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RESEARCH ON THE BUNODONT AND SECODONT TYPES OF TEETH IN DOMESTIC MAMMALS

Cristian BELU, Iulian DUMITRESCU, Bogdan GEORGESCU, Petronela Mihaela ROȘU, Anca ȘEICARU, Ștefania RAITA, Sorina Andreea MIHAI, Mădălina DOBRILĂ, Oresti MIHELIS, Gabriel PREDOI

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Abstract

The teeth are made of hard materials, they represent the most resistant organs to destruction, often being collected from archaeological sites. They play a major role in establishing the species origin of fossils or corpses. A single tooth sometimes provides clues as to where it came from, and provides information about the animal's age, diet, and behaviour. The study was performed on the skulls of pigs, canines and cats, then described the anatomical characteristics of each type of tooth, insisting on teeth that have similar anatomical aspects and can cause confusion in identification. The dentition of pigs is adapted to the omnivorous regime, the molars being of the bunodont type, compared to carnivores where all teeth are of the brachiodont type. The stricter carnivorous diet of cats, compared to that of canids, translates into the specialization of dentition to a more pronounced second type, the morphological effect being the reduction of tuberculous molars. This motivated us to carry out the work, hoping that the results could be useful for the professional activities of veterinarians, but also forensic doctors.

Key words*: carnivores, dentition, omnivores, teeth.*

INTRODUCTION

Although mammal teeth are similar from the point of view of the basic structural components, there are major differences in numbering, size and shape (Barone, 1997; Coțofan et al., 2013).

The great degree of morphological variation in mammal teeth (specifically molars) makes them easy to recognise. Thus, teeth and jaws play a major role in establishing the species origin of various fossil remains or bodies. Even a small particularity of shape or structure can have a fundamental importance in clarifying taxonomical incertitude, often being possible for a single tooth to offer clues as to which mammal species it belongs to, and moreover provide information about the age, diet and behaviour of the animal in its environment.

Dentition morphology is an extremely important chapter in legal medicine, often teeth or fragments of them being the only identifiable parts of a body. (David-Be et al., 2009; Stan, 2016.) Bibliographical sources prove that dental medicine offers enough details on the morphology of deciduous and permanent dentition in humans (Ata-Ali, 2014) However, bunodont teeth are also encountered in domesticated animals, such as swine and carnivores, species where literature data is not as abundant. (Koning & Liebich, 2015; Ungar, 2010; Lucas, 2004; Stan, 2016). This fact has motivated the choice of study, in the hope that the results will be useful not only to future veterinary dental medicine specialists, but also to medical examiners and coroners, who wish to identify samples from on-going investigations.

MATERIALS AND METHODS

The study was performed on skulls prepared through maceration, belonging to the collection of the Anatomy laboratory of the Faculty of Veterinary Medicine in Bucharest.

The pieces selected had either a complete dentition or very minor gaps. 10 skulls of swine, 10 skulls of dogs and 6 skulls of cats were analysed, originating from animals of various breeds and ages. Detailed and systematic descriptions were performed of the anatomical characteristics of each type of tooth,

insisting especially on teeth which have similar anatomic aspects and can thus generate confusion upon identification (for example secodont molars in swine which have a bunodont dentition or tuberculate molars in carnivores).
The most suggestive images on the studied material were photographed, and then edited in the Adobe Photoshop C3 program. The identification, description and homologation of the formations was done in correlation with the Nomina Anatomica Veterinaria - 2017.

RESULTS AND DISCUSSIONS

The results of the study allowed the observation of some morphological details on the basis of which teeth or tooth fragments can be identified with certitude, establishing their origin with one of the studied species, and even their position in the dental arcades when this is necessary.
The dentition of the **pig** is excellently adapted to the omnivorous regime. The dental formula is as follows: I: $\frac{3}{3}$, C: $\frac{1}{1}$, P: $\frac{4}{4}$, M: $\frac{3}{3}$.
With the exception of central incisors and inferior intermediate incisors, which are hypsodont, and the canine teeth which grow continuously, all the other teeth are brachydont.
On each side and on each maxillary three incisors can be noticed: a central one, an intermediate one and a distal or corner one. They have a different aspect ranging from one maxillary to the other, but on the same arcade. Their size decreases from the central incisor to the corner one.
The inferior incisors are pointed rostrally, following the line of the mandible. (Figure 1-1). The central incisor is strong, in contact with the one on the opposite side through the mesial side (Figure 2-1). It has an elongated crown, with prolonged eruption, supported by a strong root. The crown is flattened vestibulo-lingually, smooth on the vestibular side but with a pronounced longitudinal relief (dental tubercle) on the lingual side, delimited by two ridges. The mesial edge and the distal edge are almost parallel, thick towards the neck of the tooth. The occlusal margin is perpendicular. The neck of the tooth is not as distinct from the root, it directly lengthens the crown, very thick in its vicinity, solidly implanted in the alveoli. The intermediate incisors are similar to the central incisors (Fig. 2-2). They are however a little shorter, and their crown is thicker, almost prismatic in a triangular section, a little narrowed at the occlusal extremity. This makes the ensemble of the four central teeth (central and intermediate incisors) to appear convergent, and it gives the arcade an elongated contour, almost sharp. The corner incisor is isolated, approximately in the middle of the distance between the middle incisor and the canine tooth. It is much more reduced than the other two, with a short, narrow crown and an obvious neck, as well as a less solidly implanted root (Fig. 1-3).

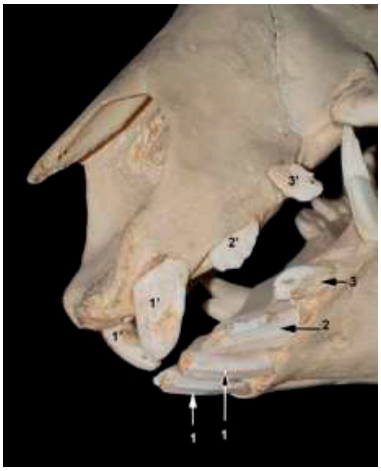

Figure 1. Incisors in pigs (lateral-rostral view) (original): 1-inferior central incisor; 1'-superior central incisor; 2-inferior intermediate incisor; 2'-superior intermediate incisor; 3-inferior corner incisor; 3'-superior corner incisor

The superior incisors are oriented less longitudinal than the inferior ones. They serve especially with food prehension, while the last ones aid in digging in the soil. The central incisor has a wide, relatively short crown, convex on the vestibular side and concave on the lingual side. The distal margin is shorter and more oblique than the mesial side. It curves a bit to the side of the opposite central incisor, the teeth being convergent through their occlusal extremities, delimiting a triangular interval between their proximal portions. (Figure 2-1'). The occlusal margin, very oblique, not very distinct from the distal

margin, is excavated towards the lingual side, where a large and relatively deep infundibulum is contoured. The neck of the tooth is net, and the root is strong, profoundly implanted in the socket. The intermediate incisors are similar to the central ones, but they are smaller and with a more compressed crown and a weaker marked infundibulum. The superior intermediate incisor is located at the level of the inferior corner incisor, with which it does not come into contact during occlusion. The corner incisor is also reduced and isolated, similar to the inferior counterpart (Figure 1-3'). The crown is flattened from one side to the other, and the occlusal extremity is trilobed, marked by two weakly evidenced incisures.

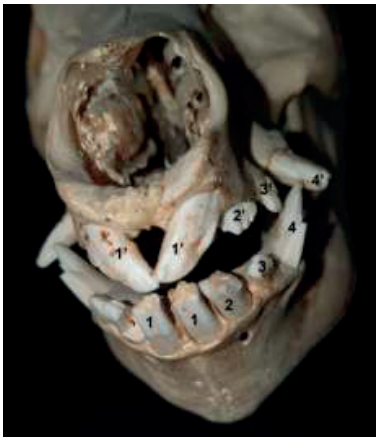

Figure 2. Incisors in pig (rostral view):
1-inferior central incisors; 1'-superior central incisors; 2-inferior intermediate incisor; 2'-superior intermediate incisor; 3-inferior corner incisor; 3'-superior corner incisor; 4-inferior canine tooth; 4'-superior canine tooth (original)

Canine teeth are very well developed in adults, where they mark a strong sexual dimorphism. They are better developed in males, where they rapidly emerge from the oral cavity, forming true "defences weapons" (Figure 3). In both sexes their growth is permanent and they do not have roots.

The inferior canine tooth is less thick but longer than the superior one. It can reach 20-25 cm in length at elderly males. It curves in such a way that the free part reaches the face of the superior canine tooth, between it and the corresponding corner incisor, lifting the superior lip. The body of the tooth is tri-faced. The distal side which occupies all its concavity does not have enamel. The other two, separated by a sharp mesial edge, only have a very thin layer. The free extremity is sharp and, just like the lingual margin, it is maintained sharp by its friction with the superior canine tooth. The alveolar extremity is wide and occupied by an open orifice which enters further and further into the bone with age until it reaches the level of the molars. In sows, the growth is more moderate, and the largest recorded length was of 3-4 cm. The calibre is more reduced too, the tooth is easily flattened from one side to the other.

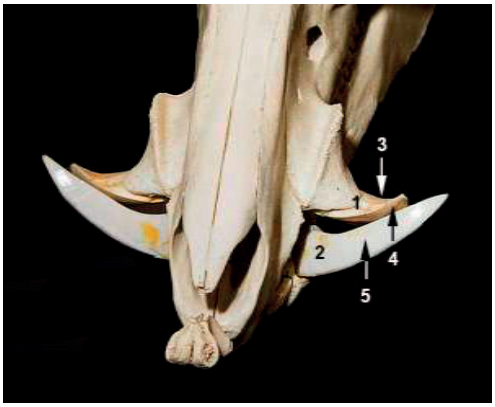

Figure 3. Canines in male pigs (rostro-dorsal view) (original):
1-superior canine; 2-inferior canine; 3-distal part of superior canine; 4-mesial part of superior canine which by friction with the inferior canine keeps it sharp; 5-mesial part of inferior canine

The superior canine tooth is thicker at the base but shorter than the inferior counterpart. The length does not exceed 12 cm in elderly males. It curves first laterally, then caudally, passing before its inferior counterpart, arising early from the oral cavity and deforming the superior lip. It has the shape of a quadrilateral pyramid, with very weakly marked angles, in an almost circular section. The distal side is concave and lacks enamel (Figure 3-3), which is very thin and often absent on the rest of the dental surface. The free extremity is blunt on the mesial side, convex and in contact with the inferior canine tooth. The alveolar extremity is open, determining a relief on the surface of the maxillary which delimits the canine fossa. Just like with the inferior counterpart, it is more reduced in sows than in male pigs and flattened from one side to the other.

On each jaw, the **premolars and molars** regularly grow in volume and complexity from the first to the last. They are all brachydont and starting from the first superior premolar and the fourth inferior premolar - purely bunodont teeth.

The inferior premolars are the simplest. They are flattened from one side to the other and almost similar to secodont teeth in carnivores. The first is separated from the others, a short distance away from the canine tooth and a reasonable distance from the second premolar (Figure 4-Pm1). It is a deciduous tooth which persists more or less in adult dentition. It falls off at a certain age and never gets replaced. The crown has little volume, being narrow and simple, with a sharp edge. It has two roots: a mesial and a distal one. The crown of the second molar is larger meso-distally and trilobed, with an intermediate lobe flanked by two reduced depressions on the vestibular side. It also has two roots. The third is similar, albeit a bit larger, the crown has ridges, better delimited on the vestibular side and two similar depressions but less profound on the lingual side. Sometimes it is three-rooted, with a bifid distal root. The fourth molar is the largest and the thickest. Being the tallest, usage quickly wipes off the details off the occlusal margin. The depressions of the lingual side and especially of the vestibular side are very profound. It has three roots.

The inferior molars continue the voluminous progression of the premolars. Their crown is low and practically cement-less, it is thick and formed of cuspids, each of them subdivided by a very complex system of ridges into primary and secondary tubercles (Figure 4-M1, M2, M3). The occlusal side is very quickly flattened by usage, though it has complicated folds of enamel which end up wiped. The first has four cuspids, which correspond to four roots: two vestibular and two lingual. The crown is twice as long as it is wide. The second molar is similar to the first but it is stronger, its lobes are better delimited and a fifth cuspid contours on the distal edge. The final molar is characteristic. It is larger in volume than the other two combined, has five cusps of which the distal one takes up almost half the tooth, and tends to divide into two portions itself: vestibular and lingual. Each cusp corresponds to a root, the distal one tending to divide into what raises their number to six.

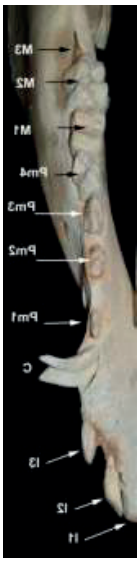

Figure 4. Inferior molars and premolars in pigs (dorsal view) (original):
I1-I3 – inferior incisors; C-canine; Pm1-Pm4 – inferior premolars; M1-M3 – inferior molars (M3 in the process of eruption)

Superior premolars are, with the exception of the first one, more complex than the inferior counterparts. The first is small, with a sharp crown and two roots. It comes in contact with the neighbouring one. The second one is also narrow, formed of two main cusps of which the mesial one is somewhat taller, and the third one is rudimentary and placed on the lingual side of the distal cusp. It is three-rooted, the two distal roots united at the base and a little divergent. The third one, more massive, has a neatly trilobed crown, the disto-lingual cusp almost as thick as the neighbouring one. The three roots are equally distinct. The fourth one has a squarer crown on a transversal section. It is formed of four massive cusps, though uncomplicated in structure, and sustained by four roots, two mesial and two distal ones. The cusps of the lingual side are not very different from one another.

The superior molars have the same organization as the inferior ones. Their crown is however thicker in a vestibulo-lingual way. The first two are also formed of four cusps and four roots. The meso-distal size is slightly

larger than the transversal size of the first one, a more neat difference in the second one which contours a supplementary distal cusp.

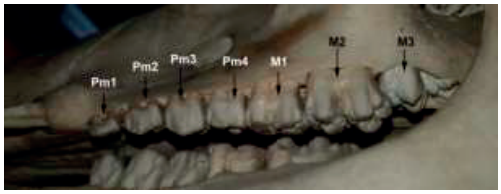

Figure 5. Superior molars and premolars in pigs (latero-ventral view) (original):
Pm1-Pm4 – superior premolars; M1-M3 – superior molars (M3 in the process of eruption)

The last one is massive, with the same conformation as its inferior counterpart, but wider and less developed backwards. The distal cusp is less strong and less bilobated (Figure 5). It does however feature 6 roots.

The molar arcades are rectilinear, with a thickness that raises progressively meso-distally. They converge slightly forward, which is better noticed at the superior maxillary. Their direction crosses the alveolar edge less. This thing becomes more visible at the mandible which appears curved towards the lingual side at the level of the molars. In occlusion, teeth easily alternate, the inferior ones a bit more rostral than the superior ones. The first inferior premolar has no contact with its superior counterpart, the last one being in contact only with the second inferior premolar.

Dentition in carnivores: The level of specialisation of carnivore dentition is somehow different, depending on the family a species belongs to. As a general rule, there are two teeth on each arcade and on each side, with a characteristic aspect (Figures 6, 7). At the entrance in the oral cavity, the canine teeth, long, powerful and sharp, and caudally, a special molar or premolar called a carnassial tooth.

The functional efficiency of the canine teeth is given by the reduction of the volume of neighbouring teeth (incisors and premolars), these being destined for catching, killing and ripping apart prey. Carnassial teeth, sharp and affronted like the blades of a scissor, serve to cut bigger pieces of meat, which are then shredded. This thing is helped by the distal teeth, also called tuberculate teeth. Unlike canine teeth which remain long and powerful at all carnivores, the carnassial teeth are those whose morphology reflects the adaptation to different alimentary regimes.

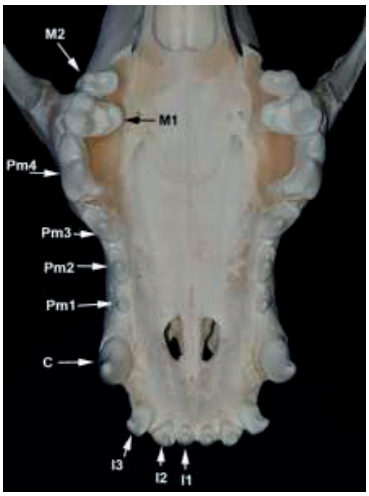

Figure 6. Superior dental arcade in dog (ventral view) (original):
I1-I3 – incisors; C-canine; Pm1-Pm4 – premolars (Pm4 – carnassial tooth); M1-M2 – molars

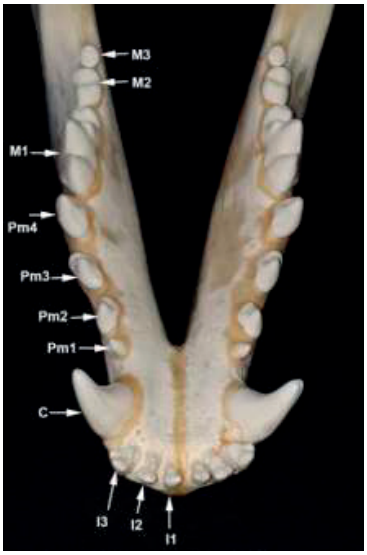

Figure 7. Inferior dental arcade in dog (dorsal view) (original):
I1-I3 – incisors; C- canine; Pm1-Pm4 – premolars; M1-M3 – molars (M1 – carnassial tooth)

Very slightly different from neighbouring teeth in *Ursidae,* where their bunodont aspect is largely correlated with the great diversity of

consumed food, in *Hienidae* and *Felidae* they exhibit an exaggerated development. At the latter, the shortage of maxillaries and by consequence of the leverages formed, gives these teeth a maximal efficacy. Canids do however have a less specialised form of these teeth. All teeth in carnivores are of the brachydont type.

The permanent dental formula of the dog is:

$$\text{I: } \frac{3}{3}, \text{ C: } \frac{1}{1}, \text{ P: } \frac{4}{4}, \text{ M: } \frac{2}{3}.$$

On each arcade, the first premolar is not replaced and, just like in swine, it persists into adulthood, which sometimes makes it considered a permanent premolar, which lost its homologous deciduous tooth.

The incisors are implanted perpendicularly on the osseous substrate; these teeth are very tiny. The crown is short, the root is long and strong.

The thick crown is flattened vestibulo-lingually near the occlusal extremity, and a bit latero-laterally near the neck of the tooth. The vestibular side is smooth, convex in all senses, narrowed towards the neck of the tooth. The lingual side is curved near the end by an obvious cingulum (Figure 10-4). It appears curved, its extremities reaching the base of the occlusal edge, where each of them will determine a small tubercle (mesial and distal) (Figure 8).

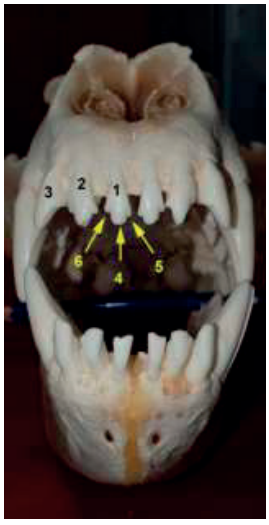

Figure 8. Incisor teeth in dogs (frontal view) (original): 1-central incisor; 2- intermediate incisor; 3-corner incisor; 4-central lobe; 5-mesial lobe (tubercle); 6-distal lobe (tubercle)

The concavity of the cingulum delimits an ample depression which will subdivide a central dental tubercle, widened and slightly relieved, but raised to the occlusal side. The contact sides, wide at the level of the neck of the tooth, rapidly narrow, in order to finish sharply at the extremities of cingulum through the two formerly mentioned tubercles. The cutting edge of the virgin tooth has the contour made up of an arcade with a sharp apex, flanked at the base by the two tubercles which mark the extremities of the cingulum, delimited by the central prominence through two reduced incisures. Thus it can be appreciated that it is trilobed, with a very prominent central lobe on the virgin tooth, contouring the so-called "club" of the tooth. This disposition will suggest the tritubercular type of the premolars and molars.

The crown is three or four times longer than the crown, very compressed from one side to the other, and finishing with an apex which closes rapidly. It is solidly implanted in the alveoli. The neck of the tooth is well marked.

The dentine is thick and fills the dental cavity very early. The enamel covers the crown totally in a thick layer. The cement is very weakly represented, absent at the level of the crown.

Usage affects initially the central lobe of the cutting margin and it shortens this margin until the level of the tubercles which flank it. Thus, the occlusal edge becomes rectilinear and thick, and the club disappears, the process being called levelling of the teeth. In the most advanced stages, the crown, very short, yellowing, separates from the neighbouring ones, reduced to a simple stump.

On each arcade, the size increases from the central incisors to the corner incisors. The difference is more poignant on the superior maxillary. The superior incisors are twice as strong as their inferior counterparts. The cingulum is better evidenced, and the central lobe is stronger and better separated from the other two. The superior corner incisor is easy to recognise: the central lobe is elongated and sharp, which gives it the aspect of a canine tooth. On the other hand, the distal lobe of the corner incisor is missing, leaving room for the inferior canine, which during occlusion comes into contact with it (Figure 9).

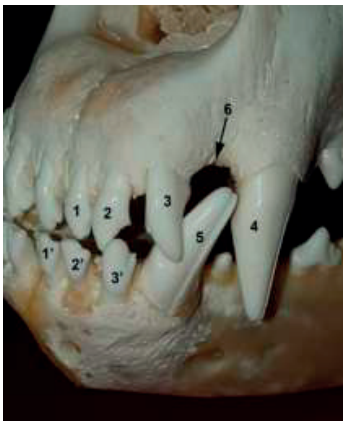

Figure 9. Incisors and canines in dogs (latero-rostral view) (original):
1, 2, 3-superior incisors; 1`, 2`, 3`-inferior incisors; 4- superior canine; 5-inferior canine; 6-diastema

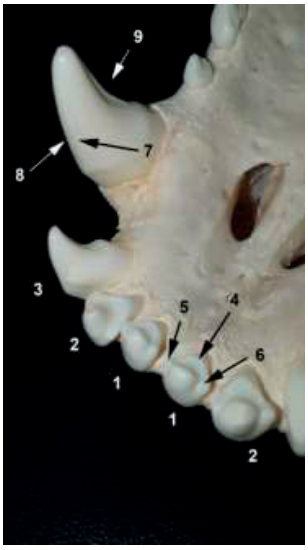

Figure 10. Superior incisors in dogs (ventral view) (original):
1-central incisors; 2-intermediate incisors; 3-corner incisors; 4-cingulum; 5-mesial tubercle; 6-distal tubercle; 7-crest; 8-mesial side of the canine; 9-distal margin of the canine

On each maxillary, the incisor arcade describes a regulated circle arc, wider in the superior side than on the mandible. The superior incisors override the inferior ones, so in the occlusion the central incisor easily masks the inferior one and the adjacent portion of the inferior intermediate incisor. The superior intermediate incisor comes to cover the occlusal margin of the two corner incisors. The superior corner incisor is located between the inferior corner incisor and the inferior canine, and a small diastema separates it from the superior canine. Dolichocephalic breeds conserve this disposition while brachycephalic breeds present an inferior prognatism, which leaves the incisors and the superior canines more or less before their counterparts, raising their efficacy.

Canines are very voluminous teeth, slightly flattened from one side to the other and slightly curved, with a distal concavity. The neck of the tooth is very weakly marked, and the root is twice as long as the crown. The latter has a vestibular side which is convex and smooth, and the lingual side is traversed by a reduced ridge limited on the mesial side by a small crest. The mesial edge is convex, the distal one is concave, and the occlusal extremity is simple and sharp (Figure 10).

The root, which is long, is the part which corresponds to the largest circumference of the section of the tooth. The vestibular side is more convex than the lingual side, which is almost flat. The apex is sharp and closes early on. The root is profoundly implanted on both maxillaries, reaching the plane of the mesial root of the second premolar (Figure 13).

Distinctive characteristics and occlusion

The superior canines are always stronger than the inferior ones. Their root is proportionally longer. Above the neck of the tooth, the superior canines have on the distal edge a prominence (cingulum) which is more obvious than in the inferior canines. During occlusion, the inferior canine is placed before the superior one, occupying a small diastema which separates the inferior canine from the pre-carnassial.

