw leaves/oral			
Juglans regia *Juglandaceae*	leaves pericarp seed	diarrhea/decoction/oral papillomatosis/cream/topical external parasites/decoction/washes antihelmintic/decoction/oral respiratory diseases/with *ţuică*/oral	8	3.37	A
Lathraea squamaria *Orobanchaceae*	rhizomes	mastitis/raw or alcoholic macerate/oral	32	13.5	D***
Levisticum officinale *Apiaceae*	leaves root	diarrhea, antihelmintic/leaves infusion or root decoction/oral mastitis/root decoction with *ţuică*/oral	85	35.86	B
Linum usitatissimum *Linaceae*	seed	indigestion/ acut meteorism/decoction/oral	2	0.84	A
Medicago sativa *Fabaceae*	aerial parts	respiratory diseases/infusion/oral	2	0.84	B
Mentha piperita *Laminaceae*	leaves	diarrhea/infusion/oral	79	33.33	A
Nicotiana tabacum *Solanaceae*	leaves	external parasites/decoction/washes	12	5.06	A
Phaseolus vulgaris *Fabaceae*	seed	mastitis/ baked seeds/oral	3	1.26	D
Plantago lanceolata *Plantaginaceae*	leaves	diarrhea/infusion/oral	6	2.53	A
Potentilla reptans *Rosaceae*	leaves flowers	diarrhea, induce oestrus/infusion/oral	1	0.42	B
Primula officinalis *Primulaceae*	flowers	respiratory diseases/infusion/oral	3	1.26	B
Prunus domestica *Rosaceaea*	leaves	diarrhea/raw/oral	21	8.86	B
Prunus persica *Rosaceae*	leaves	diarrhea, antihelmintic/raw or infusion/oral	2	0.84	B
Prunus spinosa *Rosaceaea*	fruits	diarrhea/decoction/oral	7	2.95	A
Quercus robur *Fam. Fagaceae*	bark	diarrhea/decoction with *Sallix spp* and *Robinia pseudoacacia* /oral	29	12.23	A
Robinia pseudoacacia *Fabaceae*	bark leaves fruits	diarrhea, antihelmintic/decoction/oral diarrhea/raw/oral indigestion/decoction/oral	115	48.52	A
Rubus fruticosus *Rosaceae*	aerial parts	indigestion/decoction/oral	5	2.1	A
Rumex alpines *Poligonaceae*	root seed	diarrhea, acute meteorism/ decoction or alcoholic macerate/oral	24	10.12	C
Tanacetum vulgare *Asteraceae*	flowers leaves	diarrhea/infusion/oral	17	7.17	A
Taraxacum officinale *Asteraceae*	root	diarrhea/decoction/oral	1	0.42	A
Tilia spp. *Tiliaceae*	flowers	diarrhea, respiratory diseases /infusion/oral foot disease/infusion/ washes	8	3.37	B
Salix alba, Salix capreae *Salicaceae*	leaves, bark young twigs	diarrhea, antihelmintic /decoction or raw/oral	34	14.34	A
Sambucus nigra *Adoxaceae*	bark	diarrhea/decoction/oral	1	0.42	B
Satureja hortensis *Laminaceae*	aerial parts	diarrhea/infusion/oral	7	2.95	A
Sempervivum tectorum *Crasulaceae*	leaves	mastitis, acute meteorism/decoction, alcoholic macerate, raw/oral	102	43.03	B
Ulmus montana *Ulmaceae*	leaves	diarrhea/infusion or raw/oral	2	0.84	B
Vaccinium myrtillus *Ericaceae*	fruits aerial parts	diarrhea/raw/oral diarrhea/infusion/oral	5	2.1	A
Veratrum album *Melanthiaceae*	aerial parts root	external parasites, foot disease /decoction/washes	31	13.08	B
Xanthium spinosum *Asteraceae*	aerial parts	diarrhea, antihelmintic/decoction/oral	17	7.12	A

*(U) absolute value of frequence of each plant species; ** (F) frequency (as a percentage); ***according to Lans et al. (2007)

Table 2. Cattle conditions and origin of ethno-veterinary remedies used to treat them