Premolars and molars on each arcade are systematised medio-distally in pre-carnassials, carnassial teeth and tubercled teeth. With the exception of the latter, they are all perfectly secodont teeth. It has been described that the first type is considered a permanent tooth without a precursor – however it should be considered a persistent deciduous tooth.

Upper pre-carnassials are the first three premolars. The first one is small, with a simple, ogival crown whose lingual side presents an obvious cingulum, which contours through its extremity a small distal lobe. The other two rise

in volume, become flattened from one side to the other, sharp and trilobed, with a prominent intermediate lobe, a short medial lobe and slightly detached and an elongated distal lobe (Figure 11). Each has two roots, the distal one twice as strong as the other one. The final pre-carnassial is the largest in size.

The upper carnassial is the final premolar. It is the most powerful tooth on the arcade. It exhibits three lobes whose disposition is highly characteristic. Two are very powerful and represent the body of the tooth. The most prominent one is the mesial one (Figure 11); a sharp crest connects it to the distal lobe, which is more reduced in size. The third (protoconus) is accessory; it is short, sustained by the cingulum on the meso-lingual side of the base of the main lobe. It presents three roots, two main ones and an accessory one, on the lingual side, each in direct continuation of a lobe.

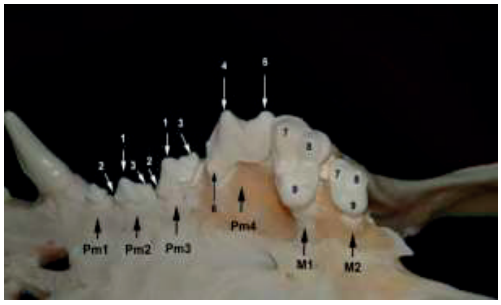

Figure 11. Superior premolars and molars in dog (ventro-medial view) (original):
Pm1-Pm4 – premolars (Pm4 – carnassial); M1-M2 – molars; 1-intermediate lobe; 2-mesial lobe; 3-distal lobe; 4-mesial lobe of the carnassial; 5-distal lobe of the carnassial; 6-accessory lobe of the carnassial 7-mesial cusp 8-distal cusp; 9-cingulum (lingual lobe) whose occlusal side is subdivided in tubercles

The upper tuberculate teeth are the two molars. They are strong, with a short and wide crown, more developed transversally than mesio-distally, strongly mamelonated. Each of them has three roots, two vestibular and one lingual. The first tuberculate tooth is very thick, slightly more reduced than the carnassial. The crown is surrounded by a cingulum which includes the lingual part of the base of two cuspids, of which the mesial one is taller. This cingulum is represented on this side by an enormous rounded and short lobe, whose occlusal side is itself divided into tubercles. The last tuberculate tooth looks like the first one, but aside from the fact that it is three or four times smaller, the two vestibular cusps are less well evidenced and the lingual lobe is not as mamelonated.

The lower pre-carnassials are the four premolars. The first one looks like its upper counterpart, but it is smaller, with a cingulum and a caudal tubercle that are more reduced in (Figure 12). The following pre-carnassials, which rise progressively, are two-rooted. The crown is trilobed, just like with the upper counterparts, but the distal lobe is stronger, slightly more pulled medio-distally. The final lobe is subdivided by a small incisure, more and more obvious from the second to the fourth tooth.

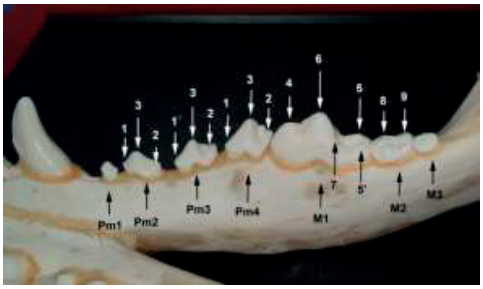

Figure 12. Inferior premolars and molars in dog (dorso-medial view) (original):
Pm1- Pm4 – premolars; M1-M3 – molars; 1-mesial lobe; 2-distal lobe; 3-intermediate lobe; 4-mesial lobe of the carnassial; 5, 5`-tubercles of the caudal lobe (distal or the heel of the carnassial); 6-intermediate lobe of the carnassial; 7-accessory lobe; 8-mesial tubercles of the first tuberculate molar 9-distal tubercle of the first tuberculate molar

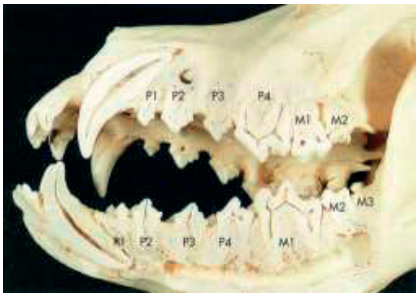

Figure 13. Section through the maxillary and mandibular teeth (lateral left view) (original)

The lower carnassial is the first molar. It is stronger than the upper one. The crown has a reduced cingulum, but it is completely trilobed.

The intermediate lobe, the strongest one, is sharp and serrated, flanked at its base by a small disto-lingual accessory tubercle. The mesial tubercle, though slightly shorter, is just as sharp; it is oriented a bit towards the lingual side; the caudal lobe (of the heel of the carnassial) is short, wide, subdivided in two secondary portions, vestibular and lingual, by a depression destined to hide a relief of the first superior tubercle. It has two roots. Very divergent, they are neatly separated, each with a salient neck.

The inferior tuberculate teeth are the final two molars. They are much smaller than the superior ones. The first one (M II) has a thick crown, slightly larger meso-distally than transversally and mamelonated on the occlusal side, the distal tubercles being the shortest. It has two roots. The final molar (M III) is very small, with a slightly mamelonated crown and a single root.

Dentition in felines: In cats, the dental formula is: I: $\frac{3}{3}$, C: $\frac{1}{1}$, P: $\frac{3}{2}$, M: $\frac{1}{1}$.

There are 30 teeth in adult dentition and 26 deciduous teeth. The reduction of the number of molars and premolars is somehow correlated to the shortening of the splachnocranium, but the conformation and the evolution of diverse types of teeth is comparable to those of canids.

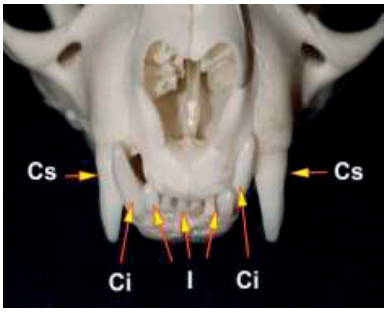

Figure 14. Incisors and canines in cats (frontal view) (original): I-incisors; Ci-inferior canines; Cs-superior canines

Incisors are very small and narrow, especially deciduous ones. They are disposed like in dogs, their size increasing from the central incisor teeth to the corner incisor teeth (Figure 14). The deciduous ones erupt near the age of three weeks and are replaced between 4 and 7 months.

Canine teeth are proportionally longer, thinner and more circular on a section, but especially sharper than in dogs. The cingulum is better marked. The vestibular side has two or three fine longitudinal ridges. The superior ones are larger than the inferior ones, and in occlusion they cover the latter partially, somewhat placed caudally to them. The deciduous ones are very thin. They erupt and are replaced at the same age as the incisors.

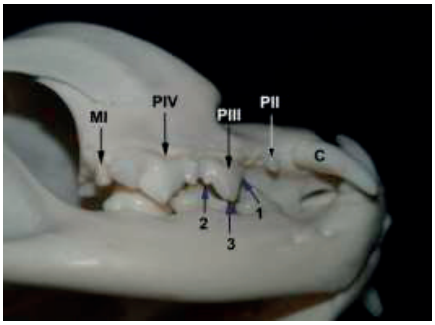

Figure 15. Premolars and molars on the left side of the cat during occlusion (latero-ventral view) (original): C-canine; PII, PIII, PIV-premolars; MI-molar (tuberculate); 1-mesial lobe; 2-distal lobe; 3-intermediate lobe

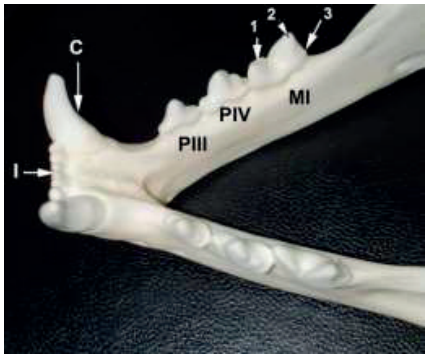

Figure 16. Inferior arcade dentition in cats (dorso-medial view) (original): I-incisors; C-canine; PIII, PIV-premolars; MI-molar; 1-mesial lobe; 2-intermediate lobe; 3-distal lobe

Premolars and molars are represented on the superior arcade by two pre-carnassials and a carnassial, as well as a tuberculate molar (Figure 15). On the lower arcade there are two pre-carnassials and a carnassial. The carnassial is thus the last upper premolar and the only lower molar. It can be noted that the first superior premolar is missing, as well as the first

two inferior premolars and the two molars on each arcade.

The first superior premolar (P II) is very small, similar to P I in dogs. The second one (P III) is clearly better represented, flattened from one side to the other and trilobed. The intermediate lobe is prominent and sharp, the mesial is less contoured, and the distal is more obvious. The carnassial (P IV) is strong and sharp. It could be compared to the one in dogs, had it not had a well contoured mesial apex, which offers it a trilobed profile. In regards to the tuberculate tooth (M I), it is very small, flattened meso-distally and practically has no contact with the inferior carnassial.

On the lower arcade, the first pre-carnassial (P III) is much more voluminous than the superior one and neatly trilobed. The second one (P IV) is just as strong as the second superior one and also trilobed. The carnassial (M I) only has a rudiment of the caudal lobe, appearing bicuspid (Figure 16). In occlusion, the final pre-carnassial and the inferior carnassial are covered laterally by their superior opposites, though they are placed slightly anteriorly.

CONCLUSIONS

The lower incisors in **swine** are easy to identify, almost rectilinear, with an elongated crown, lacing a neck and with the mesial and distal margins long and almost parallel. The corner incisor is short, with an obvious neck and reduced root. The superior central incisors are distinct through the fact that the distal edge practically continues without delimitation from the occlusal side. Both the superior central incisors and the superior intermediate incisors have a reduced infundibulum.

In **dog**, the general aspect of the crown if the incisors and the trilobed aspect of their occlusal side are distinctive characteristics. Even in the case of usage, the mesial and distal lobes disappear relatively late.

The inferior first premolar of **swine**, which can be mistaken for the superior or inferior first premolar of **dogs**, is still differentiated from the latter by the fact that in swine it has two roots (mesial and distal).In **dogs** it only has one root. Although similar to the secodont II and III premolars in dogs, the corresponding premolars in swine have two secondary depressions on the vestibular side (absent in dog). The fourth premolar in pig is tuberculate and cannot be confused for the fourth premolar of dogs.

The inferior molars are voluminous teeth in swine. They cannot be compared to the inferior tuberculate teeth of dogs, firstly because of volume, as well as due to the large number of cusps, both primary and secondary, and the greater number of roots (more than two, or one, as is the case with dogs).

The upper first premolar in pigs has two roots while it has only one in dogs. From a side view, it looks approximately sharp in dogs, while in pigs it has an elongated occlusal margin. In dogs, the three cusps of the 2-4 premolars are aligned, while in swine the distal cusp is located near the lingual side of the other two.

The multitude of cusps and the longitudinal diameter larger than the transversal, in the case of superior molars are the most obvious differences to canids.

The strictly carnivorous regime of felines compared to that of canines translates through the specialisation of dentition to a more accentuated secodont type, the morphologic effect being the accentuated reduction of the tuberculate molars.

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COMPARATIVE STUDY OF THE GESTATION AND LACTATION PERIODS IN EWES, IN TERMS OF VARIATIONS OF THE MAIN METABOLIC PARAMETERS

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Abstract

This research aims to highlight the differences between the metabolic status in gestation and lactation in ewes, translated by a series of variations of the haematological parameters. A number of 25 representative blood parameters was determined in the 90th day of gestation and 40th day of lactation. We observed similarities in these parameters' variations in both periods, but also some discrepancies - more pronounced variations in the lactation period. Regarding the biochemical profile, almost all parameters decreased in lactation; Triglycerides, Cholesterol, Albumin decreased significantly ($p<0.05$). On the contrary, the Urea increased significantly ($p<0.05$). As concerning the enzymatic activity, it was increased in both periods, but more pronounced in lactation; GGT and AST increased significantly ($p<0.05$). The calcium decreased significantly ($p<0.05$) in lactation, while the other electrolytic parameters did not show significant variations. The RBC and Hb decreased non-significantly ($p>0.05$), whereas Ht decreased significantly ($p<0.05$) in lactation. Overall, the parameters maintained the same trend in both periods, but more important variations were observed in the lactation period, indicating that, compared to gestation, lactation represents a more demanding period in this species.

Key words*: gestation, lactation, metabolic profile, ewes*

INTRODUCTION

The physiological status exerts a crucial influence on the respective individual's metabolic necessities. In order to ensure the proper health conditions of the animal, along with the appropriate resources for production and reproduction, it is mandatory to monitor the main physiological changes that occur in the body especially during demanding periods, such as gestation and lactation (Khaled & Illek, 2012).

The most relevant and conclusive method of monitoring the health status in direct correlation with the physiologic stage of the individual is by performing a series of paraclinical investigations. The haematological and blood biochemical paraclinical exams are considered by the specialty literature to be among the most useful tools in monitoring the individuals during important and extremely demanding physiological periods, namely gestation and lactation (Antunovic et al., 2011).

Therefore, during this study, the main objective was to monitor the health status of a group of ewes during gestation and, later, during lactation, using for this purpose the haematology and blood biochemistry paraclinical exams. The fluctuations of the 25 representative blood parameters that were studied in both gestation and lactation periods allows us to correctly assess the physiological processes that occur in these periods, and also to prevent the occurrence of pathological entities, our main objective being ensuring the health and wellbeing of the animals, while preserving their productive capacity at maximum desired levels.

MATERIALS AND METHODS

The present study took place in a flock of Ţurcană mixed-breed ewes. The entire herd was subjected to mandatory prophylactic programs - vaccinations and periodic deworming. For this study, 10 clinically healthy ewes were selected, forming the experimental group. The selection criteria were represented by health, age and number of previous gestations. Thus, the group consisted

of clinically healthy and without history of pathologies, approximately 3 years old, multiparous ewes.

For the gestation period, the blood sampling was performed on December 11th 2017, in the 90th day of gestation. For the lactation period, the blood sampling was performed on March 17th 2018, in the 40th day of lactation. Melet Schloesing MS-45™ haematology analyser was the device used to process the whole blood samples to determine the haematological parameters and Spotchem EZ 4430 ARK RAY analyser was used to process the plasma samples to determine the biochemical parameters.

RESULTS AND DISCUSSIONS

The variations of the main haematological parameters in lactation, compared to gestation, were predominantly not significant from a statistic point of view ($p>0.05$), with only one exception – the haematocrit, that decreased significantly ($p<0.05$) in the lactation period. The mean values of the studied haematological parameters can be observed in Table 1. Also, it is important to mention the fact that all the mean values of the haematological parameters were found within the limits of the reference ranges for this species.

Table 1. The haematological profile parameters' mean values in gestation and lactation for the studied group of ewes

	90th day of gestation	40th day of lactation	Refference range
WBC (m/mm^3)	7.66 ± 0.31	7.21 ± 0.29*	**4-12**
RBC (M/mm^3)	12.18 ± 1.51	10.51 ± 1.46*	**9-15**
Hb (g/dL)	13.40 ± 1.98	11.24 ± 1.03*	**9-15**
Ht (%)	41.0 ± 3.91	32.7 ± 2.84**	**27-45**
MCV (fl)	33.6 ± 2.31	31.7 ± 2.63*	**28-40**
MCH (pg)	10.8 ± 0.65	10.7 ± 0.49*	**8-12**
MCHC (g/dL)	32.7 ± 2.06	34.3 ± 1.99*	**29-40**

*p>0.05 – statistically non-significant differences
**p<0.05 – statistically significant differences

The overview of the haematological parameters in correlation with the physiological status - gestation vs. lactation, reveals the fact that as the lactation sets in, a slightly, statistically non-significant ($p>0.05$) decrease of the number of leukocytes (WBC), the number of red blood cells (RBC) and blood haemoglobin concentration (Hb) can be observed. Similar results were obtained also by many authors in their research regarding the hematologic parameters in this species (Antunovic et al., 2011; Bahkci et al., 2007; Liesegang et al., 2007).

The mean values of mean erythrocyte volume (MCV), mean erythrocyte haemoglobin content (MCH) and mean erythrocyte haemoglobin concentration (MCHC) tend to remain relatively constant throughout the research, with no statistically significant variations ($p>0.05$).

The only haematological parameter that decreased significantly ($p<0.05$) during lactation was the haematocrit (Ht). One hypothesis emitted by several authors for this decrease of main haematological parameters, especially of the haematocrit, is based on the fact that in gestation is physiological to find an increased oxygen requirement and also the intensification of the metabolic rate, therefore all haematological parameters connected to the oxygen transport (RBC, Hb, Ht) tend to be higher in this period compared to lactation, which does not require additional oxygen transportation and is, therefore, considered to be a less physiologically demanding period for the ewe from this perspective.

The hypothetical reason for this variations of the red blood cells number, haemoglobin concentration and, especially, of the haematocrit mean values, being that in gestation the oxygen requirement is higher than in lactation, therefore lactation is considered to be a less demanding physiological period for the mother than gestation in terms of oxygen consumption. The probable cause for the decrease of the white blood cells count mean values in lactation is represented by the migration of the leukocytes from the ewe's blood into milk, this way providing the necessary antibodies for the lamb and, also, natural protection of the mammary gland.

The variations observed in the case of the main blood biochemical parameters in the lactation period, compared to the gestation period, were, as in the case of the main haematological parameters, predominantly not significant from a statistic point of view ($p>0.05$), with only a few exceptions, namely, the calcium, the

cholesterol and the triglycerides, that decreased significantly ($p<0.05$) and the AST and GGT that, on the contrary, increased significantly ($p<0.05$) in the lactation period. The mean values of the studied biochemical blood parameters are synthetically presented in Table 2 and Graph 1. Except for the GGT, that exceeded the upper limit of the reference range in the lactation period, all the mean values of the biochemical parameters were found within the limits of the reference ranges for this species.

Table 2. The biochemical profile parameters' mean values in gestation and lactation for the studied group of ewes

	90th day of gestation	40th day of lactation	Refference range
Ca (mg/dL)	9.41 ± 2.68*	8.75 ± 2.11**	**9.3-11.7**
P (mg/dL)	4.28 ± 0.61*	5.78 ± 0.44*	**4-7.3**
K (mmol/L)	5.83 ± 0.40*	5.97 ± 0.48*	**4.3-6.3**
Na (mmol/L)	144.01±1.88*	145.82±2.04*	**142-160**
Cl (mmol/L)	112.05±1.27*	112.70±1.93*	**101-113**
ALT (U/L)	19.70 ± 3.84*	16.80 ± 3.44*	**8-57**
AST (U/L)	14.66 ± 3.32*	18.20±3.62**	**9-49**
CK (U/L)	85.80 ± 6.71*	87.10 ± 7.11*	**7.7-101**
GGT (U/L)	8.60 ± 2.19*	11.88±1.38**	**1-10**
LDH (U/L)	387.1±46.84*	397.6±41.62*	**83-476**
Glu (mg/dL)	87.4 ± 12.11*	82.2 ± 11.02*	**65-118**
Urea (mg/dL)	19.90 ± 2.59*	24.20 ± 4.01*	**10-28**
Cholesterol (mg/dL)	72.20 ± 9.91*	50.33±7.78**	**44-90**
Triglycerides (mg/dL)	15 ± 2.68**	11 ± 2.04**	-
Creatinine (mg/dL)	0.87 ± 0.07*	0.76 ± 0.02*	**0.5-1.6**
Total proteins (g/dL)	7.10 ± 0.75*	6.79 ± 0.56*	**5.4-7.4**
Albumin (g/dL)	3.69 ± 0.51*	3.28 ± 0.31*	**2.7-3.7**
Total bilirubin (mg/dL)	0.27 ± 0.06*	0.32 ± 0.07*	**0.1-0.6**

*p>0.05 – statistically non-significant differences
**p<0.05 – statistically significant differences

A statistically significant decrease ($p<0.05$) of the calcium concentration compared to the mean values obtained in pregnant ewes was observed in ewes in the 40th day of lactation. The inorganic phosphorus mean concentration shows in the lactation period a statistically non-significant increase ($p>0.05$) compared to the mean values obtained in gestation. Some authors stated that this decrease of the calcium mean concentration in ewes' blood at the beginning of the lactation period may be associated with increased calcium secretion through milk and its rearrangement in bones (Liesegang et al., 2007). Similar results in terms of calcium and inorganic phosphorus concentrations variation during lactation were often mentioned in the speciality literature (Bahkci et al., 2007). Furthermore, phosphorus and calcium are mobilized from bones using similar physiological pathways, but a much more significant amount of calcium is secreted through milk, phosphorus being also secreted, but in a much smaller amounts than calcium and, consequently, its concentration in the ewe's blood is expected to be much higher than that of calcium. In lactating ewes, were recorded higher potassium concentrations compared to pregnant ewes, the increase of the average values being, although, statistically not significant ($p>0.05$). Physiologically, maintaining the level of blood potassium as constant as possible is necessary in order to maintain homeostasis since substantial fluctuations of the electrolytes' values, especially of potassium, can lead to serious structural and functional imbalances of the muscles and the heart, aspects frequently mentioned in the speciality literature (Codreanu M.D., 2016; Călin et al., 2020; Ognean et al., 2016).

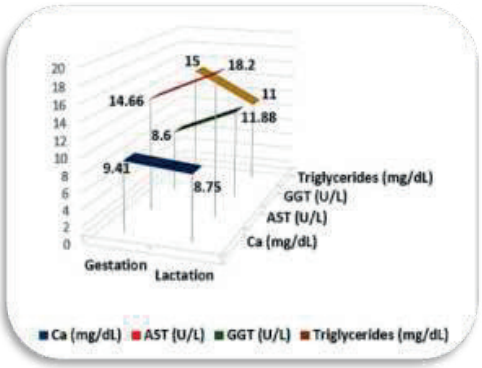

Figure 1. The dynamics of the Ca, AST, GGT and Triglycerides mean values in gestation and lactation for the studied group of ewes

The sodium and chloride concentrations mean values did not vary significantly ($p>0.05$), maintaining approximately similar mean values during the study.

Significantly increased concentrations of AST and GGT ($p<0.05$) were observed in lactating ewes. An opposite, decreasing trend was observed in the case of ALT concentration but without being statistically significant ($p>0.05$). The increased hepatic transaminases' activity,

namely, AST and GGT in lactating ewes indicates an increase of the hepatic metabolism, that can also be associated with high productivity. The results obtained in the present study are consistent with those reported in the past in the speciality literature (Antunovic et al., 2011).

The CK activity did not show statistically significant variations ($p>0.05$), although a moderate increase could be observed during lactation. Also, a more intense enzymatic activity of the LDH could be observed in lactating ewes compared to pregnant ewes, but with statistically non-significant differences ($p>0.05$). The results are consistent with the general trend of increased enzymatic activity observed in this physiological stage.

Mean plasma glucose values decreased, but non-significant ($p>0.05$) during lactation. This decrease of the mean glucose values at the beginning of the lactation period can be considered the result of a loss of energy through milk. More specifically, it suggests the increased use of glucose for the synthesis of milk lactose. The results can be also associated with an increased milk production and an intense activity of the mammary gland (Tygesen et al., 2008; Codreanu & Călin, 2018).

Elevated urea levels in lactating ewes may be the result of muscle protein catabolism when large amounts of body reserves are mobilized (Anwar, 2012). The speciality literature mentions the fact that this aspect is, most likely, directly correlated with the body status score and the body weight of the ewe, individuals with lower body status score presenting higher concentrations of urea in the blood (Khaled, 2012). Other authors have obtained similar results in their research regarding the variations of the blood biochemical profile in sheep and goats (Crivineanu et al., 2010; Bociu et al., 2015). The negative energy balance leads also to the mobilization of the body reserves and proteins catabolism, translated into increased blood urea levels.