Disease	Remedy origin			Total
	vegetal	mineral	animal	
Diarrhea	34	2	1	37
Mastitis	12	2	2	16
Internal parasites	10	3	-	13
External parasites	8	3	-	11
Acute meteorism	6	3	3	15
Foot disorders	5	4	-	9
Respiratory disorders	9	-	-	9
Indigestion	7	-	-	7
Superficial mammary wounds	4	-	2	6
Papillomatosis	4	-	-	4
Skin wounds	2	-	-	2
Imunomodulator	2	-	-	2
Eye disease	1	1	-	2
Anti- haemorrhage	1	-	-	1
Anti-anemic	1	-	-	1
	109	**18**	**8**	**135**

The aqueous extract of *Inula britannica* flowers, administered for 3-4 days to sick animals is found in all villages, also frequently used in the treatment of diarrhea in humans. From the same family, *Inula helenium* is also used, as root decoction, aqueous extract of the plant or the leaves administered in a fresh state (the plant is also used to treat mastitis). *Inula* genus has been used since ancient times in all corners of the world, from the writings of ancient Greek and Roman doctors, to traditional Chinese, Egyptian, Tibetan or Ayurvedic medicine. *Inula britannica* and *Inula helenium* are noted for their antibacterial, anti-inflammatory, anti-tumor, cytotoxic, hepatoprotective and anthelmintic action. (Amin et al., 2013; Diguta et al., 2014; Khan, 2010). Alcohol extract from the root of *Inula helenium* (harvested from Romania) had significant activity against pathogenic bacteria of animal origin and dermatophyte fungi (Diguta et al., 2014).

In the treatment of diarrhea, there are also used young willow branches and willow leaves (*Salix alba*) administered alone or together with leaves of acacia (*Robinia pseudoacacia*) or in the form of decoction. Willow can also be combined with oak leaves and bark (*Quercus robur*) or with elm leaves (*Ulmus* spp.), and acacia can be combined with lime blossoms (*Tilia flores*).

Another remedy used is a decoction made from a mixture of alder tree bark, sessile oak bark and birch tree bark, or a decoction made from peach tree bark and elderflower. Willow is known for its high salicin content, over 80% of it is absorbed and metabolized into different salicylate derivatives, it also contains polyphenols and flavonoids. Therefore, it has numerous therapeutic properties: anti-inflammatory, antipyretic, antioxidant, reduces oxidative stress, it is a cardiovascular protector, it is antimicrobial, analgesic, astringent (Amel et al., 2018; El-Mahdi, 2019).

Oak bark has antibacterial, astringent, antiseptic, anti-inflammatory effect on the skin and oral mucosa and it is recommended by EMEA for the control of light diarrhea in ruminants, horses, pigs and chickens (Deryabin & Tolmacheva, 2015; EMA, 2009). Elm contains mucilages and tannin, and is used to treat inflammation and gastrointestinal ulcers, convalescence, colitis and diarrhea (Wynn & Fougere, 2006).

Xanthium spinosum decoction, garden mint (*Mentha piperita*) or wild mint (*Mentha longifolia*) infusion, plantain (*Plantago* spp.), thyme (*Thymus vulgare*), wormwood (*Artemisia absinthum*) or marigold (*Calendula officinalis*) are often encountered in the treatment of gastrointestinal disorders of calves. *Xanthium spinosum* together with fruits of *Prunus spinosa* and *Allinum sativum* (garlic), are used in the production of an alcoholic extract that is administered in small quantities along with various herbal infusions. *Xantinum spinosum* is effective against Gram-positive bacteria (including MSSA and MRSA), and less effective against Gram-negative bacteria. In addition to its antimicrobial role, it has been demonstrated to have anti-inflammatory, antioxidant, anthelmintic and antifungal properties (Devkota & Kumari, 2015; Ginesta-Peris et al., 1994; Rad, 2013). The use of *Plantago* spp. and *Calendula officinalis* in diarrhea can be scientifically supported due to their antibacterial, antifungal and anti-inflammatory effects (Monjd Abd Razik et al., 2012; Parente et al., 2012; Shah & Williamson, 2015)

Artemisia absinthum, the plant that is used in 7 of the 14 diseases studied, is recognized by the entire medical world. Numerous *in vivo* and *in vitro* studies have been performed that prove its internal and external antiparasitic, antibacterial, anti-inflammatory, antifungal, antispastic,

antiviral, antineoplastic effectiveness. Its anti-parasitic properties are given by sesquiterpene lactones, flavonoids and artemisinin, and it is effective against *Haemoncus, Fasciola, Trypanosoma, Eimeria, Trichostrongylus, Ascaris*. In a study carried out on sheep, the efficacy of aqueous and alcoholic extract of *Artemisia absinthum* against *Haemoncus contortus* is comparable to that of albendazole (Ferreira, 2009; Moslemi et al., 2012; Tariq et al., 2009).

Externally, wormwood extract used in pig farms significantly reduced the number of *Sarcoptes scabiei* parasites in the first week after treatment, and in ruminants it was shown to be effective against *Riphicephalus microplus* (Mägi et al., 2006; Parveen et al., 2014). Wormwood extract used in the treatment of surgical wounds infected with *S. aureus* in rats has a strong antibacterial effect). *Artemisia absinthum* is mainly effective against Gram-positive bacteria (Moghaddam et al., 2016; Moslemi et al., 2012).

Highly appreciated in the treatment of diarrhea is also *Rumex alpinus*, most commonly used is the aqueous extract of the seeds, sometimes the plant as such or in the form of alcoholic extract taken from its root. Plants belonging to the *Rumex* genus are also used in other localities in Romania or in traditional medicine in Turkey to treat diarrhea, constipation and eczema (Ozturk & Ozturk, 2007; Bartha et al., 2015).

Decoction and alcoholic extract are also obtained from *Cornus mas* or *Aesculus hippocastanum* (chestnuts), which are frequently used in the treatment of diarrhea in humans. Aqueous extract from the plant or root of *Levisticum officinale* is used both for the treatment of diarrhea, mastitis, but also as an ascaricide, sometimes combined with walnut leaves (*Juglans regia*) and glass flowers (*Tanacetum vulgare*). *Levisticum officinale* essential oil obtained from leaves, flowers or fruits contains over 190 organic compounds. It has antiparasitic, antibacterial, antifungal, antiviral, antioxidant and antimicrobial action. It inhibits the development of the *Mycobacterium tuberculosis* bacterium, and the plant extract moderately inhibits the growth of *Pseudomonas aeruginosa, Acinetobacter baumannii, Escherichia coli, Salmonella enteritidis* and *Staphylococcus epidermitis*, also having a synergistic action with ciprofloxacin (Ebrahimi et al., 2016; Mirjalili et al., 2010).

The use of walnut (*Juglans regia*) in the treatment of digestive and respiratory disorders may be due to the intense antibacterial activity of all the components, leaves, bark, fruits and pericarp, having a wide spectrum of action against Gram-positive and Gram-negative bacteria. The internal antiparasitic activity is supported by many studies, comparative with that of albendazole, in vitro it is effective against the embryo eggs of *Ascaris suum*, of the larvae of *Trichostrongylus colubriformis* and of the helminths *Fasciola* spp. and *Haemonchus contortus* . It is also used as an antiviral, antifungal, hepatic and renal protector, antidiabetic, anticancer, antide-pressant, antioxidant, antirheumatic (Marhaba & Haniloo, 2018; Tajamul et al., 2014; Urban et al., 2008).

In the treatment of respiratory diseases, the fresh root of *Armoraciae rusticana*, adminis-tered in ration, or infusions of *Artemisia absinthium*, flowers of *Primula officinalis*, *Tillia* spp. and *Medicago sativa* are administered orally. It is also a common practice to place a root of *Heleborum niger* in the ear or necklace for a maximum of 24 hours. Animals have demonstrated a nonspecific immune response following the administration of *Heleborum niger* rizome or root extract. The immunomodulatory effect is based on lecocytosis, granulocytosis, increased macrophage number and neutrophil phagocytosis. The use of *Heleborum niger* in respiratory diseases in cattle is found in traditional veterinary medicine in many European countries. A root extract of *Heleborus bocconei* was tested for antimicrobial efficacy against ten bacterial strains responsible for respiratory diseases in cattle, with very good results against *Streptococcus pneumonia, Moraxella catarrhalis* and *Haemophilus influenzae* (Davidović et al., 2017; Bartha et al., 2015).