The decrease of the total protein and albumin mean values is statistically non-significant ($p>0.05$) and can be explained by the massive mobilization of plasma immunoglobulins and their transfer to milk (Cotor et al., 2011).

The mean values of the triglycerides and cholesterol are statistically significant ($p<0.05$) lower in lactating ewes, being in total accordance with the increased energy requirement and the negative energy balance of this physiological status. Other several authors reached similar conclusions (Antunovic et al., 2011; Anwar et al., 2012).

Analysing the data, we could also observe a statistically non-significant increase ($p>0.05$) of the mean concentration of total plasma bilirubin in lactating ewes. This increase may be corelated with an increased hepatic metabolism and is correlated with the results obtained in the determination of the main hepatic transaminases - GGT and AST.

The hypothesis of an increased hepatic activity was emitted by several authors, being considered a necessary condition for sustaining the milk production at normal levels and is also strengthened by the increase of the mean values of the total bilirubin and LDH, but without statistical significance ($p>0.05$).

Regarding the average plasma creatinine concentrations, no statistically significant variations ($p>0.05$) in correlation with the physiological status were observed.

CONCLUSIONS

Regarding the influence of the physiological status - gestation, respectively, lactation - on the main parameters of the haematological profile in the studied group of pregnant/ lactating ewes, the following conclusions can be mentioned:

- the variations recorded in the case of the studied haematological parameters were almost exclusively not significant from a statistical point of view ($p>0.05$) in the two physiologic periods;
- all the mean average values of the hematologic parameters were found within the reference intervals for this species;
- in lactation, the most important parameters, namely, WBC, RBC, Hb and Ht decreased, the one that decreased significantly ($p<0.05$) being the haematocrit.

As concerning the influence of the physiological status - gestation and lactation, respectively, - on the main parameters of the biochemical profile in the studied group of

pregnant/lactating ewes, the following conclusions can be mentioned:

- during the study, the values of the main biochemical parameters were generally found withing the reference range for this species, with only one exception - the GGT that slightly exceeded the upper limit of the reference interval during lactation;
- the variations of the main blood biochemical parameters were predominantly not significant from a statistic point of view ($p>0.05$), with a few exceptions, namely, the calcium, the cholesterol and the triglycerides, that decreased significantly ($p<0.05$) and the AST and GGT that increased significantly ($p<0.05$) in the lactation period;
- the only electrolyte in which case a significant variation was recorded was the calcium, that decreased significantly ($p<0.05$) in lactation, this result being expected and most likely associated with the increased milk synthesis and the excretion of calcium through milk;
- the increased energy requirements during the lactation period was translated in a significant decrease ($p<0.05$) of the triglycerides and cholesterol mean values and a not significant ($p>0.05$) decrease of the glucose mean value;
- an increased blood enzymatic activity was observed in lactation, the hepatic transaminases GGT and AST increased significantly ($p<0.05$) indicating the intensification of the hepatic metabolism in this period.

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EFFECT OF LONG-TERM COLLECTION FREQUENCY ON THE SEMEN TRAITS IN PLYMOUTH ROCK ROOSTERS

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Abstract

In this study, the effect of ejaculate sampling frequency on the morphological properties of sperm in Plymouth Rock roosters was determined. Four experimental groups were tested: of five, four, three, and two weekly ejaculates. The age of the roosters at the beginning of the experiment was 28 weeks and the duration of the experiment was 30 weeks. Increasing the frequency of semen sampling from one weekly sampling to five samplings per week had the following effects: 1. increase in ejaculate volume, but decrease in sperm density; 2. no significant changes in sperm motility; 3. increase of spermatozoa viability and 3. significant influences on the percentage of anomalies but no significant influences on the types of anomalies (the most common being the sperm head abnormalities).

Key words*: semen feature, spermatozoa abnormality, rooster.*

INTRODUCTION

Sperm morphology and morphometry were considered important techniques in the evaluation of sperm, on the occasion of the development of artificial insemination techniques and technologies. However, sperm traits and morphometry data were reported for only a few bird species, most of which were recorded for roosters, ducks, turkeys and quails. Recently, some studies have focused on wildlife (Santiago‐Moreno et al., 2016).

The intention of breeders is to use as much as possible the breeding male parents or grandparents of broiler or laying hens. A huge chapter of poultry farming has opened and developed with the emergence and expansion of artificial insemination (Bunaciu et al., 1978; Bunaciu et al., 1992). Their use has not been without effect on the physiology of the males from which the semen is collected as well as on the composition of the sperm, although the advantages of using artificial insemination in birds are unquestionable. Here are some of them: 1. reducing the number of males; 2. the possibility of improving the selection and breeding works and 3. There are indisputable economic advantages resulting from the reduction of the number of males and from the increase of the biological value of the poultry material as a result of the facilitation of the selection and breeding works (Bunaciu et al., 2009). As much as the rooster was to be protected for use in artificial insemination, the method of harvesting and the frequency of harvesting altered the processes of spermatogenesis and subsequently the biological composition and properties of sperm (Noirlaut and Brillard, 1999; Riaz et al., 2005; Racha et al., 2015).

The aim of this study was to determine the effects of different sperm sampling (ejaculates) frequencies on ejaculate volume, motility, density, viability and abnormality of spermatozoa, in roosters.

MATERIALS AND METHODS

The biological material was represented by Plymouth Rock roosters aged 28 weeks and reared in an extensive system, in individual cages 60 x 60 x 80 cm, housed in a room with a temperature of 20-28°C. The birds had free

access to forage and water and were fed on a standard commercial diet (main ingredients by %: wheat 41.9, barley 31.1, oats 11.8, soybean meal 5.5, grass meal 2.5, fish meal 5.5, dicalcium phosphate 0.5, limestone 0.8, vitamin and mineral premix 0.5). The light program was 15 hours light and 8 hours dark. Semen was collected using the method described by Bunaciu et al. (2009). The experiment was organized in 4 experimental (five roosters each) variants depending on the regime of use of breeding roosters and lasted 30 weeks, according to the following scheme:

Variant	Days between ejaculates	Day of the week						
		Mo	Tu	We	Th	Fr	St	Sa
V1	2	E	E	E	E	E	No	No
V2	3	E	E	No	E	E	No	No
V3	4	E	E	No	E	No	No	No
V4	5	E	No	No	E	No	No	No

Legend: Mo-Monday; Tu - Tuesday; We - Wednesday; Th - Thursday; Friday; ST -Saturday; Sa – Sunday; E - ejaculate; No - break day

The ejaculates were collected in transparent glass graduated collection tubes; volumes were recorded directly in the tube immediately after collection at the lower margin of the semen meniscus and are expressed in µL. Sperm motility was assessed by a wet preparation technique using a Nihon Kohden optical microscope (Sapaco 2000, Bucharest, Romania) on a warmed plate. Motility was estimated by direct observation of spermatozoa in at least five fields, using 400 × magnification and a lowered condenser to disperse the light. Motility is expressed here as the percentage of all spermatozoa showing progressive movements. Nonprogressive spermatozoa with other patterns of movement were not considered in this category. Sperm count was determined using a hemocytometer with a Nihon Kohden optical microscope. For this assessment, fresh semen samples were diluted (1:200) and fixed using neutral Hancock's solution, (62.5 mL of 37% formaldehyde, 150 mL of 1% saline, 150 mL of sodium phosphate buffer, and 500 mL of double-distilled water) and a Potain pipette. The results are expressed as the number of spermatozoa per mL. Viability of the spermatozoa was evaluated by eosin-nigrosin staining (Merck, Darmstadt, Germany) according to Kondracki et al. (2017). The results are expressed as the percentage of all spermatozoa classed as viables.

The data obtained were centralized using the Excel 2010 program and the statistical processing was performed using the GraphPad program for Windows, version 8.0.2, GraphPad Software, Inc. The correlations of the frequency of ejaculates and the sperm features were analysed by Pearson correlation coefficient. The significance between the groups was analysed by ANOVA and the the differences were considered significant for values of $P \leq 0.05$.

RESULTS AND DISCUSSIONS

The volume of ejaculate ranged between the limit values of 0.32 (V1) and 0.25 mL (V4, which represents a percentage decrease of 28%), the highest value being recorded for the group of roosters with five ejaculates per week and the lowest in the group of roosters with two ejaculates per week. The differences between the groups are significant and the decrease in ejaculate volume is strongly correlated with the number of ejaculations ($r = +0.96$). It can be seen that a higher frequency of sperm collection stimulates the increase of sperm volume, probably by increasing testicular vasodilatation and extravasation of a higher volume of water in the seminiferous tubules. The analysis of the following sperm parameters will allow the identification of the effects upon the spermatogenesis and sperm properties.

Sperm density showed an upward evolution, increasing from 1.22×10^9 (in V1) to 1.68×10^9 sperm per mL of sperm (in V4), which represents a percentage increase of 37.7%). The differences between the groups were significant ($P = 0.030$). The increase was not linear, the variant of group V3 presenting a lower value than the adjacent groups. Sperm density evolved inversely with the number of ejaculates. However, it correlates with sperm volume, the increase in ejaculate volume being based on the increase in seminal plasma volume, not on the intensification of the spermatogenesis process. This sperm parameter correlated weakly and negatively with the number of ejaculates: $r = -0.122$.

The percentage of motile sperm does not seem to be influenced by the number of weekly ejaculations: the values ranged between 55.5 (in

V4) and 57.0% (P = 0.244). Obviously this sperm parameter did not correlate with ejaculate frequency: r = 0.082.

Sperm viability was a parameter significantly altered by ejaculation frequency: P = 0.053. The values ranged from 90.52% for variant V1 with five ejaculations per week to 88.8% for variant V4 with two ejaculations per week. This parameter of Plymouth Rock rooster sperm was negatively correlated with ejaculation frequency: r = 0.076.

The percentage of abnormal sperm decreased from 99.9% to 84.4% in the four experimental variants, the differences between the variants proving to be significant: P = 0.032. This decrease was also correlated with the number of weekly ejaculations: r = +0.767.

The results obtained by us on the mentioned sampling variants are generally superposable with those reported by Schramm (2005) on Plymouth Rock roosters in two different experimental variants of frequency of sperm collection. The author shows the differences in sperm quality from roosters with three, respectively, five weekly sperm harvests. Thus, the volume of ejaculate increased as the frequency of sampling increased while the density of sperm decreased accordingly, which reveals an intensification of sperm fluid secretions (as long as they exist in birds). Sperm motility and viability were found significantly altered while the percentage of sperm with abnormalities increased significantly due to an intensification of the spermatogenesis process. Riaz et al. (2004) determined the characteristics of sperm including motility, volume, concentration and number of sperm per ejaculate in Hubart roosters subjected to a regimen of increased sperm sampling frequency. Sperm motility was not affected by the collection interval, but sperm volume was smaller. Sperm concentration also decreased significantly with increasing sampling frequency. Mkpughe and Bratte (2015) determined the effect of breed and sperm sampling frequency on indigenous Nigerian roosters: sperm concentration and sperm count per ejaculate were the only attributes of sperm affected by ejaculation frequency and increased significantly (P <0.05) with increasing frequency of ejaculation, which is not in agreement with our results but can be interpreted as a stimulation of the spermatogenesis process.

The effects on sperm morphology in our study were analyzed at the end of the experimental period, respectively in the last week of sperm collection. The results of this study are summarized in Table 1. Among the types of sperm abnormalities were: twisted sperm, localized abnormality in the head, followed by bent sperm, cytoplasmic drop sperm (immature sperm, Figure 1), and broken sperm (without acrosome and tail). Regarding the percentage of sperm with abnormalities, the analysis of the data presented in Table 2 shows that the percentage of abnormal sperm (calculated on the total sperm examined) varied between a minimum of 13.12% in V2 and 14.12% in V3. For the other variants, the values were very close: 13.66% in V1 and 13.84% in V4, respectively). Statistical analysis of differences between groups showed no significant values (P<0.05). Head abnormalities were most common.

Table 1. Comparative presentation of the sperm traits from roosters subjects of different weekly ejaculates

Item	UM	Number of ejaculates per week				SD	P
		V1 (five ejaculates)	V2 (four ejaculates)	V3 (three ejaculates)	V4 (two ejaculates)		
Ejaculate volume	mL	0.32^{b}± 0.01	0.32± 0.03	0.28± 0.07	0.25^{b}± 0.07	0.04	0.044
Spermatozoa density	$x10^{9}$ /mL	1.22^{c}± 0.34	1.55± 0.23	1.20± 0.21	1.68^{c}± 0.54	0.05	0.030
Spermatozoa motility	%	57.0± 4.5	55.9± 3.0	55.2± 3.2	55.5± 4.0	2.27	0.244
Spermatozoa viability	%	90 .52± 5.89	90.9± 4.87	89.0± 5.08	88.8± 3.09	4.21	0.053
Normal spermatozoa	%	88.9^{a}± 11.0	88.7± 9.09	86.0± 13.3	84.4^{a}± 14.0	6.46	0.032

The values are mean ± standard error of mean.
Each value represent the mean of 4-5 ejaculated samples
Values with the same superscript on the same row differ significantly
SD = standard deviation
P calculated by Tukey test

An important study on the effect of sperm harvesting frequency on roosters on sperm morphology was undertaken by Noirault and Brillard (1999) in turkeys (study lasting 10 weeks, from 30 to 40 weeks).

Table 2. The effect of ejaculate frequency on the type and frequency of spermatozoa abnormalities in Plymouth Rock roosters (percentage of sperm with abnormalities in the total sperm examined)

Type of anomaly	V1	V2	V3	V4	Total (%)
1- cytoplasmic drop (immature)	1.44	1.14	1.24	1.18	5.00
2- boselates (acrosome anomaly, head and intermediate piece)	1.06	1.11	1.05	1.18	5.40
3- bosses (anomaly in the head and the intermediate piece)	1.07	1.1	1.06	1.01	5.24
4- twist, head anomaly	1.65	1.74	1.76	1.79	7.94
5- whip, anomaly at the intermediate piece	1.17	1.33	1.11	1.13	5.74
6- club (anomaly at the acrosome, head and intermediate piece	1.05	1.03	1.11	1.09	5.28
7- rosette (acrosome abnormality, head, intermediate piece, and tail)	1.01	1.03	1.05	1.04	5.13
8- long (anomaly in the head and the intermediate piece)	1.03	1.01	1.01	1.01	5.06
9- bend (anom. at the head)	1.4	1.38	1.61	1.58	6.97
10- broken	1.01	1.01	1.01	1.01	5.04
11- vacuolised	1.03	1.01	1.01	1.01	5.06
Percentage of total types of abnormalities	13.66	13.16	14.12	13.84	

Note: Values are presented as mean

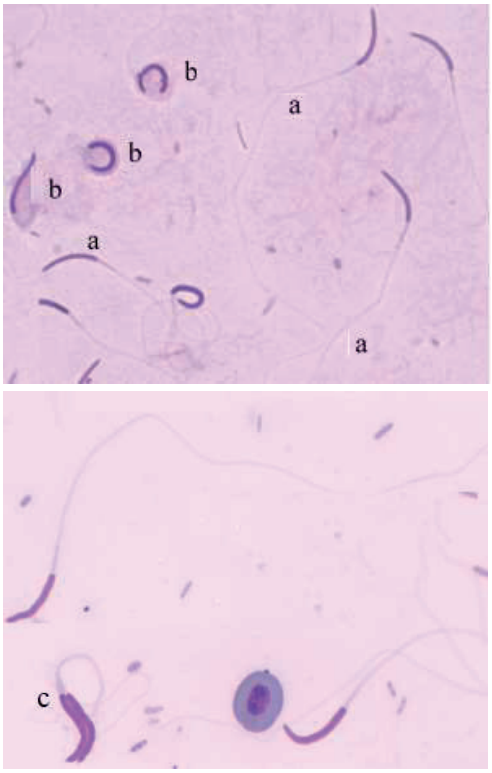

Figure 1. Spermatozoa abnormalities in rooster: a- normal; b- protoplamsmatic dropplets; c- double headed

The turkeys were distributed in groups from which semen was collected once every two weeks, weekly, twice a week, three times a week, five, six and seven times a week (respectively).

Apparently paradoxically, the authors conclude that turkeys produce better quality sperm, parametrically speaking, when the harvesting frequency is higher than when the harvesting frequency is lower. The results obtained by us on Plymouth Rock roosters are thus consistent with those reported by these authors in turkey. Malecki et al. (1998) investigated the effects of sperm sampling frequency on emu sperm morphology: emu sperm is characterized by a high sperm denial. However, the increase in ejaculation frequency did not affect their morphology. A study on the impact of harvest frequency on sperm traits was conducted by Rakha et al. (2015) on Red Jungle hen (*Gallus gallus murghi*), an endangered species native to South Asia: the number of live sperm cells was higher ($P>0.05$) in the case of short-range harvests (12 hours), compared to 24, 48 and 72. The research carried out by Bunaciu et al. (1978) on three turkey breeds: White large type, White small type and Bronze breed showed that the percentage of dead and abnormal sperm can increase as the males get older, respectively in the breeding season. Marques and Ogasawara (1974) in a study on turkey semen showed that in this species, the semen, yellow in color, contains a large number of abnormal sperm compared to roosters, in correlation with a lower ability to fertilize semen. Our research reveals differences in rooster sperm abnormalities compared to other species as well as the weak influence of ejaculations on this aspect of sperm. Values of the evolution of the normal percentage of sperm in the ejaculate were

revealed by Siudzinska and Lukaszewicz (2008) in a study performed on several cock breeds of different sizes. Three of these breeds did not show significant differences in the percentage of normal spermatozoa in the sperm (values between 70.5 and 69.1, which is in agreement with the results of our research). Only one breed, the Italian Partridge, which is a light-sized breed, had a much lower percentage, at 54.0%.

CONCLUSIONS

Our study on Plymouth rock roosters subjected to a sperm sampling regimen 5 to 2 times a week revealed changes in the type and frequency of different types of abnormalities. Increasing the frequency of ejaculation to five per week does not negatively affect the mor- phological properties of sperm, which can be taken into account in determining the use of these roosters.

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THE DEVELOPMENT OF DAIRY FARM LEVEL MULTI-ACTOR TEAMS TARGETING REDUCED ANTIBIOTIC USE IN ROMANIA

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Abstract

The Multi-actor Farm Health Teams (MAFHT) developed for dairy farms are teams that include farmers, veterinarians, and advisors covering complementary areas of interest in the field. The main objective of MAFHT is the design of the Multi-Actor Farm Health Plan (MAFHP) of actions by using the DISARM model. This model uses a participatory, farmer-led approach that was used previously in Denmark and the UK. This paper aimed to describe the particularities of MAFHP's in five Romanian dairy farms designed to improve animal health and to reduce the need for antibiotic treatment. Farmers usually face management and/or health problems in correlation with the age category and physiological condition. The most common calf diseases were respiratory and enteric, but these problems did not create severe outbreaks of disease. Large dairy farms are more common with hoof disorders while small dairy farms are more exposed to udder diseases. All teams demonstrated their ability to identify farm practices to reduce bacterial disease and the need to use antibiotics.

Key words*: antibiotic resistance, livestock farming, best practices, innovation, precision farming.*

INTRODUCTION

In the context of reducing antimicrobial use (AMU) in food animal production, strategic objectives to optimise the use of antimicrobial medicines in human and animal health were developed in the global action plan of the World Health Organization (WHO, 2021), Also, the European Union (EU) approved new measures to fight antimicrobial resistance (AMR), mainly regulations on veterinary medicines and medicated feed (EU, 2019a; 2019b). These regulations provide an important foundation for the preservation of the antimicrobial's efficacy. To date, several EU Member States have been implemented a broad series of changes with substantial progress on AMR and AMU (More, 2020).

Moreover, AMR and AMU must not be the sole responsibility of the medical staff, and they should hold all actors directly or indirectly responsible for human and animal health. To increase the awareness and involvement of all actors involved in animal husbandry, several innovative approaches have been described. These innovative approaches are farmer-led (Morgans et al., 2021), based on the Danish stable schools (Vaars et al., 2007; Bennedsgaard et al., 2010), and/or focused on developing multi-actor groups of farmers and other stakeholders (see https://disarmproject.eu/what-we-do/farm-health-teams/).

In the participatory, farmer-led approach to changing practices around antimicrobial use, Morgans et al. (2021) promoted a novel

application in the context of reducing AMU on UK dairy farms by prioritizing and promoting farmer expertise in identifying and solving farm-specific challenges (Morgans et al., 2021).

The innovative "Danish stable" schools promote the development of the individual farmer and dairy production in a 1-yr process of study with a small group of farmers by using farmers' motivation and experience-based learning process: one farmer and farm are analysed and advised by the farmers' group in a cyclical process in which all farmers take both roles. Participating farmers must have a common goal, let others get information about their farm, create an agenda to direct as a host farmer, and be equal in the sense that all the experiences and opinions of one farmer are accepted by the other farmers. In this type of schools, Vaars et al. (2007) and Bennedsgaard et al. (2010) proved the ability of farmers to reduce the use of antimicrobials in their herds without negative effects on production and herd health or in phasing out antibiotics from organic dairy herds (Vaars et al., 2007; Bennedsgaard et al., 2010)

The interest in finding the best innovative solutions to reduce the need for antibiotics in animals was also manifested in Romania, where the involvement of farmers, veterinarians, and feed advisors in Disseminating Innovative Solutions for Antibiotic Resistance Management (DISARM) thematic network was remarkable (see: https://disarmproject.eu/).

In this context, the paper presents an analysis of the evolution of the Multi-actor Farm Health Teams (MAFHT) developed for dairy farms in Romania, and the design of the Multi-Actor Farm Health Plan (MAFHP) of actions by using the DISARM model. The paper highlights the particularities of MAFHP's in five Romanian dairy farms designed to improve animal health and to reduce the need for antibiotic treatment, activities carried out within the DSARM project.

MATERIALS AND METHODS

In order to characterize the evolution of the Multi-actor Farm Health Teams (MAFHT) developed for dairy farms in Romania, five Romanian dairy farms were chosen. In the selection of dairy farms, we were focused to identify heterogeneous herds sizes with different operating technologies.

Dairy Farms

Dairy Farm 1 (DF1) has been 27 adult cows, 1 calf, 6 heifers, and 15 calves. The key person in the management of the dairy farm is a young farm health manager and veterinary doctor with 11 years of experience in dairy farming. He is directly responsible for the health of the herd and manages all activities of surveillance and control of animal diseases. Also, he is responsible for implementing the best measures to manage the herd health.

Dairy Farm 2 (DF2) has been 1006 adult cows, 349 heifers, and over 750 calves. The key person in the management of the dairy farm has 40 years of experience in the field of dairy farming. The farm has its staff, including veterinarians and zootechnical engineers, but for some specific disease problems, the owner also hires external consultants (e.g., veterinarians and feed advisors).

Dairy Farm 3 (DF3) has been 678 adult cows, 125 heifers, and over 200 calves. The key person in the management of the dairy farm is a zootechnical engineer responsible for raising and animals' welfare. The farm has its staff, including veterinarians and zootechnical engineers, but for specific veterinary medical activities, the owner also hires external consultants (e.g., veterinarians and feed advisors).

Dairy Farm 4 (DF4) has been 720 adult cows, over 150 heifers, and over 600 calves. The key person in the management of the dairy farm is an entrepreneur with great experience in agriculture which developed in just 14 years one of the most modern dairy farms in the region. The dairy farm has its veterinarians and zootechnical engineers and sometimes requests advice on nutritional issues from international specialists. In this farm, the management of the herd health and breeding is done through veterinarians and zootechnical engineers under the direct coordination of the owner.

Dairy Farm 5 (DF5) has been 24 adult cows, 10 heifers, and 6 calves. The key person in the management of the dairy farm is a young

entrepreneur and veterinarian who began to develop a dairy farm with his family.

BioCheck scoring tool

All dairy farms were scored before the design of MAFHPs by using the biosecurity scoring system Biocheck.UGent™ to quantify biosecurity in cattle production. The scoring system consists of one questionnaire that contains 124 questions. The system provides various biosecurity scores and allows for benchmarking of farms and herd-specific advice for improvements (Damiaans et al., 2020).

MAFHT

The Multi-Actor Farm Health Teams brings together the owner or manager of the dairy farm and his veterinarian, who are supported by other health and feed specialists. Each team from the five dairy farms included in this analysis has a facilitator who organizes the team's activity, supports the team in drafting the action plan, and identifies specialists who can provide information specific to the health of dairy farms.

MAFHT is in charge to diagnose the dairy farms' main points of improvement that will increase the herd health status that can result in a reduced need for antibiotics and a lower potential for antibiotic resistance on the farm.

MAFHP

The Multi-Actor Farm Health action plan is based on the Planning-Do-Check-Adjust (PDCA) cycle. MAFHP is focused on (1) listing and determining goals and points of improvement, (2) SMART (Specific/ Measurable/Acceptable/Realistic/Time-specific) definition of each goal, (3) detailed description of the action plan to achieve each goal (each action of the plan is described, has a project owner and a project implementer, and clear deadlines for starting, running, and completing), (4) Monitoring execution of actions for each goal, and (5) adjusting action plan and/or goals.

RESULTS AND DISCUSSIONS

The analysis of farmer's profiles revealed that three farmers have long experience in raising dairy cows, one dairy farm health manager has over 11 years of experience and one dairy farm owner has only one year of experience. Farmers with experience in this sector of activity and who have large dairy farms, promote the concept of teamwork, and have veterinarians, zootechnical engineers, and agronomists employed within the company.

All farms expressed interest and responded positively in developing their productive performance by increasing or maintaining the health of dairy cows and reducing the need to use antibiotics. Moreover, large dairy farms had already implemented programs to monitor the use of antibiotics in lactating dairy cows. Testing of antibiotic residues in milk was already implemented as a common practice and part of the commercial relations with milk processing factories. One of the dairy farms has shown interest in developing the veal sector.

To improve the health of dairy cows, the MAFHTs of all five farms propose the introduction of measures to reduce bacterial infections and antibiotic use on dairy farms. To reduce the consumption of antibiotics, the dairy farms will identify risk factors of the bacterial disease's emergence that require the excessive use of antibiotics as the only solution for healing and animal welfare.

MAFHP of DF1

The BioCheck scoring tool revealed values around 50% in the subcategory "Purchase and reproduction" (47%) of the external biosecurity and the subcategories "Calving management" (43%) and "Dairy management" (53%) of the internal biosecurity (Figure 1). In the light of the biosecurity scoring, MAFHT provided advice on the management of cattle breeding and health on the dairy farm to avoid the unjustified use of antibiotics. Also, in this farm was identified the opportunity to organise a "Stable school" by using Vaarst et al. (2007) model.

The MAFHP of DF1 has the following goals: (1) Identification of factors that may promote the occurrence and spread of bacterial diseases; (2) Identification of solutions for optimizing animal husbandry management; (3) Identification of solutions for optimizing animal health management.

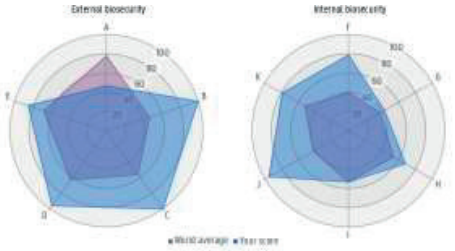

Figure 1. Subcategories scores of the internal and external biosecurity obtained by using the biosecurity scoring system Biocheck.UGent™ in DF1 (A. Purchase and reproduction; B. Transport and carcass removal; C. Feed and water; D. Visitors and farmworkers; E. Vermin control and other animals; F. Health management; G. Calving management; H. Calf management; I. Dairy management; J. Adult cattle management; K. Working organisation and equipment)

Identification of factors that may promote the occurrence and spread of bacterial diseases included four actions:

1. *Determination of possible non-compliance with biosecurity measures when people and vehicles access the dairy farm*. In addition to the biosecurity practices already implemented in the farm, the farm's biosecurity plan has been strengthened to minimize the risk of transmitting infectious diseases to employees and visitors. During the period monitored by MAFHT, the movement of visitors and staff employed on the dairy farm was substantially reduced due to restrictions on the movement of persons during the COVID-19 pandemic. The COVID-19 epidemiological context has significantly increased the responsibility of its staff and visitors to comply with existing biosecurity measures.
2. *Milking monitoring*. During the monitoring period it was found that the personnel involved in this activity comply with the hygienic and technological stages of milking. Also, the milking stages carried out by the staff involved in this activity were completed with steps to reduce the risk of transmitting infectious diseases to employees and visitors.
3. *Colostrum quality assessment*. Possible introduction of a colostrum quality analysis method using a refractometer.
4. *Establishing the role played by the respiratory complex of calves, enterocolitis in calves, pododermatitis, and mastitis in herd pathology*. This action should be adjusted to correlate bacterial infectious diseases with diseases of nutrition and metabolism.

Identification of solutions for optimizing animal husbandry management included three actions:

1. *Feed management optimization solutions*. Continuous evaluation of the feed management.
2. *Solutions for optimizing the management of shelter hygiene*. Continuous evaluation of the shelter hygiene management.
3. *Milking management optimization solutions*. Continuous evaluation of the milking management.

The farm health team has established that the activities of this objective must focus on obtaining information that answers the following questions:

- To what extent can some identified technological deficiencies be factors favouring bacterial diseases?
- How often can these deficiencies be present?
- Which people should be held accountable for remedying or preventing the occurrence of these deficiencies?
- Is it necessary to set a deadline for meeting these objectives or should they be applied continuously?
- What is the best way to monitor deficiencies in animal husbandry management?
- What target value is realistic and achievable and in what time frame?

Identification of solutions for optimizing animal health management included four actions:

1. *Solutions for optimizing the health management of lactating cows*. Continuous evaluation of the health management of lactating cows. To improve the health of dairy cows on the farm, the farm's health team proposes the introduction of measures to reduce the number of cows with milk fever and reduce the risk of injury that requires treatment with antibiotics. Milk fever management will involve investigating farm risk factors through stable analysis. Based on the number of cows with this manifestation, it will be decided at a later stage, if and when recommended to apply preventive

measures. To reduce the risk of accidents that require treatment with antibiotics, it will be necessary to identify the stable factors that favour the occurrence of these events.

2. *Solutions for optimizing the health management of cows during the dry period*. Continuous evaluation of the health management of cows during the dry period.
3. *Solutions for optimizing the health management of cows during the transition period (antepartum and postpartum)*. Continuous evaluation of the health management of cows during the transition period. The transition period can negatively influence the subsequent lactation, with implications on production and reproduction performance. The quality of the health management during the transition period is reflected in the frequency of postpartum disorders (e.g., milk fever, dysplasia of the abomasum, and placental retention).
4. *Solutions for optimizing and managing the health of calves and heifers*. Continuous evaluation of the health of calves and heifers.

MAFHP of DF2

The BioCheck scoring tool revealed values around 50% in the subcategory "Calving management" (26%) of the internal biosecurity. The MAFHT provided advice to improve the management of calving and calf in the specific MAFHP developed for DF2 (Figure 2).

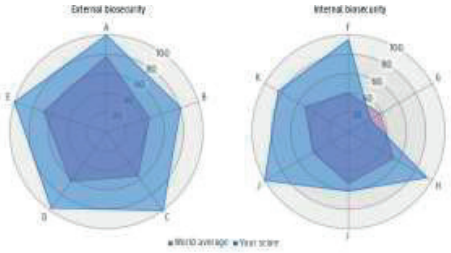

Figure 2. Subcategories scores of the internal and external biosecurity obtained by using the biosecurity scoring system Biocheck.UGent™ in DF2 (A. Purchase and reproduction; B. Transport and carcass removal; C. Feed and water; D. Visitors and farmworkers; E. Vermin control and other animals; F. Health management; G. Calving management; H. Calf management; I. Dairy management; J. Adult cattle management; K. Working organisation and equipment)

The MAFHP has the following activities:

1. *Analysis of the possible transmission of diseases through direct and indirect contact*. Calves will receive the feed on their own bucket that will be personalised with the number of the calve accommodation box. The hygienic measures will be applied after each feeding by cleaning the buckets and preventing the contamination of buckets with dust, insects, and dirty water. Calves will receive colostrum only from the farm's cows and those who have not been treated recently with antibiotics (Dewolf & Van Immerseel, 2019).
2. *Evaluation of the intake of maternal antibodies administered through colostrum in the first hours of life*. Enough colostrum (200 grams of IgG antibodies) should be administered within 6 hours of birth. Mother's colostrum from the first milking is preferred to that of other cows. Due to the low capacity of the abomasum, colostrum will be administered frequently in small quantity feedings. Colostrum will be refrigerated between feedings, bottles, and tubes for colostrum administration will be cleaned and disinfected after each use (Dewolf & Van Immerseel, 2019). The farm implemented a protocol for determining the serum protein in the blood at 72 hours after birth by using a refractometer.
3. *Assessment of calf housing conditions*. In accord with Dewolf & Van Immerseel (2019), MAFHT recommended that the calves will be housed in individual calf boxes or hutches in the first weeks of life and regrouped in pens of 7-10 calves of the same age. The spreading of urine and faeces between boxes or hutches must be avoided and the surfaces must be easily cleaned. The contact between different calf groups will be avoided.

MAFHP of DF3

The BioCheck scoring tool revealed values around 50% in the subcategory "Transport and carcass removal" (46%) of the external biosecurity and in the subcategories "Health management" (53%), "Calving management" (10%), and "Working organisation and equipment" (17%) of the internal biosecurity (Figure 3). The MAFHT of DF3 provided

advice to improve the internal and external biosecurity into the MAFHP.

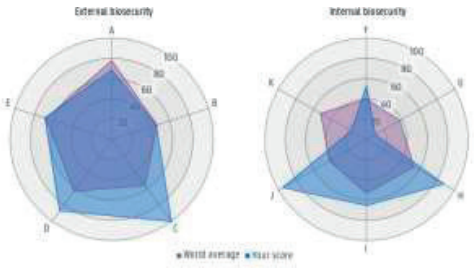

Figure 3. Subcategories scores of the internal and external biosecurity obtained by using the biosecurity scoring system Biocheck.UGent™ in DF3 (A. Purchase and reproduction; B. Transport and carcass removal; C. Feed and water; D. Visitors and farmworkers; E. Vermin control and other animals; F. Health management; G. Calving management; H. Calf management; I. Dairy management; J. Adult cattle management; K. Working organisation and equipment)

The goals of the MAFHP are (1) Identify issues that may affect calving and calf management, and (2) Identify deficiencies in work organization and equipment that may affect internal biosecurity.

The identification of the issues that may affect calving and calf management has brought together three activities:

1. *Analysis of the possible transmission of diseases through direct and indirect contact*. Each individual box will have its own bucket that will be cleaned and disinfected after each use. Colostrum from other farms will not be used. Colostrum should not be used in cows treated with antibiotics.
2. *Evaluation of the intake of maternal antibodies administered through colostrum in the first hours of life and assessment of calf housing conditions*. A minimum of 200 grams of IgG antibodies should be in colostrum administered (in small and frequent feeding) in the first 6 hours after calving. Colostrum from the calf's mother will be preferred in feeding. Calves will be housed in individual boxes or hutches in the first weeks of life and regrouped in pens of 7-10 calves of the same age. All surfaces of the boxes and hutches must be easily cleaned. Leakage of urine and faeces from one box to another should be avoided (Dewolf & Van Immerseel, 2019).
3. *Assessment of the transition period in dairy cows*. The activity will cover the three weeks before calving and three weeks after calving. In the last three weeks of gestation, the cow's body is subjected to the pressure given by the rapid growth of the foetus and the synthesis of milk components for the next lactation. At the beginning of lactation, cows mobilize body reserves (5-8% of birth weight), appetite is low and capricious (intake decrease with 45%).

The identification of the deficiencies in work organization and equipment that may affect internal biosecurity involved two activities:

1. *Identify deficiencies in work organization that may affect internal biosecurity*. To prevent or reduce the risk of diseases transmission by direct and indirect contact, calves and adult cattle will be housed in different stables, or they will be completely separated, without physical contact and at a distance of at least 3 meters between boxes. To prevent the continued spread of pathogens among calves, they will be grouped according to age and not by growth rate and weight. In a stable, the calves will be positioned so that the direction of propagation of the air flows will be from the younger calves to the older animals. The changing clothes and washing hands between each age group of animals will be developed in a way to increase physical barriers from an age group to another.
2. *Identify equipment deficiencies that may affect internal biosecurity*. Feeding tools are cleaned and disinfected after each use. The farm will use specific equipment for each age group and will not share tools with other farms.

MAFHP of DF4

The BioCheck scoring tool revealed values around 50% in the subcategory “Feed and water” (50%) of the external biosecurity and the subcategory “Calving management” (20%), of the internal biosecurity (Figure 4). MAFHT provided advice on the feed and water biosecurity and calving management when the activities of the MAFHP goals were established.

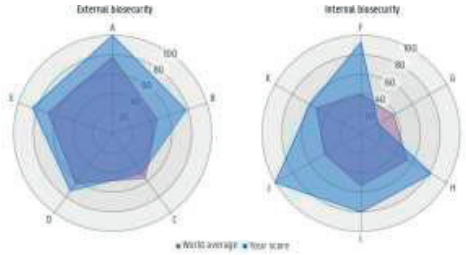

Figure 4. Subcategories scores of the internal and external biosecurity obtained by using the biosecurity scoring system Biocheck.UGent™ in DF4 (A. Purchase and reproduction; B. Transport and carcass removal; C. Feed and water; D. Visitors and farmworkers; E. Vermin control and other animals; F. Health management; G. Calving management; H. Calf management; I. Dairy management; J. Adult cattle management; K. Working organisation and equipment)

The MAFHP of DF4 has the following goals: (1) Feed and water biosecurity and (2) Management of calving and calf.

The activities of the goal Feed and water biosecurity were:

1. *Monitoring the risk of manure contamination during the crop or pasture fertilization*. The farmer will continuously check the fertilization of the land used for fodder production. It is considered that the contamination of feed with pathogens and/or (myco-) toxins can occur at all stages of feed production and storage. The feed can also be contaminated with manure during their fertilization or adjacent pastures. All feeding tools should be cleaned after each use to remove debris.
2. *Monitoring of manure storage (platform), place and form of storage of feedstocks*. The farmer will continuously check the storage of manure (platform), the place and form of storage of feedstocks. To avoid contamination of feed and water by rodents, birds, dogs, and cats, access to the stables, manure storage facility and feed storage facility will be limited.
3. *Monitoring the risk of manure contamination of feed purchased from other producers*: The farmer will continuously check the fertilization of the land used for fodder production and will assess the risk of contamination with manure from neighbouring lands.

MAFHP of DF5

The BioCheck scoring tool revealed values around 50% in the subcategory "Vermin control and other animals" (28%) of the external biosecurity and all subcategories of the internal biosecurity (Figure 5). MAFHT provided advice to improve internal biosecurity measures and recommended the organisation of a "Stable school" by using Vaarst et al. (2007) model.

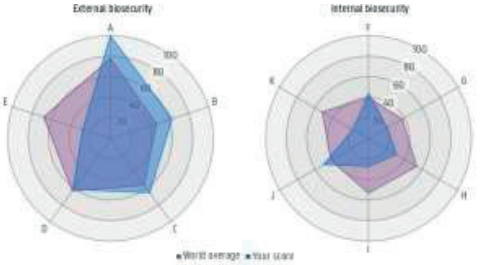

Figure 5. Subcategories scores of the internal and external biosecurity obtained by using the biosecurity scoring system Biocheck.UGent™ in DF5 (A. Purchase and reproduction; B. Transport and carcass removal; C. Feed and water; D. Visitors and farmworkers; E. Vermin control and other animals; F. Health management; G. Calving management; H. Calf management; I. Dairy management; J. Adult cattle management; K. Working organisation and equipment)

MAFHP's goals are (1) to identify issues that may affect the management of calving and calving and (2) identify deficiencies in work organization and equipment that may affect internal biosecurity.

The identification of the issues that may affect the management of calving and calving was done in the following activities:

1. *Analysis of the possible transmission of diseases through direct and indirect contact*. More attention will be paid to biosecurity measures that avoid direct contact between animals (Wells et al., 2002; Dewolf & Van Immerseel, 2019). The farm will evaluate the risk of disease transmission through direct and indirect contact between calves, young stock, and adult cattle: building a new stable or the compartmentalization of existing ones, using farm-specific boots and clothing, washing hands, use of disposable syringe and needles.
2. *Evaluation of the intake of maternal antibodies administered through colostrum in the first hours of life*. Farm must provide calves with a good volume of clean, high-quality colostrum within the first six hours of life (Godden et al., 2019). The mother's

colostrum obtained from the first milking will be the first option in the feeding of calves, and the use of colostrum from other farms will not be advised (Dewolf & Van Immerseel, 2019).

3. *Assessment of calf housing conditions*.

The identification of the deficiencies in work organization and equipment that may affect internal biosecurity was done in the subsequent activities:

1. *Identify deficiencies in work organization that may affect internal biosecurity*. Treatment of sick or injured animals will be done at the end of the daily routine.
2. *Identify equipment deficiencies that may affect internal biosecurity*: The farm will use age-specific materials and feeding tools. Labelling the materials and feeding-specific tools is proposed by MAFHT.

MAFHT's practitioners considered the general recommendations regarding the holistic approach to disease control, considering the epidemiology of diseases and the specific situations of each farm including the risks and perceptions of risk by decision-makers (Dargatz et al., 2002; Wells et al., 2002; Dewolf & Van Immerseel, 2019). In this study, al MAFHTs identified the farm practices to reduce bacterial disease and developed MAFHP tailored to specific situations in correlation with the results of the Biocheck.UGent™ and teams' meetings.

CONCLUSIONS

Farmers usually face management and/or health problems in correlation with the age category and physiological condition. The most common calf diseases were respiratory and enteric, but these problems did not create severe outbreaks of disease. Large dairy farms are more common with hoof disorders while small dairy farms are more exposed to udder diseases. All teams demonstrated their ability to identify farm practices to reduce bacterial disease and the need to use antibiotics.

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RETROSPECTIVE STUDY ON PATENT DUCTUS ARTERIOSUS (PDA): SURGICAL LIGATION IN SELECTED DOGS NOT TREATED BY AMPLATZER OCCLUDER

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Abstract

Patent ductus arteriosus (PDA) is the most common congenital heart disease in dogs and can lead to heart failure. The left-to-right PDA can be treated by minimally invasive procedures or open thoracotomic surgery. Intravascular techniques for PDA occlusion in our Hospital involve the use of vascular occluders, a device that expands within the ductus lumen to close the PDA. In small breed dogs, due to the small diameter of the femoral artery or due to the shape of the ductus itself, intravascular access is not achievable. In these patients, open surgery with ductus ligation is the proper approach. The surgical procedure is influenced by the characteristics of the ductus. When the ligature of the ductus appears difficult or too risky because of the presence of multiple adherences and adhesions on the medial part of it or because the extremely reduced size of it, the Jackson-Henderson technique is a valid alternative. The appropriate technique is chosen for each patient after considering the possible complications, the risk of dissection, the surgical timeline and the outcome. Our study aimed to evaluate all the intra- and postoperative complications associated with thoracotomic PDA-ligation in a definite category of affected dogs characterized by higher risk factors than the average of the surgery-treated ones.

Key words: Patent ductus arteriosus, PDA, surgical ligation, PDA complications, dogs.

INTRODUCTION

Patent ductus arteriosus (PDA) is the most common congenital cardiovascular disease in dogs occurring in about 30% of cases (Buchanan, 2001).

In this cardiovascular pathology, the ductus arteriosus fails to close in the immediate post-natal period, due to a reduced percentage of muscle fibers in the ductus wall, especially near the aorta where they may be absent (Buchanan & Patterson, 2003).

Some breeds appear to be highly predisposed to PDA and inheritance has been demonstrated in Poodles and Welsh Corgis (Patterson et al., 1971; Oswald & Orton, 1993). Females appear to be more predisposed to the development of the defect than male dogs (Buchanan, 2001).

The presence of the PDA implies the existence of a shunt between systemic and pulmonary circulation, in which the flow direction is determined by either the size of the ductus or the difference between the systemic vascular resistance (SVR) and the pulmonary vascular resistance (Moïse and Short, 1987).

Under physiological conditions, the resistance in the aorta is greater than that in the pulmonary artery, therefore usually (at least in the initial stages of the disease) the shunt is directed from left to right. If not treated, left-to-right PDA may result in congestive heart failure by 1 year of age (Eyster et al., 1976).

Transcatheter occlusion with an ACDO device is the treatment of choice for the majority of dogs with left-to-right shunting PDA (Singh et al., 2012), but when the dog is too small (<2.5 kg) or the femoral artery is not suitable for the egress of the catheter or when the ductus is characterized by an excessive minimum ductal diameter (MDD) or by a cylindrical morphology with a variation in its diameter <20%, ACDO occlusion is not achievable (Blossom et al., 2010; Wesselowski et al., 2017; Orton, 2017).

In all these circumstances surgical ligation is the only solution to resolve the pathology.

Different approaches for dissection around the ductus and safe passage of the ligature material have been reported, like Standard ligation and Jackson-Henderson techniques (Jackson & Henderson, 1979; Parchman, 1991; Downs et al., 1995).

An appropriate technique is chosen for each patient after considering all the risk factors correlated to the most frequent complications.

It's essential to proceed with surgery as soon as possible, due to the increase in fibrousness of the periductal tissue and the reduction in elasticity and friability of the ductus in relation to the age of the animal (Breznock, 1975; Eyster et al., 1976).

The most serious complication associated with thoracotomic ligation is a potentially fatal hemorrhage (Brockman, 2016).

It's common to deal with large and short ducts, characterized by a greater degree of abnormality of the duct and therefore greater fragility (Buchanan, 2001). Considering also the greater perimeter to be explored with the dissection and the abundance coalescences, the surgical dissection may be quite difficult. To avoid blind dissection medial to the ductus, the Jackson-Henderson technique is a valid alternative but is linked to a higher risk of postoperative residual flow than standard ligation (53% vs 21%) (Stanley et al., 2003).

Another challenge related to the surgical approach is represented by the small size of the patient that imposes a very restricted operating field and requires a skilled surgeon.

Our aim was to evaluate all the intra and postoperative complications associated with thoracotomic PDA-ligation in a selected category of cases in which the execution of minimally invasive procedures was limited, and compare our results with the literature.

MATERIALS AND METHODS

The data for this study were collected from the database of dogs referred at the University Veterinary Teaching Hospital (OVUD) of the University of Perugia between January 2009 and February 2021, for a PDA surgical treatment. All clients were informed of therapeutic options and formally consented to the therapeutic plan.

Eighteen dogs underwent surgical ligation because they were excluded from minimally invasive techniques. Three of them were previously attempted to close with ACDO, with no success, while the others were immediately destined for surgical ligation due to their small size or the morphology of the ductus. All dogs were diagnosed with PDA by correlating physical examination, radiography, and transthoracic echocardiography (TTE). A complete TTE study was performed with echocardiography using electronic sector-scanning transducers (frequency range: 2-11 MHz).

Ductal shape and minimal ductal diameter at the pulmonic ostium were assessed by TTE. All imaging studies were submitted for evaluation to a single cardiologist (FP) who then determined the ductal measurements and ACDO size for each dog.

A complete blood count and biochemical and coagulation profile were performed in all patients.

According to these clinical findings, the patients included in the study represent a heterogeneous population in which 3 were mixed-breed and 15 breed dogs (Pomeranian, Chihuahua, Maltese, German Shepherd, Dachshund, Miniature Poodle, Lagotto, Deutsch Kurzhaar, Deutsche Spitz). Dogs had a bodyweight ranging between 0.8 kg and 15.8 kg, aged between 2 months and 2 years old. Of the 18 dogs evaluated, 13 were females and 5 were males (see Table 1 for demographic data).

All surgical procedures were performed by the same surgeon. Dogs were pre-oxygenated (flow-by; 3 to 5 L/min according to the size of the dog) before induction of general anaesthesia. Dogs were premedicated with intramuscular (im) opioids (such as pethidine 4-5 mg/kg or methadone 0.1 mg/kg) and midazolam 0.2-0.3 mg/kg, induced with lidocaine (Lidocaina 2%, Esteve Spa, Italy) (1.5 mg/kg) and propofol (Proposure, Merial Italia Spa, Italy) intravenous (iv) to effect (Cerasoli *et al*. 2016) and maintained with isoflurane (Isoflo, Esteve Spa, Italy) in 100% oxygen and sufentanil (Disufen, Angenerico Spa, Italy) constant rate infusion (CRI) (Bufalari et al. 2007).