Plants of the genus *Allium* (*Allium sativum, Allium cepa* and *Allium ascalonicum*) in the form of aqueous extract, tincture or fresh form, are used for their anthelmintic, antiseptic, antifungal, antibacterial, antioxidant, anticoagulant, anti-cancer, hepatoprotective and immunomodulatory action. They are some

of the most studied plants in the world, information about their use in therapy has been found since 5000 years ago, in Sanskrit writings (Londhe et al., 2011; Shari-Rad et al., 2016; Sonia et al., 2018).

The present study revelead their use in the treatment of interdigital dermatitis, mammary papillomatosis, indigestion, tympanism, mastitis or for their anthelmintic effect.

For the treatment of mastitis, 13 plants were identified, of which four plants are found in all the investigated areas.

Sempervivum tectorum is cultivated by humans on the roof of animal shelters, being used especially in the treatment of mastitis. It is administered in a raw state or as an alcoholic extract. It is a plant used throughout the Balkan area, but still insufficiently studied. In other ethno-veterinary studies it is used as ruminative and digestive in cows, and in human medicine it is used in the treatment of otitis, insect bites, burns and ulcers. The aqueous extract has proved to be effective against antibiotic resistant *E. coli strains,* an action due to polyphenols that oxidize and/or hydrolyze the bacterial cell wall and their plasma membrane (Di Sanzo et al., 2013; Muselin et al., 2014; Rovcanin et al., 2015). *Lathraea squamaria* is the most prized and praised by farmers, who assign it miraculous powers. During the spring they go into the mountains to collect it, and use it exclusively in the treatment of mastitis. It is administered 2-3 rhizomes in a raw or dry state, for 3-4 days. In the literature there is no data to confirm the effectiveness of the plant, but we cannot doubt its activity without scientific support.

Hylotelephium spectabile and *Hylotelephium telephium* are two other plants that are frequently used to treat mastitis. In traditional medicine it is also used in Spain for the treatment of infected wounds and inflammation of the skin and in Serbia for the treatment of diphtheria, intestinal worms, scurvy and various skin conditions. The *Hylotelephium spectabile* extract has moderate antibacterial activity against *P. aeruginosa, S. aureus, Bacillus subtilis* and *Salmonella typhimurium* (Stojanovic et al., 2014), but without in vitro and in vivo studies the efficacy of the two plants in treating mastitis cannot be questioned.

To remedy superficial mammary wounds, ointments prepared according to different recipes are used, most of them are based on medicinal plants to which fir resin, wax, honey or eggs are added in varying proportions, and as excipients lard, sheep/goat fat or butter are used. *Callendula officinale* is a plant that appears in all ointment recipes, along with the addition of *Artemisia absinthum* leaves, walnut leaves (*Juglans regia*), *Chelidonium majus, Hypericum perforatum* or hazel kernels (*Corylus avellana*). The creams that have the composition of *Chelidonium majus* or *Juglans regia* leaves are also used to treat mammary papillomatosis.

For the control of external parasites, the animals are bathed using decoctions made from *Nicotiana tabacum* (tabacco), *Cannabis sativa* (hemp), *Atropa belladona, Chelidonium majus, Eqiusetum arvense* or *Veratrum album*. An in vivo study demonstrated the strong effect of *Nicotiana tabacum* in rabbits infested with *Sarcoptes scabiei*. The rabbits were treated locally by applying a decoction on the affected areas, leading to the complete healing of the lesions; also for a month and a half no reinfestations were observed. Tabacco extract is effective against ticks *Rhipicephalus sanguineus* and *Rhipicephalus appendiculatus*, but has a reduced action against lice (Nouri et al., 2014; Schorderet Weber et al., 2019). The decoction from the dried root of the *Veratrum album* in combination with the leaves of *Brassica oleracea* is used against lice in small ruminants (El Mahdy et al., 2017). *Veratrum album* is also used as anthelmintic, anti-inflammatory, antiseptic, or antipyretic (Grobosch et al., 2008). Fresh leaves of *Chelidonium majus* or dry plant powder are applied to lesions in the treatment of scabies in cattle.

In addition to the external antiparasitic effect, *Chelidonium majus* is also used as an anthelmintic, antibacterial, antifungal, antiviral, antiprotozoal, hepatoprotective, immunomodulatory, anti-inflammatory (Chakraborty & Kanti Pal, 2012; El Mahdy et al., 2017). The extract of *Atropa belladonna* at a concentration of 20% was lethal to the ticks *Rhipicephalus microplus*, and against the larvae it was effective at a concentration of 10% (Godara et al., 2014). All plants used as treatment for external parasites are toxic to

ruminants if the therapeutic doses are exceeded, and animal breeders, aware of this fact, usually only allow certain people with experience to perform treatments.

Statistical data analysis

Of the total number of plants identified in this study, according to the classification made by Lans et al., 2007, the majority were classified in categories A and B (Figure 3), in the specialized literature, there being sufficient information regarding their chemical composition and their therapeutic efficacy.

Only about *Lathraea squamaria* and *Phaseolus vulgaris* used to treat mastitis pharmacologically relevant information has not been found, but without in-depth studies the therapeutic effect cannot be questioned.

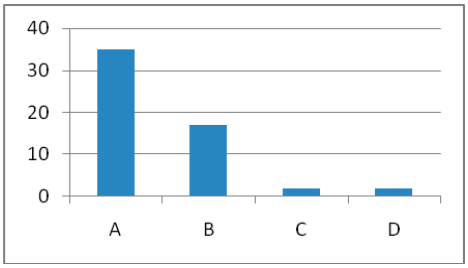

Figure 3. Classification of the plants (according to Lans et al., 2007)

The plants with the highest frequency of occurrences in all the investigated area were acacia (*Robinia pseudoacacia*) used to treat diarrhea, indigestion or anthelmintic effect, with 115 occurrences (48%), followed by pelin (*Artemisia absinthium*) with 114 occurrences used in most of the conditions investigated (diarrhea, indigestion, skin wounds and foot disorders, internal and external parasites) and *Sempervivum tectorum*, identified 102 times, used to treat mastitis and acute meteorism.

Other remedies

In addition to the plants analyzed above, there are also a number of remedies of mineral or animal origin. Some of them, such as fine powdered sugar administered intraocular, are found to be a common practice in the treatment of eye disorders in many countries of the world, others, such as hedgehog or badger skin pig or bear bile administered in mastitis appear to be a part of ancient local rituals.

CONCLUSIONS

The area of the Curvature Subcarpathians remains an inexhaustible source for documentation on ethno-veterinary medicine and a very important source of medicinal plants. The investigation carried out in this study highlighted the ethnotherapeutic use of 135 herbal remedies, both of mineral and animal origin.

Gentiana lutea was the most widely used plant, mainly the root and the rhizome, in adult bovine diarrhea, formulated as an alcoholic extract, macerated or decocted. His effectiveness is scientifically proved.

Alcohol extract from the root of *Inula helenium* is used in the treatment of diarrhoea, in mixture with *Salix alba* young branches and leaves, administered alone or together with leaves of *Robinia pseudoacacia,* or in the form of decoction.

The plants with the most frequent use in the investigated area were: *Robinia pseudoacacia* (acacia) used to treat diarrhoea, indigestion or internal parasites (48%), *Artemisia absinthium* to treat diarrhoea, indigestion, skin wounds and foot disorders, internal and external parasites (48,1%) and *Sempervivum tectorum* used to treat mastitis and acute meteorism (43.03%)

For the treatment of mastitis, 13 plants were identified, of which four plants are found in all the investigated areas. *Sempervivum tectorum*, *Lathraea squamaria*, *Hylotelephium spectabile* and *Hylotelephium telephium* are the plants frequently used to treat mastitis, but there are no scientific studies to support their therapeutic efficacy.

Ethno-medicine, beyond remaining an alternative for small farmers who practice traditional agriculture or for the farmers who practice organic farming, can provide the solution to balance the abuse of antibiotics.

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