Table 1. Breed, Gender (M - Male; F - Female), Age and Weight of 19 cases of PDA ligation.
Case 13 and 14 were the same dog, which required a second surgery due to the total ductus recanalization

Cases	Breed	Gender	Age (months)	Weight (kg)
1	**Miniature Poodle**	**M**	4	**2,7**
2	**German Shepherd**	**F**	4	**15,8**
3	**Deutsche Kurzhaar**	**F**	2	**3,3**
4	**Lagotto**	**M**	3	**4**
5	**Pomeranian**	**F**	7	**1,5**
6	**Maltese**	**F**	8	**2,1**
7	**Mixed breed**	**F**	2,5	**1,2**
8	**German Shepherd**	**F**	3	**6,8**
9	**Chihuahua**	**F**	3	**0,8**
10	**Deutsche Spitz**	**F**	9	**4,3**
11	**Pomeranian**	**M**	6	**2,3**
12	Mixed breed	M	3	2,6
13	Dachshund	F	12	4,4
14	Dachshund	F	13	3,8
15	Chihuahua	F	12	1,7
16	Pomeranian	F	3	2,8
17	Maltese	M	7	2,6
18	**Mixed breed**	**F**	24	**13**
19	Pomeranian	F	5	1,4

For those who required it, dobutamine or colloidal solution to counteract hypotension and lidocaine to control arrhythmias were used. All dogs received a pre-operative and post-operative intercostal nerve block with bupivacaine, lidocaine or ropivacaine. Carprofen (4 mg/kg iv) (Rimadyl®, Zoetis, Italy) (Bufalari et al. 2012) and buprenorphine (10 µg/kg iv) (Buprenodale, Dechra Veterinary Product Srl, Italy) were administrated as postoperative analgesic drugs.

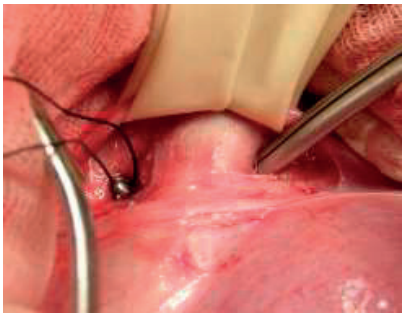
HR (beats/min); electrocardiogram; respiratory frequency; systolic, diastolic and mean non-invasive arterial blood pressures; peripheral capillary oxygen saturation; end-tidal carbon dioxide partial pressure; end-tidal isoflurane and rectal temperature (Multiparameter Monitor HB100; Foschi) were monitored continuously.

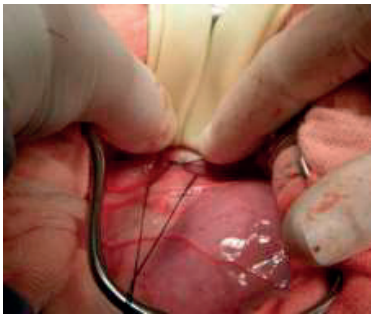
The surgical approach to the ductus was through a left, fourth intercostal thoracotomy. Dissections were either intra- or extrapericardial, with identification and careful preservation of the vagus, phrenic and left recurrent laryngeal nerves. We can identify two groups according to the technique used: Group S (Standard Dissection Technique) and Group JH (Jackson and Henderson Dissection Technique).

Group S

After caudal retraction of the cranial lung lobe, the vagus nerve was dissected free from the pericardium and retracted dorsally or ventrally using stay sutures or a silicone vessel loop. The phrenic nerve and recurrent laryngeal nerve were identified and avoided. A right-angled dissection instrument was then used to dissect around the aorta caudal to the ductus but cranial to the first intercostal artery. Therefore, a Penrose drain was placed around the aorta as a noose with its ends secured in a forceps. Slight traction of the loop allowed to open the space caudal to the ductus between the aorta, ductus and pulmonary artery trunk and to extend it medially by gentle dissection under direct visualization (Brockman, 2016). Exposure of the cranial aspect of the ductus was facilitated by cutting the loose connective tissues between the ascending aorta and ductus with blunt-tipped scissors, then a right-angle forceps was passed between the aorta and ductus at a 45° angle to the transverse plane (Orton, 2017). Afterwards, a blind dissection medial to the duct was carried out from caudal to cranial to complete the duct dissection. This step was undertaken with care because of the possible fragility of the medial wall of the ductus and proximity of the right pulmonary

artery. The dissection was performed using blunt-tipped right-angled forceps like Satinsky. Once the passage was created, a loop of ligature was passed from cranial to caudal (Figure 1). The loop was divided to form 2 individual strands. The ligature at the aortic end of the ductus was always tied first (Figure 2).





Figures 1, 2. Standard dissection technique. To the left (1): the right-angled forcep is passed from caudal to cranial, medial to the ductus to grasp the loop of ligature. To the right (2): The ligature at the aortic end of the ductus is tied first

Group JH

After caudal retraction of the cranial lung lobe and preservation of the nerves (as above), the mediastinal pleura dorsal to the aorta was incised with Metzenbaum scissors and delicately blunt dissection of the medial side of the aorta was performed, using both fingers and Satinsky forceps (Figure 3).

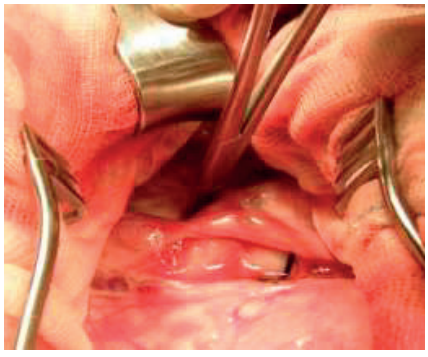


Figure 3. Jackson and Henderson dissection technique. The area dorsal and medial to the aorta has been dissected

A gentle exploration cranial and caudal to the ductus was carried out.

A right-angled forceps was then inserted immediately cranial to the ductus and passed around the aorta from ventral to dorsal while gently elevating the aortic arch with a finger or whit a Penrose drain aortic noose (as above). A loop of ligature was passed from the dorsomedial aspect of the aorta to the cranial aspect of the ductus, ventral to the aorta. The same procedure was repeated around the aorta from ventral to dorsal on the caudal margin of the ductus to pick up the 2 free ends of the ligature. Ventral traction was applied on the ligatures to draw them down slowly from the dorsomedial aspect of the aorta to the medial aspect of the ductus. The loop was divided to obtain two individual strains and the ligature at the aortic end of the ductus was always tied first.

In all dogs, ligations were performed using silk suture. Overall the closure took place in a time ranging from 5 to 7 minutes, in order to reduce the impact of a possible Branham reflex (De Monte et al., 2017). The ductus, aorta, and pulmonary arteries were then palpated for the presence of fremitus and to evaluate the appearance of aortic aneurysm dilation. After repositioning the cranial lung lobe and making sure of its complete reperfusion and physiological re-expansion, the thoracotomy was closed in layers. Negative pressure was delicately and progressively restored by a temporary small thoracic drainage that was removed after closure of the muscle layer. Perioperative antibiotic coverage was provided to all patients. First-generation cephalosporins or amoxicillin associated with clavulanic acid were administered, starting at induction and every 90 minutes during surgery, then continued postoperatively every 12 h for five days.

All dogs were echocardiographically evaluated 24-48 hours after surgery and 15 days after surgery, while the subsequent checks were set according to the clinical conditions of the individual patient.
Complications, either intra- or postoperative, were classified as:

Severe:
- Severe bleeding (Brockman, 2016);
- Suture suppuration;
- Total ductus recanalization (Brockman, 2016);
- Secondary chylothorax (Brockman, 2016);
- Cardiac arrest (Van Israel et al., 2002; Saunders et al., 2014).

Medium:
- Branham sign (De Monte et al., 2017);
- Transient or permanent post ligation aortic aneurysm dilation (Brockman, 2016);
- Iatrogenic pulmonary injury (Brockman, 2016);
- Iatrogenic left recurrent laryngeal nerve injury (Brockman, 2016);
- Partial ductus recanalization (Brockman, 2016);
- Hypertension (Brockman, 2016);
- Minor bleeding (Brockman, 2016).

Mild:
- Slight residual flow (Brockman, 2016);
- Cutaneous suture infection/dehiscence (Alison Moores, 2016);
- Weakness of the left forelimb (Alison Moores, 2016).

Associated risk factors were analyzed for each complication. Initial procedural success was defined as patient survival without need for a second surgery. Dogs that survived the initial procedure but died from an unrelated cause were considered to have a successful procedure.
Procedural mortality was defined as death within 14 days of the procedure.

RESULTS

Ligation was carried out in 95% of cases (n=18/19): in one dog the presence of numerous medial adhesions to the ductus and the aneurysm of the pulmonary artery were considered too high risks to proceed with the ligature. The same dog had already undergone an attempt at occluding with ACDO, whose access was considered impossible.
We considered 19 as denominator as one dog was counted twice since a second surgery was required.
On the other hand, the patient in which the ligation was not completed was not considered in the calculation of the procedure times and intra and postoperative complications rates.
Surgeries lasted from 75 to 275 minutes, with an average of 142 minutes.
Standard ligation was performed in 88% of cases (n = 16), Jackson-Henderson technique in 12% of cases (n = 2).
The silk threads used for the ligature were USP 1 in 7% of cases, USP 0 in 65% of cases, USP 2/0 in 14% of cases and 3/0 in 14% of cases.
A graphic illustration of the complications that occurred is shown in Figure 4.

Severe complications
Severe complications occurred in 11% of dogs (n = 2).
Severe I.O. complications included severe bleeding, that happened in 5.5% of dogs (n = 1): first, a hemorrhage medio-cranial to the ductus occurred, then, at the time of ligating the PDA, severe pulmonary artery bleeding occurred, which resulted in the patient's death.
Severe P.O. complications included total ductus recanalization, which occurred in 5.5% of dogs (n = 1), due to the failure of the silk threads. This dog required a second surgery to close the duct, 1 month later (surgery was postponed due to respiratory infection by *Bordetella bronchispeptica*, not related to the first surgery). At the time of the second surgery, it was possible to verify that the knot was intact, while the thread was totally worn out. The size of the thread used in the first surgery was a 2/0 USP in diameter. Probably, due to the high pressure reached in the postoperative phase, the thread had suffered a fatigue failure.
Medium complications
Medium complications occurred in 89% of dogs (n = 16).
The I.O. ones included Branham reflex that showed in 55.5% of dogs (n = 10) and transient

aortic aneurysm dilation, occurring in 22.5% of dogs (n = 4).
In dogs that exhibited Branham reflex, atropine 20 μg/kg iv was administered with a complete return to baseline values of the heart rate within few seconds.
In these dogs, the average MDD was 3.87 mm and the average Ampulla diameter was 8.56 mm.
Aortic aneurysm dilation is caused by the increase in pressure triggered by the ligation of the ductus. High-pressure blood flow acts on the thin walls of this anatomical structure causing its dilation, which can be transient and reduce with the reestablishment of blood pressure or persist into the postoperative period.
In one dog (5.5%) permanent aortic aneurysm dilation occurred. It led to the formation of a thrombus inside of it, which required low dose

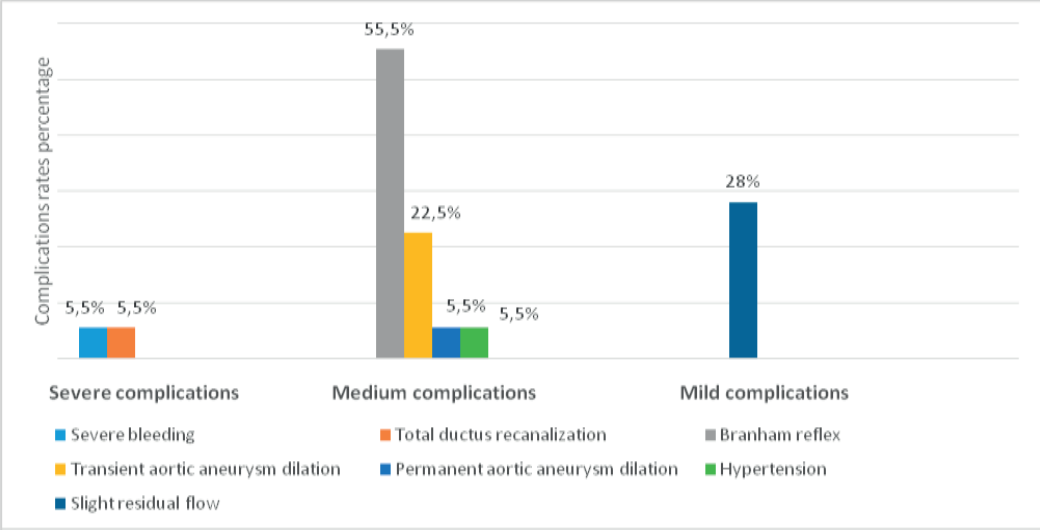


Figure 4. Graphic illustration of the complications, and related rates, we encountered in our study

acetylsalicylic acid treatment until the thrombus disappeared, which was confirmed two months after surgery follow-up.
In dogs that showed aortic aneurysm dilation, the average MDD was 4.3 mm and the average Ampulla diameter was 10.6 mm.
Other P.O. complications included hypertension, presenting in 5.5% of dogs (n=1).
Furosemide (2 mg/kg EV BID) was administered in this dog; the same dog underwent ductal recanalization due to the fatigue failure of both sutures which required a second surgery.

Mild complications
Mild complications occurred in 28% of dogs (n = 5) which presented slight residual flow.
In one of them, the 4 months after surgery echocardiographic control decreed the absence of the flow. In the other patients, subsequent checks confirmed the persistence of a slight but insignificant residual flow with no repercussions on cardiac hemodynamics.
The initial success rate was 94.5% because 17 of 18 dogs didn't require a second surgery.
The procedural mortality rate was 5.5%, due to the only patient who died intraoperatively due to severe arterial bleeding.

DISCUSSIONS

In this study, we evaluated the results of PDA ligation in a group of high-risk patients selected for their particular small size and morphology of the ductus, which limited the execution of minimally invasive procedures. Starting from this assumption, the results obtained were considered good and comparable with those obtained by other authors who had a larger cohort of non-selected patients.

The anatomical features of the ductus influence the choice of surgical technique, as they also influence complications rate. In this regard, the studies of James Buchan (Buchanan 1978, 2001) illustrate the anatomical basis that explains the fragility of the ductus or aorta experienced during dissection in some animals. His studies demonstrated that certain regions of the ductus and the section of the aortic wall through which the ductus courses (which Buchanan termed the "ductus aneurysm") were very thin compared with the normal aorta. In addition, certain anatomic types, which are ever excluded from minimally invasive techniques (like type III ducts according to Miller classification) (Miller, 2006), might be even more challenging to dissect around to the point. In this respect, according to some authors, the best choice may be not the ligation of the ductus; some authors prefer to perform its incision followed by suturing the two ends (Buchanan, 1996; Rodriguez Gomez et al., 2013).

To plan the proper surgery, careful preoperative measurement of the MDD, of the diameter and length of the ampulla is essential. This data can be obtained by magnetic resonance (RMI), angiotomography (Henjes et al., 2001) or transesophageal echocardiography (TEE) which provides more accurate results than TTE (Saunders et al., 2019).

In our study, diagnostic tools for preoperative measurement only included TTE which unfortunately can overestimate the size of the duct (Saunders et al., 2007), often leading us to understand the real conformation of the duct only in the intraoperative setting.

The major complication we encountered, was severe bleeding which led the dog to sudden death. The patient was a 3 months old Chihuahua, with a weight of 800 grams. Its duct had a MDD of 2.8 mm and the ampulla was 4.1 mm long. The dog experienced severe ductal bleeding during the dissection of the duct; at first, the bleeding was controlled and stopped. Unfortunately, during the following duct ligature, an unrecoverable hemorrhage of the pulmonary trunk caused the animal death. The ligation, in consideration of the small size of the dog, was attempted with a USP 3/0 silk thread; we may speculate that the size of the thread was too small and contributed to tearing the tissue apart, which was already very fragile in itself. The choice of suture material and size may be very important. Unfortunately, since there are no specifics about it, the choice of the size of the thread depends on the surgeon's experience. Silk is a great option as it is a braided suture that has greater strength and allows a higher knot tightness than a monofilament one, although it's more prone to bacterial nesting. A smaller USP suture allows to tighten the knot better but has a more relevant abrasive action that can lead to a progressive laceration of the vessel; moreover, it's less resistant and can break. On the other hand, a higher USP suture has greater resistance, less abrasive effect but can untie easily and its thickness could create difficulties in the complete closure of the duct and could increase the risk of the permanence of a residual flow.

When ductal hemorrhage is minimal, a change in the direction of dissection may lead to the completion of dissection (Brockman, 2016). Other authors like Hunt (2001) have described the clinical use of the temporary total cardiac outflow occlusion, as advocated by Eyster (1985). This technique would either allow further dissection or facilitates accurate clamp placement in the event of ductal hemorrhage (Hunt et al., 2001). To realize it, pericardium must be incised and vascular clamps are placed simultaneously across the aortic and pulmonary roots. In addition, making traction on the aortic noose (method described above) will limit backflow of blood down the aorta and allow to expose the caudal ductus further to facilitate clamp placement. In our study, extrapericardial access was used for most of the subjects as the duct was outside the pericardial membrane and clearly visible. According to some authors (Selmic et al., 2013), intrapericardial access would allow also to deal with a smaller amount of tissue and reduce the dissection times.

Based on previous studies, severe bleeding during PDA ligation has been estimated at 6-10% in dogs (Eyster et al., 1976; Ackerman et al., 1978; Hunt et al., 2001; Birchard et al., 1990) leading to a 1.6-11% mortality risk (Van Israel et al., 2002; Hunt et al., 2001; Eyster et al., 1976; Bureau et al., 2005; Birchard et al., 1990); that means our results fall within the incidence ranges described in the literature. The

most important risk factor linked to intraoperative bleeding is the blind dissection medial to the duct, principally required in the Standard technique: care must be taken not to execute a shallow dissection in relation to the ductus because this could lead the instrument directly into the medial ductus wall and, at the same time, it's imperative not to continue dissection too deeply because the right pulmonary arterial branch is vulnerable in this position. In addition, the risk is increased by the type of instrument used and the age at the time of the procedure. Very small curved forceps are often required (Figure 5) (especially when the patient is small), the ends of which are pointed, increasing the risk of perforation of the vessel walls. While in older subjects, especially in those over two years of age (Rodriguez et al., 2013), the risk increases due to the greater fibrousness of the periductal tissue (Breznock, 1975) and the friability and the lower elasticity of the duct (Eyster et al., 1976). For this reason, it is important to proceed with surgery as soon as possible. In our series, the average age of the operated subjects was 6.4 months, with 61% ≤ 6 months and 21% ≥ 12 months.

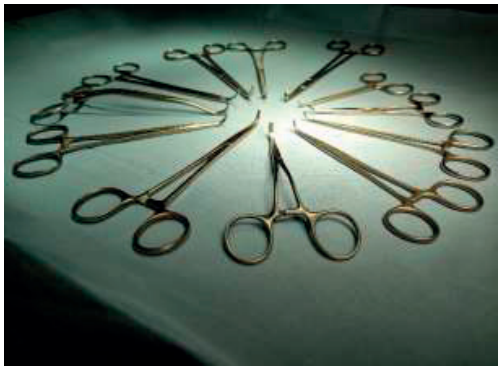


Figure 5. Several types of Satinsky hemostats were used in our study for the dissection of the patent ductus arteriososus

Jackson & Henderson technique (Jackson & Henderson, 1979) was specially designed to avoid blind dissection medial to the duct and thus reduce the risk of hemorrhage. A randomized, prospective study on 35 dogs revealed that the risk of residual postoperative ductal flow is higher for this technique (53%) than for the Standard one (21%), due to greater inclusion of periductal tissue in the ligature (Stanley et al., 2003). We noted a similar slight residual flow (mild complication) in group S (19%, n = 3/16) but a higher incidence in group JH (100%, n = 2); it must be considered that in our study the JH technique was performed only in 2 patients, therefore the number of subjects included is small in order to be able to compare this data with other reports. To avoid residual ductal flow, authors like Brockman (2016), propose a third ligation with polypropropylene suture, transfixed to the duct and positioned between the two traditional ones.

If the residual flow is evaluated as insignificant from a haemodynamic point of view, not only it is not necessary to perform a second surgery but it is even not recommended (Brockman, 2016). Surgical revisions are inherently risky due to the development of adhesions between the duct and surrounding structures that make dissection difficult and ligation dangerous as they increase the risk of vascular laceration. Sometimes the risk is so high that division of the duct and pulmonary lobectomies may be required (Eyster et al., 1975).

The major cause for concern with residual shunting is apparent recanalization of the ductus, which is reported to occur in approximately 1%-2% of cases (Eyster et al., 1975; Birchard et al., 1990), whose risk increases according to the extent of the residual flow and in case of hypertension (Brockman, 2016).

In our study, 1 dog (5.5%) had postoperative hypertension (due to the initial closure of the duct) that caused the breakage or failure of the suture. It led to duct recanalization and so to a second surgery. In the first surgery, ligation was realized with a 2/0 USP silk suture; during the revision surgery, we opted for a 0 USP for the reasons described above. The same dog also required a partial pulmonary lobectomy of the left cranial lobe which was severed during the access to the thoracic cavity due to a complete adhesion with the left rib wall.

Speaking of Branham reflex, it consists in the lowering of the heart rate (by at least 5-6 bpm) (Muir, 2007) as a consequence of an increase in mean and diastolic arterial pressure, following the closure of the duct, both by surgical ligation and by catheter occlusion (Hellyer, 1992).

According to the study by De Monte et al. (2017), the only variable capable of predicting the haemodynamic reactions following the ligation of the duct would be the diastolic velocity of blood flow in the duct, as it has a moderate inverse correlation with the increase in mean arterial pressure.

The entity of a flow through a duct is directly proportional to the pressure gradient existing at its ends and inversely proportional to the resistance encountered. In small ducts, the resistance is highs, as is the flow velocity, but the volume of blood passing through them is modest. For this reason, a less pronounced Branham reflex should be expected following the occlusion of smaller diameter ducts.

In the dogs which exhibited Branham reflex in our study, ducts were characterized by a medium to large MDD and a large ampulla. One of them is the one who developed hypertension and total ductal recanalization (MDD = 4.2 mm; Ampulla diameter = 10 mm).

Many factors related to anesthesia and surgery can lead to changes in heart rate and blood pressure. In our case, sufentanil, isoflurane, as well as pain, surgical manipulation and hypothermia may have played a role in influencing these parameters (Bufalari et al., 2007).

Finally, post ligation aortic aneurysm dilation: in our series, we have found that dilation is very common, especially in dogs with large ducts, in which the increase in pressure following the ligation of the ductus is greater.

We have more frequently noticed a transient intraoperative dilation, which however retains a high risk of rupture, described by some authors in the postoperative period. Unfortunately, is not possible to avoid its rupture (Brockman, 2016), but perhaps it could be possible to predict its formation based on the characteristics of the duct.

CONCLUSIONS

Reducing the risks associated with surgical ligation is possible thanks to a multimodal approach which consists in planning the surgery as soon as possible in young animals, in the use of diagnostic imaging tools like RMI, angio-TC or TTE that give precise and reproducible results and allow to know the characteristics of the ductus before surgery, in order to choose the most suitable technique, as well as the most suitable dissection forceps and suture for ligation. Last but not least, the surgeon's skill and experience on dissection and familiarity with the techniques usedare of the utmost importance

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PASSIVE TRANSFER OF IMMUNOGLOBULINS FROM EWE TO LAMB

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Abstract

The neonatal period in small ruminants in the Northern Hemisphere usually starts in December. It is around this critical period that 10-12% of lambs and kids die in the first 72 hours of life because of poor colostrum quality and quantity. Concerning this fact, it is of importance that all pregnant ewes have adequate access to forages for a good colostrum quality, indispensable for the lasting growth of newborns lambs. To demonstrate the passive transfer of immunoglobulins from the colostrum, 10 samples of colostrum were collected from 10 individual ewes, followed by 10 samples of serum from their resulting lambs in the first day after lambing and at 8 days of age. The colostrum and protein serum levels were tested with: refractometry, using zinc sulfate turbidity test, qualitative sodium sulfite turbidity test. From all the colostrum samples collected, just 2 of them presented an acceptable quality of immunoglobulins. By negative correlation, the lambs' levels of serum protein in day 1 and day 8 were high in all newborns. Although the current preliminary results are somewhat inconclusive, they outline the importance and practical significance of colostrum quality monitorization in ewes.

Key words: *colostrum, ewes, immunoglobulins, lambs, protein levels.*

INTRODUCTION

For decades it is known that the ruminants' synepitheliochorial placenta is very similar to the equines and swine epitheliochorial one. Thus, the six tissue layers of these types of placenta prevent the passage of maternal antibodies to the fetus (Vejlsted, 2010; Borghesi et al., 2014). Even new world camelids, like llama and alpacas that are not considered true ruminants, present failure of passive transfer due to their particular type of placenta (Weaver et al., 2000). Consequently, the immune system of newborns ruminants for defending against microorganisms is received throughout colostrum ingestion, passively (Maden et al., 2003).

The term "colostrum" represent the first lactic secretion enriched with elements of blood serum, including antimicrobial proteins such as lactoferrin, lactoperoxidase, lysozyme, proline-rich polypeptides and immunoglobulins (Igs) (Tyler et al., 1999; Loste et al., 2008; Vejlsted, 2010; Lopreiato et al., 2017). Colostrum acts as an important nutritive source, due to the high content of vitamins and minerals, having a laxative effect (Boucher, 2014).

In order to reduce the failure of passive transfer, and at the same time morbidity and mortality in foals, piglets, calves, lambs, kids and alpaca crias, colostrum ingestion and absorption immediately after birth is requires (Nowak & Poindron, 2006), due to the drastic decrease over time of Igs content (Weaver et al., 2000). Therefore, for a maximum efficacy, the offspring must ingest the colostrum during the first two hours of life, as Santiago et al. (2020) reported. These antibodies are absorbed via pinocytosis by epithelial cells in the jejunum and ilium, and then they are transferred by the vascular system into the thoracic duct (Riddle, 2003).

There are five classes of immunoglobulins known, however a high importance for passive immunity belongs to IgG, IgM and IgA. Out of these, IgG_1 levels decrease significantly within the first 10-12 hours post lambing and after 24 hours postpartum, the IgG levels become unsatisfactory (Santiago et al., 2020).

The yellowish color and viscosity of the colostrum can be used as indicator of the colostrum IgG concentrations (Meo-Scotoni & Machado Neto, 1992), nevertheless, a more precise method is to evaluate the total protein content of colostrum (Borghesi et al., 2014).
To evaluate the total protein content of the colostrum and the lambs blood serum, Quigley et al. (2013) proposed the use of Brix refractometer as a tool. Few years later, Alves et al. (2015) evaluated the passive transfer of immunity from ewes to lambs using indirect enzyme-linked immunosorbent assay (ELISA) test and the enzymatic colorimetric kits.
Currently, in practice there are used different methods of passive transfer evaluation like: radial immunodiffusion of IgG_1 concentration, sodium sulfite turbidity assay, zinc sulfate turbidity assay, ELISA, refractometry, glutaraldehyde coagulation test etc (Tyler et al., 1996; Tyler et al., 1999; Boucher et al., 2014; Alves et al., 2015).
According to Vatankhah (2013), the immune-globulin levels that are present in the ewes colostrum are directly correlated with the immunoglobulin levels found in lambs blood serum. Up-to-date, few studies have been conducted in order to estimate the optimal passive immunity transfer in lambs, to the best of our knowledge, being the first study on this subject from Romania.
The main goal of this paper, was to compare the results of passive transfer of immunity in newborn lambs, evaluated throughout techniques of refractometry, qualitative zinc sulfate turbidity test, and qualitative sodium sulfite turbidity test, to conclude if there is a correlation between them, and which of these could be reliable in practice at farm level.

MATERIALS AND METHODS

The authors of this study respected all rights of animals' welfare in correlation to European Union legislation (Directive 2010/63/EU), and none of them suffered of any painful procedures.

Animals
For this experiment, 10 crossbred ewes were used (F_1 Texel x Țurcană) together with their resulting 10 lambs, belonging to a 200 heads herd from Poiana Marului, Brasov county. All ewes were primiparous, at a proper body condition score, ranging between 2.5-3 BCS.
The ewes were naturally breeded during late August, and they were confirmed pregnant two months later after transabdominal ultrasonographically examination (Tringa Linear VET®, Esaote, The Netherlands), as Jones et al. described in 2016. All ewes taken into the study were carefully monitored to ensure that none was affected by any pathological condition. At the same time, each animal received dried hay (7 kg) and corn grains (500-700 g), twice per day, and the water intake was *ad libidum*.

Experimental design
Based on aspects such as udder shape and consistency, used as prodromal signs of lambing, the ewes were separated around ten days before parturition of other flockmates. Between January and February 2020, all ewes had monotocous eutocic deliveries, without the need of assistance. In maximum 4 hours after lambing, from each ewe were collected 20 ml of colostrum into 30 ml vials (Urocultor, EasyCare®, Romania), and from each lamb blood samples were taken by jugulare vain puncture into 4 ml cloat activator vials (Vacutest, Kima®, Italy).
Using the same protocol, eight days later, another 10 blood samples were collected from neonatal lambs.

Colostrum assessment
Each colostrum sample was evaluated in the first hour after milking, looking for the color, consistance and IgG level. To determine IgG level of each colostrum sample, it was used a Brix refractometer that was calibrated before. According to Bielmann et al. (2010), the Brix refractometer is not sensitive to the temperature of the colostrum at the time of analysis.
After a gentle mixture of each colostrum sample, it was pipetted one drop from each sample, and after it was covered with the refractometer prism, the value was read using natural light. For precise results, this procedure was repeated 30 minutes later, considering that during this time, the colostrum composition will not be affected.

The obtained values were expressed in Brix percentages that later were converted into mg/ml according to statement of Hameed et al. (2019) witch said that a Brix value of 22% corresponds to 50 mg/ml of IgG.

Blood samples evaluation
After sampling, blood was kept around one day at room temperature for clot sedimentation, then centrifugated for 10 minutes at 500 rpm, then the serum was separated into Eppendorf vials and freezed untill all samples were obtained.

1. Refractometry
To assess total serum protein level, an ordinary refractometer was utilized in the same manner as the Brix refractometer. Serum IgG_1 (being the most abundant in serum) concentration could be estimated based on serum total protein concentration using the following formula:

$$\textbf{Serum IgG1}\ \left(\frac{mg}{dl}\right) = -3615 + \left[901\ x\ total\ serum\ protein\ \left(\frac{g}{dl}\right)\right]$$

2. Zinc sulfate turbidity test
A 0.1 ml aliquot of each serum sample was added to 6 ml of zinc sulfate solution in 10 ml sterile tubes. The solution was mixed carefully, then incubated for 1 hour at 23°C, and placed in front of a text. Positive results were considered if text was not legible through the sample tube, turbidity confirming the presence of IgG into the serum by precipitation of gamma globulin.

3. Sodium sulfite turbidity test
Using distilled water, sodium sulfite solutions were prepared at14%, 16% and 18%. A quantity of 0.1 ml of serum aliquot was mixed with 1.9 ml of each concentration of sodium sulfate. Tubes were mixed, incubated for 15 minutes at 23°C, and evaluated in the same manner as the previous test was performed. The test results were recorded on a 0 to 3 scale, where 0 represented no turbidity in all 3 tubes, 1 for turbidity in 18% solution tube, 2 for turbidity in 18% and 16% solution tube, and 3 for turbidity in all tubes. All assays were investigated by the same reader.
The results obtained from the turbidity tests were compared and correlated with the refractometry results using Office Excel 2016.

RESULTS AND DISCUSSIONS

None of the sheep was excluded from this study due to pathological conditions or other nonmedical causes. At the same time, all ewes registered a BCS according to their condition, ranging between 2.5 and 3.

Colostrum assessment
After the evaluation of colostrum quality by Brix refractometer, using the rule of three, it was calculated the level of IgG (mg/ml) (Figure 1) for each of the ten samples. Values between 29.55 and 53.41 mg/ml were obtained, results that coincide with a good quality colostrum in ewes, similar to the report of Alves et al. (2015) that used in their study the ELISA method.
Berge et al. (2018) showed that from frozen-thawed Awassi ewes, the colostrum values range between 14.40 and 17.10 Brix %, compared to the present study, where data ranged between 13 and 23.5 Brix %. By comparison, Boucher et al. obtained in 2014, higher values in Marino breed ewes (from 92.32 to 131.90 mg/ml), and in Dorper breed (from 75.69 to 81.20 mg/ml) supplemented with wheat or canola.

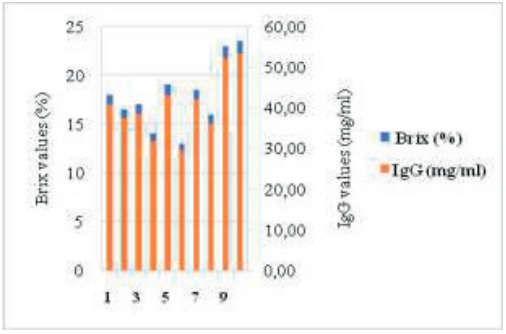

Figure 1. Graphic representation of colostrum quality expressed in Brix % and the values of IgG (mg/ml)

Recently, Kessler et al. (2019) observed in Santa Ines ewes, IgG colostrum values ranging between 1.2 and 60.7 mg/ml. Massimini et al., (2006) showed that serum proteins concentrations range from 4.0 to 8.2 mg/dl, signicatvely smaller values compared to our results.

Serum refractometry
Even if by testing the serum refractometry, only IgG_1 results were obtained, it can be

concluded that this is the main component of IgG, from all four subtypes (IgG_2, IgG_3, and IgG_4), thus the data is not quite pertinent to extrapolate the whole IgG levels of maternal colostrum.

Although we evaluated just the colostrum of first lactating ewes, the current results suggest that this is method is proper for the use in assessing the passive transfer of IgG in newborn lambs. The highest value registered was in ewe number 10, and the minimum value, observed in ewe number 6, this values were not correlated with abnormalities during their pregnancy or lambing, showing that animals from the same flock can present a large difference in the values of IgG, and at the same time, to be physiological sound.

Colostrum ingestion took place before blood sampling in 6 out of the10 lambs, according to belly palpation and refractometry results (Figure 2). The thawing of serum samples took place at room temperature for one hour, then samples were evaluated using refractometry.

Values of serum total protein (Table 1) were converted using the above formula.

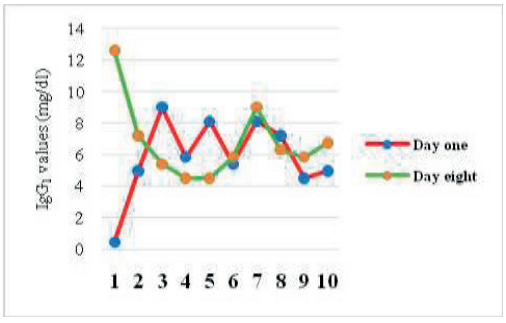

Figure 2. IgG_1 values obtained from blood samples collected from lambs on day 1 and 8 of life

Table 1. Serum proteins values obtained from lambs at 1 day and 8 days of life

Serum proteins (g/dl)	
1st day of life	8th days of life
4.5	18
9.5	12
14	10
10.5	9
13	9
10	10.5
13	14
12	11
9	10.5
9.5	11.5

Table 2. Comparative data of IgG concentration colostrum and serum IgG_1

Serum IgG_1 values (mg/dl) in lambs		Colostrum IgG values (mg/dl)
1st day	8th days	
0.44	12.60	4,091
4.94	7.20	2,727
9.00	5.40	2,273
5.85	4.49	2,045
8.10	4.49	2,045
5.40	5.85	2,386
8.10	9.00	3,182
7.20	6.30	2,500
4.49	5.85	2,386
4.94	6.75	2,614

Thus, evaluating Table 2, it can be observed that in most lambs, a very small amount of IgG_1 was absorbed from the total volume of IgG from the maternal colostrum. Boucher et al., metioned in 2014, concentrations ranging between 700 and 1100 mg/dl of the serum IgG on 2 days old lambs, and Alves et al. (2015) found in Santa Ines lambs values of serum IgG of 182 mg/dl in males and 293 md/dl in females, using refractometry. For the same breed, Kessler et al. (2019) showed values ranging between 120 and 6070 mg/dl.

According to Massimini et al. (2007), an increased risk of illness and death is associated with the concentration of IgG. In their work, the concentration of IgG serum 24 hours after birth ranged from 0 to 524 mg/dl. Similar results were obtained by Hashemi et al., in Karakul breed, with an average of260 mg/dl.

Recently, Gokce & Atakisi (2019) registered serum IgG concentration, 24 hours after birth at a mean of 2198 mg/dl.

Moreover, Daniels et al. (2000), working on supplementing vitamin E in ewes, registered 1.96 mg/dl for serum IgG concentration in lambs from ewes that received vitamin E supplementation one month prior to lambing. Stewart et al. (2013), after supplementing ewes before lambing with selenium, estimated that IgG concentration of lamb serum was at a mean of 2670 mg/dl.

Zinc sulfate and sodium sulfite turbidity tests

Regarding the zinc sulfate test, the turbidity was present for all samples both in the first day and in the eight day after lambing, suggesting that IgG was present in the serum of the lambs. The score registered by the sodium sulfite test

for al serum samples is in contradiction with the previous test results, because on the first day of the lambs life, it was registered the maximum score of 3+ just in one serum sample, for other two was 0.

Even after eight days, the results of both tests were still in contradiction, this time the serum samples presenting in the first day the score 0 for the sodium sulfite test, presented during the eight days interval the score 3+, respectively 1+, however, the turbidity of the zinc sulfate test was still observed at this moment for all samples (Table 3).

Brujeni et al. (2010), same as Demis et al. (2020) used in lambs a quantitative zinc sulfate turbidity test to estimate the total immunoglobulin levels, however, not for IgG concentrations. Comparing to the recent literature, it seems that this is the only study on lambs, that used a qualitative zinc sulfate test, could be reliable for use at farm level.

Tabel 3. Qualitative score of sodium sulfite and zinc sulfate test used for first and eight day serum samples in newborn lambs

First day of life		Eight day of life	
Sodium sulfite turbidity test	Zinc sulfate turbidity test	Sodium sulfite turbidity test	Zinc sulfate turbidity test
0	Turbid	3+	Turbid
0	Turbid	1+	Turbid
1+	Turbid	0	Turbid
2+	Turbid	0	Turbid
2+	Turbid	0	Turbid
2+	Turbid	0	Turbid
3+	Turbid	0	Turbid
2+	Turbid	0	Turbid
2+	Turbid	0	Turbid
0	Turbid	0	Turbid

Based on the good quality of the colostrum in this study, it can be estimated that there is a strong correlation with a good IgG absorption by the lambs small intestines established by the positive score of zinc sulfate test.

CONSLUSIONS

All of the described and used assay methods from this study need rigorously handling, however, require minimal instruments.

The IgG colostrum quantity and levels of absorbtion can be easily estimated throughout the use of Brix refractometer and by zinc sulfate turbidity test, respectivelly. The clear relation between these two methods, recommends them to be performed in a routine manner for the perinatal management of lambs at farm-level.

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EVALUATION OF AN ANAESTHESIA PROTOCOL FOLLOWING TRANSLOCATION OF FERAL HORSES OUTSIDE THE LETEA FOREST

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Abstract

This study was performed in order to evaluate an anaesthetic protocol used for remote chemical immobilization of ten free ranging feral horses (Equus ferus caballus) for the following purposes: translocation, health assessment and/or contraceptive immunization. Horses were immobilized using a Ketamine/Medetomidine combination delivered remotely with a 6 mL dart syringe from a dart gun. A mean ($\bar{X}$) induction time of 7 minutes (SD = 2.82) with a $\bar{X}$ = 83.3 minutes of recumbency was recorded using $\bar{X}$ = 2.37 mg/kg Ketamine (1.89-2.81 mg/kg) and $\bar{X}$ = 0.09 mg/kg Medetomidine (0.08-0.1 mg/kg). Heart rate ($\bar{X}$ = 38.43), respiratory rate ($\bar{X}$ = 26.14), rectal temperature ($\bar{X}$ = 35.12), relative arterial oxygen hemoglobin saturation ($\bar{X}$ = 83.11) and capillary refill time (1-1.5 s) were monitored during anaesthesia, every 10 minutes after induction until recovery. This protocol was suitable for the chemical immobilization of the feral horses from the Danube Delta - Romania providing a good anaesthesia depth and muscular relaxation. Nevertheless, supplementation of Oxygen is recommended for the resulting hypoxia.

Key words*: chemical immobilization, feral horses, ketamine, medetomidine, translocation.*

INTRODUCTION

This current study is part of ARCA's Feral Horse Birth Control Program in collaboration with Danube Delta Biosphere Reserve Authority and Romsilva Tulcea and took place in Letea Sandbank, Tulcea County, Romanian Danube Delta. Letea Sandbank consists mostly by forests and grassland (app. 10.000 ha), from which 2825 ha is represented by the strictly protected Letea Forest which is surrounded by a fence. According to ARCA's aerial census form 2019, up to 600 feral horses roam free in this area, from which 300 live in the Letea Forest (Roșu, 2017).

Due to the potential negative impact on the vegetation done by the rising number of horses in the Letea Forest, a need of both physical removal and reproduction control was necessary. Therefore, during ARCA's Birth Control Program, a protocol of chemical immobilisation was evaluated for ten horses from/near the Letea Forest with the purpose of translocating them outside the forest and/or contraceptive vaccination with Porcine Zona Pellucida (PZP) (Roșu et al., 2014; Roșu, 2017).

MATERIALS AND METHODS

The study was carried out from January to November (2019-2020). Ambient temperatures ranged from -4ºC to 6ºC (average of 1.75ºC), with an average wind speed of 5.1 m/s and a maximum of 6.6 m/s. No immobilizations had been carried out during snowfall or heavy rain. During heavy wind, the immobilization occurred in the wind shadows of the forest. Seven mares and three stallions were part of the study. They were either translocated (or already outside/near the Forest), microchipped, ear tagged and the mares received an immunocontraceptive vaccine with PZP (Roșu, 2017). All ten horses were healthy based on their physical appearance and behaviour and were members of established harem groups. The procedures involved physical restraint and transportation of eight horses outside the Forest. The car travelled each time about 3-4 km distance on a forest road. The two remaining horses of the study were immobilized only for reproduction control and did not need translocation, being outside the forest. For remote chemical immobilization, Ketamine dry powder (Ketamine 1 g, Kyron

Laboratories, Johannesburg, South Africa) diluted with Ketamine 100 mg/ml (Ketamidor®, Richter Pharma ag, Wels, Austria) and Medetomidine 40 mg/ml® (Kyron Laboratories) were used. The substances were delivered by Pneu-Dart® type "U" through a compressed air tranquilizer dart-gun Pneu-Dart® X-Caliber. The chemical immobilizations were done using a single disposable 6 ml dart, with a standard combination of 775 mg/horse Ketamine (K) and 30 mg/horse Medetomidine (M). A combination of Ketamine powder, diluted with a solution of Ketamine and Medetomidine was calculated and divided for 6 ml darts, according to the following formula: Ketamine dry powder 1000 mg + 2100 mg, injectable Ketamine 100 mg/ml (21 ml) +120 mg Medetomidine (3 ml)-total of 24 ml, 6 ml/dart used for anaesthesia.

Horses were darted from 15 to 40 m distance in the rump or cervical region. First signs of induction were noticed when mild ataxia and stilted gait occurred. The induction time was evaluated form the time of successful dart placement to lateral recumbency. Once induced horses were blindfolded and placed with the head and lower forelimb extended at the beginning of anaesthesia then restrained for transportation. All hands-on procedures were done in a safe zone behind the horse's spine to prevent any possible injury (Gimenez R. et al., 2008). During anaesthesia, heart rate, pulse rate (evaluated using a stethoscope or with the pulse oximeter Nonin® 2500 A; Minneapolis, USA with the probe attached to the tongue), respiratory rate (evaluated using a stethoscope or by observing the thoracic movements), rectal temperature (digital thermometer), oxygen haemoglobin saturation - SpO_2 (Nonin® 2500 A; Minneapolis, USA) and capillary refill time were monitored every ten minutes after induction, until recovery (Muir WW, Hubell JAE, 2009). For the transportation, the following restraint method was used: the horses in lateral recumbency were pulled on the rescue glide (8' x 4' Assisting Glide, L.A.R.G.E Inman, South Carolina, USA) through the backside manner technique, legs were pulled and tied together by hobbles (Nylon Hobbles, L.A.R.G.E Inman, South Carolina, USA) at the pastern level and secured to the glide holes by one 2 m long strap (Gimenez R. et al., 2008). The head was fitted with a halter and secured to the glide and the glide front was attached to the car by a 1 m long stap. The horses were monitored during transportation from the back of the car through the glass window, respiratory frequency was evaluated every ten minutes by counting the thoracic movements or the warm air coming out the nostrils. At the end of anaesthesia 25 mg Atipamezol (Antisedan® 5 mg/ml, Orion Corporation Animal health, Turku, Finland)/horse was administrated iv.

During the recovery phase the horses were monitored based on the behaviour changes during sternal recumbency (calm/increased muscle tension/padding), on the transition to sternal recumbency with the number of attempts to recover the standing position, after stimulation (calm, well-coordinated/ difficult/ rolls to the other side/immediately tries to stand), description of sternal recumbency, transition to standing position, balance and coordination while standing, an overall impression and the final score.

Total body weight was measured with the tape measure technique. For the tape technique the horses were measured around the girth (times 2) while recumbent, then the value was multiplied by the body length measured from point of shoulder to point of the ischial tuberosity in cm, and divided by a standard number, resulting the weight in kilograms.

The set-out formula to asses body weight in horses (Elizabeth L. Wagner et al., 2011) is:

weight (kg) = [(heartgirth^2 × body length)/ (11,880 cm^3)].

RESULTS AND DISCUSSIONS

Two groups of horses were established according to their weight: 200-300 kg, 5 horses, > 300 kg, 5 horses.

Seven out of ten horses were immobilized after administering anaesthesia through a single dart and three needed a supplemental standard combination dart (775 mg/horse Ketamine (K) and 30 mg/horse Medetomidine (M) since they were not induced/or presenting any signs of induction at 15 minutes after a fully discharged dart.

For induction of anaesthesia the following total dosages were used in this study: $\bar{X}$ = 2.37 mg/kg Ketamine (1.89-2.81 mg/kg) and $\bar{X}$ =

0.09 mg/kg Medetomidine (0.08-0.1 mg/kg). The mean ($\bar{X}$) induction time was 7 minutes (SD = 2.82) with a $\bar{X}$ = 83.3 minutes of recumbency after induction. Heart rate ($\bar{X}$ = 38.43), respiratory rate ($\bar{X}$ = 26.14), rectal temperature ($\bar{X}$ = 35.12), relative arterial oxygen haemoglobin saturation ($\bar{X}$ = 83.11) and capillary refill time (1-1.5 s) were monitored during anaesthesia, every 10 minutes after induction until recovery (Table 1).

Table 1. Physiological parameters measurements (given in mean ± SD and range) - Temp. = temperature; SpO_2 = relative arterial oxygen haemoglobin saturation; CRT = capillary refill time, K = Ketamine (additional intravenous dose administered), A = Atipamezole (a total intravenous dose administered), T1 = induction time, T2 = recumbency time, n = number of animals evaluated

Variable	n	200-300 kg	n	>300 kg
HR (bpm)	5	36.58 ± 5.95 (31-54)	5	39.78 ± 10.78 (28-68)
RR (breaths)	5	26.12 ± 4.28 (20-36)	5	26.97 ± 4.6 (18-40)
Temp (°C)	5	34.83 ± 1.14 (32.7- 36.6)	3	35.42 ± 0.73 (34.1-36.8)
SpO_2 (%)	5	83.87 ± 5.88 (71-96)	5	82.6 ± 7.93 (64-97)
CRT (sec)	5	1.28 ± 0.26 (1-1.5)	5	1.25 ± 0.24 (1-1.5)
T1 (minutes)	5	7.2 ± 2.38 (4 – 10)	5	6.8 ± 3.49 (2-11)
T2 (minutes)	5	74.2 ± 14.21 (62 – 95)	5	92.2 ± 44.66 (64-169)
K (mg) iv	0	-	3	600 ± 264.5 (400.0-900.0)
A (mg) iv	3	25	5	25

During recovery all horses experienced a certain degree of ataxia. All horses were provided with good myorelaxation during transportation and the medical assessments. Three horses required supplemental doses of ketamine (top up administered iv in the jugular vein) due to signs of spontaneous recovery or incomplete immobilization. Mean translocation time ($\bar{X}$) of 24 minutes was recorded (SD = 9.94 minutes). The horses immobilised to the rescue glide attached to the car were extracted outside the Letea Forest fence, one by one, using the forest roads trying to protect the surrounding vegetation. Field conditions (the area where the anesthetised horse fell asleep and the difficulty of finding it, fences, trees, bad weather: heavy rain, fog) made animal extraction challenging in some situations, thus the duration of transportation longer. One stallion (exception) with the duration of recumbency of 169 minutes received two intramuscular darts (the second one was given because the stallion was in a standing position after the first dart, although already sedated) and needed supplementation of anaesthesia (two top ups). It took 25 minutes to pull the glide with the horse and attach it to the car so it could be translocated (first top up of Ketamine during the extraction). During the transportation (total of 35 minutes) it showed signs of spontaneous recovery (ear twitching, muscle contraction, head tilt). The horse managed to get up and rolled over while connected to the rescue glide with the straps and hobbles. The car was stopped, the anaesthesia was supplemented IV and the hobbles and straps securely repositioned. The recovery was prolonged and in standing position the stallion was ataxic and unstable for about 10 minutes. At the end of anaesthesia eight of the horses were antagonized with 25 mg Atipamezole/horse iv as a standard dose, while two recoveries were spontaneous. The horses that received antagonization, were assisted recoveries (the horses needed help transitioning from lateral recumbency to sternal recumbency, then to standing position) by hand.

CONCLUSIONS

The standard combination used proved to be effective for the remote chemical immobilization of the feral horses, providing an appropriate degree of anaesthetic depth and myorelaxation.

All the horses showed a slight increase in cardiac and respiratory frequency, with the decrease of tissular oxygenation and temperature. The resulting peripheral hypoxia can be countered with the supplementation of oxygen which was difficult to do in field conditions. One factor that influenced the respiratory frequency and amplitude was the restraint and transportation of horses on the glide.

An advantage of the anaesthetic combination is the possibility of antagonizing one of the components (M).

To the best of the authors' knowledge this is the first case of equine transportation by car with the rescue glide under general anaesthesia for the purpose of translocation under continuous monitoring. This procedure can be potentially

dangerous for the animal and it has to be carefully monitored.

All the procedures were conducted with safety measures taken at each step. No mortalities were recorded.

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DIAGNOSIS AND TREATMENT OF ACQUIRED MYASTHENIA GRAVIS IN AN AMERICAN STAFFORDSHIRE TERRIER DOG

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Abstract

Myasthenia gravis is an immune-mediated disease that affects the neuromuscular junction due to the increased production of antibodies directed against the acetylcholine receptors of skeletal muscles. The consequence is the impairment of action potential transmission from nerve to muscles. Although in human medicine, Myasthenia gravis is a well-known condition with diagnostic protocols and several therapeutic strategies, in veterinary medicine diagnosis and therapy for this condition are still challenging. This study presents the case of a 5-year-old male American Staffordshire Terrier referral to the Clinic of Faculty of Veterinary Medicine in Bucharest for a neurological consultation due to a history of fatigue, limb tremor and reluctance to exercise. After the complete physical and neurological examination, the findings were consistent with Myasthenia gravis, so neostigmine methylsulfate was administered intravenously to confirm the diagnosis. Considering the positive clinical reply that was obtained after 15 minutes, a long-term therapeutic scheme was established and the patient was reassessed periodically.

Key words*: myasthenia gravis, neuromuscular disease, neostigmine methylsulfate, weakness, veterinary neurology.*

INTRODUCTION

Acquired myasthenia gravis (MG) is an immune-mediated disease that implies the production of autoantibodies that act against acetylcholine receptors of skeletal muscles (Shelton, 2002; Plat & Olby, 2004). In dogs, this pathology is documented by several case studies and retrospective case series, and it exhibits many similarities to the corresponding disorder of people (Dewey et al., 1997).

From the clinical perspective, the symptomatology of MG is subsequent with a generalized peripheral nervous system (PNS) disorder and very often its diagnosis is a challenge even for experienced neurologists. The difficulty lies in the variability of PNS symptoms that do not follow a clear pattern, considering that they are influenced by factors like the moment of onset, clinical course, severity and extent of the disease (Shelton et al., 1997). However, most systemic neuropathies, including MG, are characterized by an insidious onset and a chronic course, so whenever a disease of the peripheral nervous system is suspected, a differential diagnosis between polyradiculoneuritis, MG, megaoesophagus or disorders of neuromuscular transmission like tick paralysis, botulism or organophosphate toxicity must be performed (Platt & Olby, 2004). Although the gold standard for the diagnosis of MG is the detection of serum autoantibodies against muscle acetylcholine receptors by immunoprecipitation radioimmunoassay, in the absence of this method, other testing procedures can be used (Conti-Fine, 2006). An example is a pharmacological test that implies the administration of an ultra-short acting anticholinesterase drug. A positive response with an obvious improvement of muscle strength within several minutes of administration is very suggestive for MG (Shelton, 2010).

This study presents the clinical signs, the diagnostic approach and the therapeutic management of a 5-year-old American Staffordshire Terrier dog with a presumptive diagnosis of MG.

MATERIALS AND METHODS

The dog included in this case study was referred to the Clinic of the Faculty of Veterinary Medicine of Bucharest during 2018

for a neurological assessment. The evaluation was performed according to the protocol already implemented in our clinic that involves strict follow-up of the following stages: animal signalment, history, physical and neurological examination, neurolocalization of the disease, differential diagnosis using the acronym VITAMIND (vascular, inflammatory/ infectious, traumatic, anomalous, metabolic, idiopathic, neoplastic, degenerative), recommendations of paraclinical investigations, diagnostic and treatment (Thomas, 2010; Dewey & da Costa, 2016).

After the positive response obtained at the pharmacological test, for this patient, the therapeutic goal was to improve muscle strength and to minimise the adverse effects of the medications until remission of the disease was obtained, so we use pyridostigmine bromide as an acetylcholinesterase inhibitor (Dissanayake et al., 2006; Engel et al., 2015; Stanciu & Solcan, 2016). In addition, we completed the treatment with a product that supports the function of the liver (administered to diminish the adverse side effects of pyridostigmine bromide) and Omega-3 supplementation for its effect on functional brain activation and reduction of inflammation in autoimmune diseases.

For two years and a half, the dog was reassessed every 3 months, even after complete remission of the disease. In addition, the treatment dose was adjusted periodically according to the patient's evolution.

Animal signalment and history

A 5-year-old dog, intact male, American Staffordshire terrier was referred for neurological consultation. The dog came to the Clinic of the Faculty of Veterinary Medicine of Bucharest in September 2018. After the discussion with the owner, we discover that ten days before consultation, the patient started to manifest difficulty in getting up and walking, a need to rest after minimal efforts and stiffness of the limbs (first the hind limbs were affected and after several days the same symptoms were observed on the thoracic limbs, too). The appetite for food and water remained unchanged during this period and the macroscopic aspect of urine and faeces was normal. Vaccination and deworming schemes were completed and updated according to standard protocols. The history did not reveal signs of another recent illness (coughing, lack of appetite, vomiting, inactivity or agitation), trauma or exposure to toxins. Initially, the owner suspected an orthopaedic problem and asked for a surgeon opinion, at another clinic. No abnormalities of the locomotory system were detected, so the dog was referred for a neurological examination. The treatment received after the initial evaluation consisted of supplementation with B vitamins, in injectable form, as an attempt to reduce the weakness manifested throughout the patient's body. The evolution of the disease was progressive and the owner did not observe any improvements after the administration of the vitamins.

Because the clinical presentation was compatible with a neuromuscular disease, we performed a full physical examination, that was completed by neurological assessment to identify and establish the localisation of the lesion within the nervous system. Based on the corroboration of the results, we requested a series of paraclinical investigations for diagnostic confirmation. Findings were recorded in the neurological examination sheet, which has been used to monitor the subsequent evolution of the case. Finally, we established the treatment protocol and we settled on the following check-ups.

RESULTS AND DISCUSSIONS

Physical and neurological examination findings

Physical examination revealed a normal temperature (38.8°C), a heart rate of 87 beats per minute and a synchronous femoral pulse, present bilaterally. The respiratory rate was mildly elevated (43 respirations per minute), but we associated this value with the stress trigger by the environmental change, considering that at home the dog's respiratory rate was normal. All palpable lymph nodes were mobile, painless and normal size. The colour of the mucous membranes was pale pink and we obtained a capillary refill time of 1.5 seconds. The patient did not express pain when the abdomen was deeply palpated. The physical examination was followed by neurological examination, which included an evaluation of

the mental status, posture, cranial nerves, proprioception, gait, spinal reflexes and sensory testing to establish the localization of the lesion within the nervous system. For this dog, neurological evaluation showed several deficits:

- The **posture** was characterised by permanent sternal recumbency. However, when the patient was encouraged to move, he could support his weight and walk only for a short period (seconds), after which he displayed progressively over flexion of the joints and a crouched stance that forced him to rest and recover the strength needed to walk again (Figure 1).

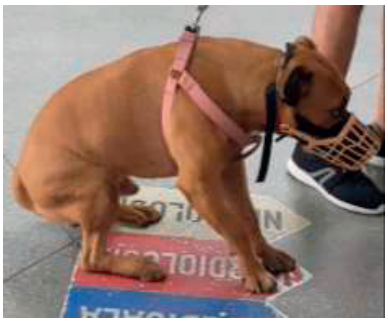

Figure 1. The dog showed progressive overflexion of the joints and crouched stance

- Regarding the gait, we observed tetraparesis, a narrow base of support on the thoracic limbs and the tendency to step on the dorsal surface of the paw on the forelimbs. The examination showed also hypometria, short steps with hyperflexion of the joints, dragging of the nails (with consequent noise of rubbing the nails on the ground), crouched stance with lowered tail, emprosthotonus and decreased ability to support the weight. Also, during gait, the whole musculature of its body was tense.
- Postural reactions were difficult to be assessed due to the patient's inability to support his weight.
- Mental status, behaviour, cranial nerve, spinal reflexes and sensory testing were normal for this dog.

The next step after the neurological examination was to establish the localization of the lesion within the nervous system. For this case, our differential diagnosis was made between a C1-C5 lesion (that would have evolved also with tetraparesis) and a peripheral nervous system lesion (which could have affected the nerve, the neuromuscular junction or the muscle).

Taking into consideration the acute onset, the deteriorating clinical course, the symmetry of the deficits, the lack of pain involvement and the signalment of the patient, according to, "VITAMIND" acronym, we ruled out most of the causes that could have generated exercise intolerance, but instead, we kept the suspicion of congenital or metabolic causes. The differentiation between the two will be made based on paraclinical investigations.

Paraclinical investigations and diagnosis

To obtain an aetiological diagnosis, we recommended a complete cell blood count (CBC), a serum chemistry panel, a cardiologic examination and radiography of the cervicothoracic chest to rule out megaoesophagus. No abnormalities were detected on blood analysis and the cardiologist did not identify any modification of the heart that could have produced the symptomatology.

On radiological examination of the cervical spine at the level of C1-C5 area was excluded any radiological signs such as narrowing of the intervertebral space, mineralization of the discs or other signs consistent with initial suspicion.

The radiological signs specific for megaoesophagus such as ventral deviation of the trachea, the radiolucent band superimposed on its projection area with the highlighting of the oesophageal wall in the form of a narrow radiopaque band, well delimited, which surrounds the radiolucent area resulting from aerophagia were not visible in this case and ruled out.

Due to the suspicion of acquired Myasthenia gravis, we decided to perform a pharmacological test by the administration of 1 ml neostigmine methylsulfate intravenously (iv) (Miostin® - 0.5 mg/ml) as an acetylcholinesterase inhibitor. Although the recommended substance for this test is edrophonium chloride, this drug is not available in Romania, so we use the alternative anticholinesterase agent.

Before the Miostin® administration, the dog was reluctant to move and had difficulty sustaining its bodyweight. Approximately 5

minutes later, he left decubitus and started to move and after another 10 minutes, we observed an obvious improvement of strength and better movement coordination.
Considering the positive response to the pharmacological test, the suspicion of Myasthenia gravis was confirmed.

Treatment and follow-up

Established treatment consisted of an oral anticholinesterase drug (pyridostigmine bromide - Mestinon®) at an initial dose of 2 mg/kg every 8 hours. In addition, we added silymarin for his role in diminishing hepato-toxic reactions and Omega-3 supplementation for its effect on functional brain activation and reduction of inflammation in autoimmune diseases.
The owner was informed that in this early stage of the disease, the prognosis is still guarded and a recidivation can occur even though remission is obtained (Mao et al., 2010). He agreed to follow our recommendations and to come back for a check-up after 2 months of treatment.

Figure 2. First evaluation after the onset of treatment - The posture was improved and the dog was able to walk without any signs of fatigue

On November 11, the dog came back for revaluation and an important progression was noticed. The patient could stand and walk without stopping and without any signs of fatigue. The mental status was normal, alert, the appetite for food and water remained unchanged and no adverse side effects of the pyridostigmine bromide have been reported by the owner (Figure 2).
Under these circumstances, the treatment recommendations remained the same, and the next visit was scheduled after another 8 weeks.
In January 2019, we noticed the same positive evolution, except for the occurrence of vomiting episodes (white foam) at intervals of about 2 weeks. The serum chemistry panel was repeated and we found a mildly increased level of the enzyme γ-glutamyl transferase and a high level of creatine kinase (1220 U/l) - which could be explained on the basis of the generalised muscle inflammation produced by the autoimmune disease (Garlepp et al., 1984). On the same day, abdominal ultrasound was performed and no other internal abnormalities have been found. We added at the previous therapeutic protocol a product containing L-Ornithine Aspartate, L-Arginine Hydrochloride, L-Citrulline and Acetyl Methionine (Ornitil®) as a hepatic metabolism aid. After one year of treatment, the dose of pyridostigmine bromide was reduced to 1 mg/kg every 12 hours. No other side effects have been reported during treatment.
Over the next two and a half years, periodic checks were performed every 3 months. The result was that from the onset of the disease, the dog did not show any episodes of relapse and its general condition remained unchanged.

CONCLUSIONS

1. For this case, the diagnosis of Myasthenia gravis was based on the typical clinical signs: reluctance to exercise, chronic hindlimb's weakness, tetraparesis, hypometria, progressive over flexion of the joints and a crouched stance during the walk.
2. The pharmacological test with neostigmine methylsulfate showed an obvious improvement of strength and better movement coordination, so the initial diagnosis was confirmed.
3. Proper cooperation between doctor and owner is essential since the owner must evaluate the results of treatment with pyridostigmine bromide, whose dose was adjusted according to patient evolution and

no significant side effects have been reported.

4. From the onset of the disease until the present, the patient was reassessed every three months and the evolution remained favourable, without any other episodes of relapse.

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URINALYSIS IN THE DIAGNOSTIC WORKUP - A CASE SERIES

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Abstract

This study demonstrates the utility of performing a relatively simple, inexpensive test during the diagnostic workup of several canine patients presented in the Internal Medicine Clinic of the Faculty of Veterinary Medicine of Bucharest. Although universally recommended, urinalysis is infrequently used in our clinic when not diagnosing and monitoring lower urinary tract disease. The cases presented will reinforce the necessity of performing a simple urinalysis and various pathologies that induce alterations of the urine, either in its chemical or cellular constituents or by interfering with the ability to concentrate urine. However, urinalysis is not diagnostic in every patient - it should be interpreted in light of the clinical presentation and of the other laboratory tests. The use of this test in the diagnosis of most non-urinary diseases requires serial examinations to emphasize persistent alterations and demonstrate the need for a diagnostic workup.

Key words*: urinalysis, dog, diagnosis, screening.*

INTRODUCTION

Urine is the product of glomerular filtration, followed by tubular resorption and secretion. It can provide essential information about renal and systemic diseases; thus, a complete urinalysis should be part of the minimum database for all patients (Callens & Bartges, 2015). It is indispensable for patients with urinary tract signs, kidney injury or disease, polydipsia, and polyuria. It also has several advantages: it does not require technical expertise and it has a high diagnostic significance for a low cost.

Urinalysis comprises physical examination (color, turbidity, and urine specific gravity, abbreviated USG), chemical analysis (particularly pH, protein, glucose, ketones, bilirubin, and blood/heme), and microscopic examination. Some urine dipsticks also measure urine creatinine and microalbumin (urine albumin values under 30 mg/dL), which can assist in identifying early proteinuria (Lees et al., 2005; Elliot et al., 2017). USG measurement using the dipstick is unreliable in small animals; it should be measured using a refractometer (Stockham & Scott, 2014). The microscopic examination of the sediment describes and characterizes cells (erythrocytes, leukocytes, and epithelial cells), bacteria, casts, and crystals present in the sample.

This study supports the use of urinalysis whenever possible by presenting different pathologies and the utility of different urine abnormalities in their diagnosis.

MATERIALS AND METHODS

Basic urinalysis was performed in the diagnostic workup of most of the dogs seen in the Internal Medicine Clinic by the first two authors in 2019. For each of these patients, the complete history (signalment, past complaints, environment, diet, reproductive history, vaccination status, and current and past medication), the reason for presentation, general appearance, vital signs, and physical examination findings were noted.

Several criteria were used to select relevant cases. First, the owners' compliance, adherence to recommendations, and assent to return for follow-up examinations and laboratory testing had to be satisfactory. Second, the diagnosis had to be either definitive or to have a high degree of confidence. Thirdly, the patients had to be monitored for at least 3 months. Finally,

four different presentations were chosen to exemplify a few common urine abnormalities and situations in which urinalysis is invaluable.

RESULTS AND DISCUSSIONS

The first case is a common presentation in a veterinary clinic – an 8-year-old male intact mixed-breed dog presented for acute vomiting and malaise. It was an indoor pet fed a high-quality diet; dewormed, fully vaccinated, with no relevant prior diseases. The dog was not receiving any drugs or supplements, and its appetite had varied recently. It had vomited several times over the past few months; on this occasion, it was undigested food and bilious material.

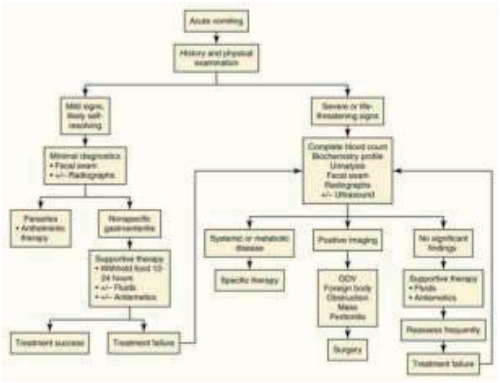

Figure 1. Algorithm to approach acute vomiting. Urinalysis is part of the minimum database. GDV, Gastric dilation-volvulus. From Ettinger et al., 2017

Clinical examination identified mild pain in the cranial abdomen, dehydration, and low normal body temperature. The initial evaluation included a CBC, serum biochemistry, and urinalysis (Figure 1). The CBC and blood smear described an inflammatory leukogram, while the biochemistry revealed increased total protein, liver enzymes (alanine aminotransferase, ALT and alkaline phosphatase, ALP), a slight increase in blood urea nitrogen and creatinine, and low normal glucose. Urinalysis of free-catch yellow urine showed 1.040 USG, 6.5 pH, 6 mg/dL bilirubin, 30 mg/dL protein, and negative glucose, urobilinogen, blood, and leukocytes. The urine sediment was inactive (Reppas & Foster, 2016; Stockham & Scott, 2014): <5 RBCs (erythrocytes) and <5 leukocytes per hpf (40x objective), no casts, small numbers of amorphous crystals, and numerous bilirubin crystals were observed. Despite applying pressure to the venipuncture site, it bled for over 5 minutes. Thus, a coagulation panel was recommended and the results were prolonged prothrombin time and partially activated thromboplastin time, normal thrombin time, and increased fibrinogen.

Urinalysis is essential to evaluate patients' renal function and any interpretation of blood urea nitrogen and creatinine should be interpreted along with the USG of a urine sample obtained at the same time (Pressler, 2013; Elliot et al., 2017). In this patient with azotemia, USG confirmed prerenal azotemia (dehydration due to emesis) and excluded overt kidney disease. It also identified the presence of bilirubinuria. Bilirubinuria with normal CBC, increased liver enzymes, and prolonged coagulation time supports a diagnosis of liver disease. The next steps were abdominal ultrasonography and SNAP cPL (canine pancreas-specific lipase) evaluation. Ultrasonographic examination identified diffuse hepatic hypoechogenicity, thickened biliary ducts, and biliary sludge. There was no identifiable pancreatic disease and the qualitative cPL was normal. The tentative diagnosis was liver disease due to cholangiohepatitis and secondary coagulopathy. The owner refused a hepatic biopsy. The dog was treated with fluid therapy, antimicrobials, analgesics, antiemetics, ursodiol, vitamin K1, antioxidants, and diet. It evolved favorably and the serum chemistry, urinalysis, and coagulation parameters normalized; however, biliary sludge was still present at subsequent ultrasonographic evaluations. In this patient, repeated urine chemistry evaluations were used to monitor bilirubin. Increases in urine bilirubin appear earlier than in plasma, and significantly earlier than bilirubinemia can be identified as icterus (Stockham & Scott, 2014).

Another common presentation in the internal medicine clinic is the polydipsic polyuric dog. A 13-year-old male intact Chihuahua presented to the clinic for increased excessive water consumption and urine production of unknown duration. It was up to date on vaccination and parasite prevention, fed a mixture of home-

cooked food and a high-quality kibble; it was not receiving any drugs. The owner noticed a variation in appetite (alternatively increased or decreased). The clinical examination revealed a dry, thinning, and lusterless hair coat, thinning of the hair on the tail ("rat tail"), a reduction in muscle mass, and a heart murmur over the mitral valve. The rest of the clinical examination was unremarkable.

The work-up began with the examination of a sample of free-catch urine to evaluate the USG and confirm polyuria (Figure 2). It was light yellow and slightly turbid. The urinalysis results were USG 1.025, pH 7, glucose 1000 mg/dL, protein 30 mg/dL, ketones 15 mg/dL and bilirubin 0.5 mg/dL. Glycosuria increases USG by 0.004-0.005 for each 1 g/dL (Stockham & Scott, 2014); in this situation, polyuria can coexist with an increased USG. The other dipstick parameters were negative. The sediment examination revealed active sediment (<5 RBC and more than 5 leukocytes per hpf and <2 hyaline casts per lpf). The urine culture was negative.

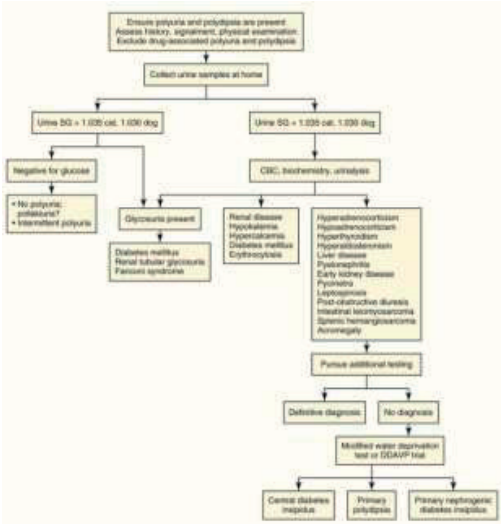

Figure 2. Algorithm for polyuria and polydipsia in dogs. Urinalysis is essential for a diagnosis.
From Ettinger et al., 2017

The data confirmed polyuria and identified significant glycosuria, which occurs in diabetes mellitus, renal tubular glycosuria, and Fanconi syndrome (Ettinger et al., 2017; Bruyette, 2003). The Chihuahua had hyperglycemia, increased liver enzymes (ALP>700 U/L, ALT 250 U/L), triglycerides, and cholesterol; uremia was not present. Fructosamine was also increased in this patient (450 μmol/L). CBC and blood smear revealed an inflammatory leukogram and normocytic normochromic anemia of chronic disease. An autoimmune polyendocrine syndrome was suspected, but the owners refused further testing; the dog was administered long-acting insulin 2x/day. The owners were encouraged to use urine dipsticks at home to monitor glycosuria and present the animal for a glucose curve if an insulin dose change was required based on worsening clinical signs and presence of significant and persistent glycosuria. Its evolution was favorable.

A clinical presentation in which urinalysis is irreplaceable is the cat presented for lower urinary tract disease. A 7-year-old neutered female shorthair was presented for discolored urine, dysuria, and periuria (house soiling). The history revealed a history of recurrent urinary tract infections treated symptomatically (with compliance on behalf of the owner) and intermittent urinary tract disease signs for the past years. A thorough history identified several stress factors: a 2-year-old baby in the family, changes in its environment, and imposed restrictions. The cat was fed dry kibble and occasionally wet food. Its appetite, defecation, and fecal appearance were normal. The only significant findings on the physical examination were an empty bladder and thinning hair in the perineal region. The kidneys had a normal size, shape, and position.

To obtain a urine sample, intravenous fluids were administered; cystocentesis was performed after a few hours. Ultrasonography was also completed, describing an irregular hypoechoic thickening of the urinary bladder wall; no anatomical defects, crystalluria, or calculi were observed. Urinalysis is essential for the diagnosis of lower urinary disease, to which cats are particularly susceptible (Figure 3). Physical examination and abdominal ultrasonography findings, as well as CBC and biochemistry results in the reference range ruled out systemic diseases in this patient.

The urine was light red due to hematuria, confirmed by centrifugation of the sample. The USG was 1.040. The dipstick was positive for heme (a speckled pattern on the reagent pad

that results from intact RBCs), protein (100 mg/dL), urobilinogen (1 mg/dL); the other tests were negative. It is important to stress that leukocyte esterase evaluation with a dipstick is unreliable in veterinary medicine, particularly for cats (Reppas & Foster, 2016).

Microscopic evaluation identified active sediment, and numerous RBCs, leukocytes, epithelial cells, rods, and struvite crystals. The urine culture was positive for a multi-drug resistant beta-hemolytic Escherichia coli, possibly because of the previous antimicrobial therapies. This was a complicated urinary tract infection (Weese et al., 2011), thus the antibiotic choice was based on the susceptibility results and administered for 4 weeks. Analgesics and a diet for cats with urinary tract disease living in stressful environments were also recommended. At 6 weeks, all clinical signs except periuria had abated; another urine culture was negative. The repeated urinalysis identified active sediment, a sign that inflammation was still present. The presumptive diagnosis was idiopathic cystitis, and the therapeutic options were presented to the owner. The cat improved with multimodal environment modification.

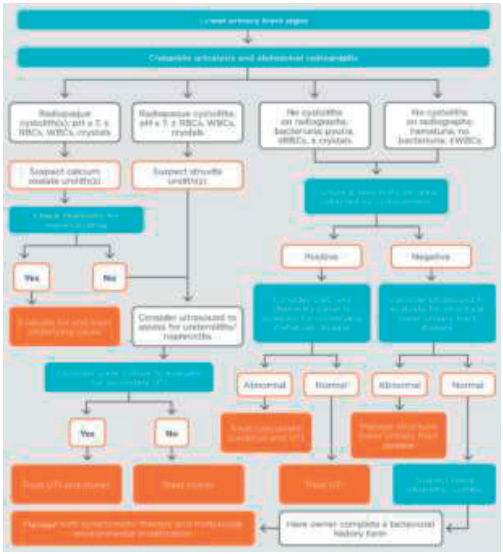

Figure 3. A diagnostic approach for the cat with non-obstructive lower urinary tract signs.
From Heseltine, 2019

A common finding on urinalysis is proteinuria. Persistent proteinuria should be investigated after concurrent inflammation in the lower urinary or genital tract is addressed. Its significance is greater in patients that present with inactive sediment or low USG. A 7-year-old outdoor neutered female German Shepherd was presented for progressive weight loss, decreased appetite, and reduced activity levels of several months' duration. The physical examination identified a right-sided heart murmur, normal lung sounds, and a body condition score of 2/5, with a noticeable loss of muscle mass. The vital signs were in reference intervals for large breed dogs. The minimum database, in this case, was composed of a CBC, biochemistry, urinalysis, as well as radiological and cardiological examinations. CBC revealed an increased number of reticulocytes, mature neutrophilia, and eosinophilia; microfilariae were detected in the direct blood smear and stained smear. A rapid enzyme-linked immunosorbent assay for Dirofilaria immitis antigen was positive. Blood chemistry identified low normal albumin, high-normal globulin, decreased albumin/globulin ratio, slight increases in alkaline phosphatase, alanine aminotransferase, blood urea nitrogen, and creatinine. The thoracic radiographs were normal and the cardiologist identified right ventricular hypertrophy. The dog was diagnosed with moderate heartworm disease. However, there was concern over the significant weight loss and the increased renal parameters.

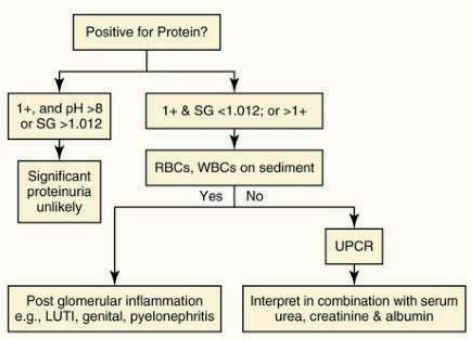

Figure 4. Interpretation of dipstick positive results for protein. From Ettinger et al., 2017

Urinalysis identified significant proteinuria (300 mg/dL) with inactive sediment and lipid droplets. The USG of 1.015 confirmed kidney disease. Proteinuria was further investigated by

measuring the urine protein to creatinine ratio (UPCr) on a fresh sample collected in an EDTA tube (Figure 4). The value obtained was 2.5; in an azotemic dog, this is suggestive of glomerular disease (Lees et al., 2004; Elliot et al., 2017). This created an index of suspicion for glomerulonephritis due to circulating immune complexes or microfilariae antigens. Urine protein electrophoresis was not available. The dog was referred for heartworm treatment with Melarsomine, and 6 months later proteinuria levels had decreased to values of 30-100 mg/mL, while the UPCr decreased to 1.3. Repeated evaluations of the degree of proteinuria and the UPCr are useful to evaluate the progression of renal disease and the patient's response to therapy (Elliot et al., 2017).

CONCLUSIONS

In veterinary medicine, urinalysis provides useful information at a low cost. The quantity produced and the macroscopic evaluation of the sample are highly informative through specific findings such as anuria or polyuria, color, and turbidity. The USG (that evaluates tubular function) and the presence of proteinuria (frequently more severe in glomerular disease) are essential to exclude or diagnose and stage kidney disease in small animals. The presence of glucose, bilirubin, protein, and ketones can also signal the presence of organ or system disease, and are essential in the diagnosis of most metabolic diseases. The sediment is invaluable in characterizing lower urinary tract disease and be helpful in diagnosing other pathologies (e.g. Ethylene glycol poisoning, proximal tubular disease, portosystemic vascular malformation). Finally, serial measurements of specific parameters can be used to monitor disease progression and adjust therapy accordingly.

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ASSESSMENT OF LEAD, CADMIUM, AND MERCURY TOTAL CONCENTRATIONS IN CATS BASED ON THEIR LIFESTYLE AND FEEDING CONDITIONS

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Abstract

Heavy metals are more ubiquitous as their uses have grown over the years. This research aimed to assess the total concentrations of some heavy metals (Pb, Cd, Hg) using cats' fur as an indicator, while also taking into consideration the age, living and feeding conditions of the cats. The fur samples were analysed by Inductively Coupled Plasma Mass Spectrometry. Generally, fur samples from cats that lived outdoors and that ate commercial food had higher total concentrations of heavy metals. The only exception is the total concentration of Cd, which was higher in the case of samples taken from cats living indoors compared to those living outdoors. In addition, samples taken from cats above the age of 5 had statistically significant higher Hg total concentrations compared to samples taken from cats between 3-5 years old. The findings of this research support the assumption that cats which are raised outdoors, in a polluted environment, accumulate higher total concentrations of some heavy metals. In addition, total concentrations of heavy metals also rise as the cats get older.

Key words*: lead, cadmium, mercury, fur, cats.*

INTRODUCTION

In high concentrations, heavy metals have proven toxic effects in all live organisms. However, because of their benefits in various manufactures, they are still used, and exposure to heavy metal sources can cause intoxications.

Pb can be used for the manufacture of various household appliances, pipes, or paints, or as protection against X-rays (De Francisco et al., 2003; Gulbinska, 2014; Jensen, 2013; Rădulescu & Lundgren, 2019).

Absorption of Pb occurs in the small intestine, especially in the duodenum (Conrad & Barton, 1978).

Pb excretion occurs mainly in the urine. Other routes of excretion include bile, sweat, and saliva (Conrad & Barton, 1978; Saran et al., 2018).

Pb can be stored in bone in an inert form (Conrad & Barton, 1978).

Pb bone deposits can supplement circulating Pb long after exposure has ended (Fleming et al., 1997; Smith et al., 1996).

Pb has numerous toxic effects, including inhibition of heme synthesis, by blocking Fe incorporation (Haeger-Aronsen, 1960). Pb also decreases erythrocyte lifespan, being able to cause anaemia (Hernberg, 2000; Schwartz et al., 1990).

Due to its high pollutant potential, the use of cadmium (Cd) has decreased, but Cd is still used for alloys, pigments and, most commonly, NiCd batteries (Huff et al., 2007; Morrow, 2004).

After absorption, most Cd is bound to a cysteine-rich protein called metallothionein (Nordberg, 2004). Cd can also bind to cysteine, albumin, glutathione and other proteins with sulfhydryl groups. Metallothionein synthesis can be stimulated by Cd, over 90% of the amount of Cd in the intestinal cytosol being bound to metallothionein (Klaassen et al., 2009; Waalkes, 2003).

Cd accumulates mainly in the liver and kidneys, which are organs with high levels of metallothionein (Klaassen et al., 2009).

Finch et al. (2012) performed a study on cats and concluded that Cd is involved in the occurrence of hypertension, because hypertensive cats had higher urinary Cd levels compared to normotensive cats.

Cd can interfere with Ca metabolism and cause a decrease in bone density and a predisposition to fractures (Martelli et al., 2006; Huff et al., 2007).

Mercury (Hg) is the only metallic element in liquid form at room temperature (Blum, 2013; Senese, 2018).

Hg is used in several manufactures, such as manufacture of thermometers, barometers, dental amalgams, or liquid mirror telescopes (Akhavan, 2011; Hammond, 2000; Hickson & Lanzetta, 2003; Srivastava, 2008; Watt, 2005).

It is not completely understood by which mechanism Hg is absorbed in the gastrointestinal tract. Hg absorption appears to be related to thiol-containing compounds to which it can bind, and can be absorbed in the small intestine due to the amino acid and peptide transporters in enterocytes (Bridges & Zalups, 2005; Dave et al., 2004; Foulkes, 2000).

Methylmercury, an extremely toxic organic Hg compound, undergoes intensive enterohepatic recirculation, 90% being excreted in the faeces. Inorganic Hg salts are excreted in the urine and faeces (Goran & Crivineanu, 2016; Liu et al., 2008).

Hg, in any form, whether it is organic or inorganic, binds to sulfhydryl groups. Thus, Hg has the potential to affect any tissue, but mainly targets the brain (Bernhoft, 2012).

Hg can cause acute and chronic intoxication (Rustagi & Singh, 2010), and organic Hg compounds are more toxic than inorganic ones (Sin et al., 1983).

In case of acute intoxication with Hg salts, vomiting, haemorrhagic diarrhoea and necrosis of the intestinal mucosa can occur.

Chronic Hg salt intoxication is rare, with renal tubular necrosis or autoimmune glomerulonephritis being observed (Barnes et al., 1980).

In cats intoxicated with methylmercury, ataxia, weakness, tremor, and convulsions were observed (Chang et al., 1974; Charbonneau et al., 1974).

This researched aimed to assess the total concentrations of some heavy metals (Pb, Cd, Hg) using cats' fur as an indicator, while also taking into consideration the age, feeding conditions, and lifestyle of the cats.

MATERIALS AND METHODS

A total number of 69 cats were used for the purpose of this study.

The cats were further divided into categories based on their age, type of feed they receive, and conditions in which they are raised, as shown in Table 1.

Table 1. Number of cats from each category used in the research

Age (years)	< 3	12
	3-5	27
	> 5	30
Food type	Commercial	37
	Homecooked	10
	Combined	22
Lifestyle	Indoor	43
	Outdoor	26
Total		69

In this sense, cats were divided into groups of individuals below the age of three (n = 12), between the ages of three and five (n = 27), and above the age of five (n = 30).

The cats were also divided into groups based on the type of feed they were receiving from their owners, either commercial food (n = 37), homecooked food (n = 10), or combined food, a mix of both commercial and homecooked food (n = 22).

Lastly, the lifestyle of the cats was taken into account, whether they were raised indoors (n = 43) or outdoors (n = 26).

For this study, from each cat a fur sample was collected. The fur samples were collected from the flank region and were placed in disposable paper envelopes. The envelopes were labelled and transported to the laboratory. Upon analysis, the samples were removed from the envelopes and placed in polypropylene test tubes. Each sample weighed roughly 0.5 g. The samples were digested using 5 ml HNO_3 and 1 ml HCl fuming. The samples were then diluted to 10 ml with ultrapure water. The total concentrations of Pb, Cd, and Hg were determined using Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

Statistical analysis was performed using VassarStats: Website for Statistical Computation (http://vassarstats.net/). One-Way ANOVA was performed for all samples.

RESULTS AND DISCUSSIONS

Several individuals had total concentrations of either Pb, Cd or Hg below the detection limit of the method. Of all cats, 2 cats had total concentrations that were below the detection limit for Pb, 6 for Cd, and 21 for Hg.
The mean Pb, Cd, and Hg total concentrations for the cats used in this study are shown in Figure 1.

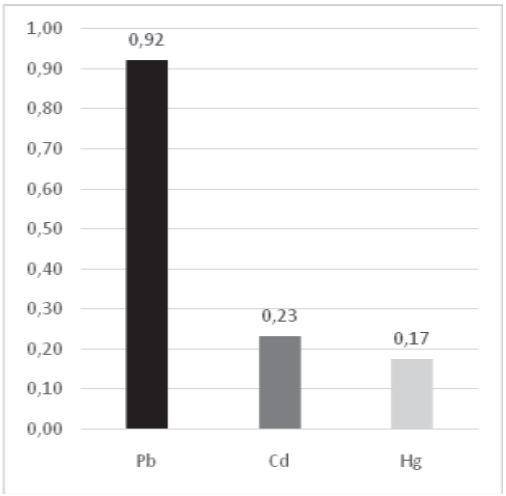

Figure 1. Mean Pb, Cd, and Hg total concentrations (mg•kg^{-1}) for all cats

Regarding all the cats used in this study, the mean total concentrations of Pb were 0.92 mg•kg^{-1}, mean total concentrations of Cd were 0.23 mg•kg^{-1}, and mean total concentrations of Hg were 0.17 mg•kg^{-1}.
Sakai et al. (1995) determined the Hg total concentrations in cat fur samples and found a concentration of 7.40 mg•kg^{-1} and 7.45 mg•kg^{-1} male and female cats, respectively.
Pb, Cd, and Hg mean total concentrations found when dividing the cats based on their age are shown in Figure 2.
Cats below the age of three had the lowest total concentrations of Pb (0.77 mg•kg^{-1}), cats between 3-5 years old had 0.9 mg•kg^{-1}, and cats above the age of five had the highest total concentrations of Pb (1 mg•kg^{-1}), however the differences are not statistically significant.
Cats below the age of three had the lowest total concentrations of Cd (0.1 mg•kg^{-1}), cats between 3-5 years old had 0.19 mg•kg^{-1}, and cats above the age of five had the highest total concentrations of Cd (0.32 mg•kg^{-1}), however the differences are not statistically significant.
Cats below the age of three had 0.11 mg•kg^{-1} Hg. Cats between 3-5 years old had the lowest total concentrations of Hg (0.09 mg•kg^{-1}), and cats above the age of five had the highest total concentrations of Hg (0.38 mg•kg^{-1}). The difference is statistically significant, at $p < 0.05$.

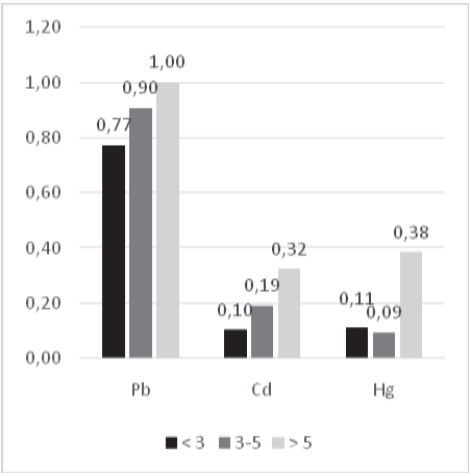

Figure 2. Mean Pb, Cd, and Hg total concentrations (mg•kg^{-1}) based on the cats' ages (expressed in years)

Sakai et al. (1995) determined the Hg total concentrations in cat fur samples based on their age, and found 5.50 mg•kg^{-1} in cats aged less than 1 year and 17.99 mg•kg^{-1} in cats over 3 years old, the difference being statistically significant ($p < 0.01$).
Park et al. (2005) performed a similar study on dog fur samples. In dog fur samples, the mean Cd concentration was 0.03 mg•kg^{-1} for dogs below one year, 0.07 mg•kg^{-1} for dogs between 1-2 years old, and 0.14 for dogs over 2 years. The mean Hg concentration was 0.33 mg•kg^{-1}, 0.67 mg•kg^{-1}, and 0.73 mg•kg^{-1}, respectively. The mean Pb concentration was 0.85 mg•kg^{-1}, 1.21 mg•kg^{-1}, and 1.35 mg•kg^{-1}, respectively. Park et al. (2005) also observed and increase in the concentration of heavy metals with age, but there were also no statistically significant differences in heavy metal concentrations among the groups.
Pb, Cd, and Hg mean total concentrations found when taking into consideration the

feeding conditions of the cats are shown in Figure 3.

Cats eating commercial food had the highest total concentrations of Pb (1.06 mg•kg^{-1}) compared to cats eating homecooked food (0.54 mg•kg^{-1}) and cats eating combined food (0.86 mg•kg^{-1}), however the differences are not statistically significant.

Cats eating commercial food had the highest total concentrations of Cd (0.27 mg•kg^{-1}) compared to cats eating homecooked food (0.17 mg•kg^{-1}) and cats eating combined food (0.20 mg•kg^{-1}), however the differences are not statistically significant.

Cats eating commercial food had the highest total concentrations of Hg (0.23 mg•kg^{-1}) compared to cats eating homecooked food (0.02 mg•kg^{-1}) and cats eating combined food (0.12 mg•kg^{-1}), however the differences are not statistically significant.

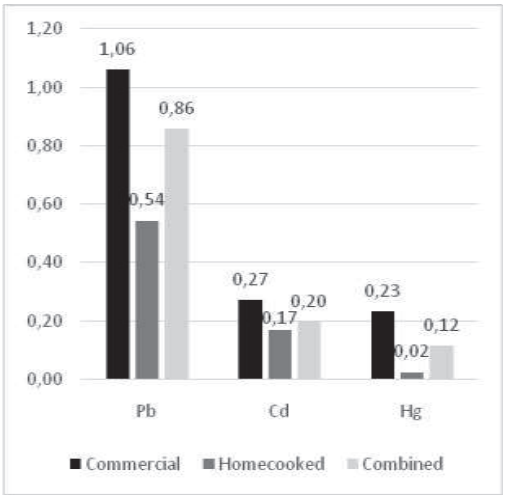

Figure 3. Mean Pb, Cd, and Hg total concentrations (mg•kg^{-1}) based on the cats' feeding conditions

Park et al. (2005) also determined the total concentrations of Pb, Cd, and Hg in dog fur samples taking into account their food type, dividing the dogs in two groups: dogs fed commercial food and dogs fed combined food. Therefore, the mean Pb concentration was 1.15 mg•kg^{-1} for dogs eating commercial food and 0.93 mg•kg^{-1} for dogs eating combined food. The mean Cd concentration was 0.09 mg•kg^{-1} for dogs eating commercial food and 0.02 mg•kg^{-1} for dogs eating combined food. The mean Hg concentration was 0.83 mg•kg^{-1} for dogs eating commercial food and 0.32 mg•kg^{-1} for dogs eating combined food. A statistical significance was found for Cd concentrations in dogs eating commercial food compared to dogs eating combined food ($p < 0.01$).

Pb, Cd, and Hg mean total concentrations found when taking into consideration the lifestyle of the cats are shown in Figure 4.

Cats living indoors registered lower total concentrations of Pb (0.74 mg•kg^{-1}) compared to cats living outdoors (1.22 mg•kg^{-1}), however the difference is not statistically significant.

Cats living indoors registered higher total concentrations of Cd (0.32 mg•kg^{-1}) compared to cats living outdoors (0.11 mg•kg^{-1}), however the difference is not statistically significant.

Cats living indoors registered higher total concentrations of Hg (0.25 mg•kg^{-1}) compared to cats living outdoors (0.03 mg•kg^{-1}), however the difference is not statistically significant.

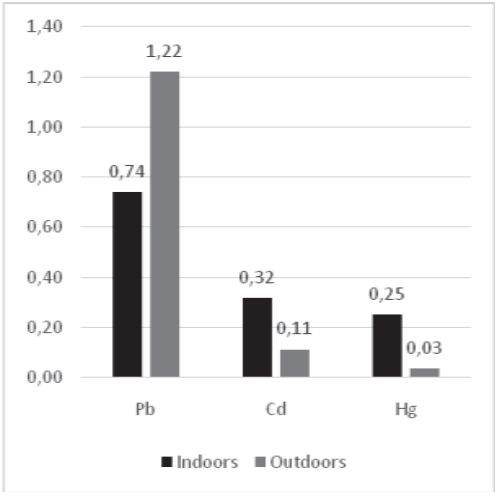

Figure 4. Mean Pb, Cd, and Hg total concentrations (mg•kg^{-1}) based on the cats' lifestyle

Skibniewski et al. (2013) performed a study to determine the total concentrations of Pb in cat fur samples based on the lifestyle of the cats, and found 1 mg•kg^{-1} in pet cats and 2.89 mg•kg^{-1} in feral cats.

Sakai et al. (1995) determined the Hg total concentrations in cat fur samples based on their feeding conditions, and found a concentration of 5.61 mg•kg^{-1} in cats eating commercial food and 12.11 mg•kg^{-1} in cats eating homecooked food.

Park et al. (2005) also determined the total concentrations of Pb, Cd, and Hg in dog fur samples taking into account their lifestyle. Thus, in dog fur samples, the mean Cd concentration was 0.05 $mg \bullet kg^{-1}$ for dogs living indoors, 0.07 $mg \bullet kg^{-1}$ for dogs living outdoors (cement), and 0.15 $mg \bullet kg^{-1}$ for dogs living outdoors (sand). The mean Hg concentration was 0.75 $mg \bullet kg^{-1}$, 0.17 $mg \bullet kg^{-1}$, and 0.19 $mg \bullet kg^{-1}$, respectively. The mean Pb concentration was 1.12 $mg \bullet kg^{-1}$, 0.77 $mg \bullet kg^{-1}$, and 0.85 $mg \bullet kg^{-1}$, respectively. No statistical significance was found for Pb, Cd, or Hg concentrations between these groups.

CONCLUSIONS

The mean Pb, Cd, and Hg total concentrations found in this study were similar or lower compared to other total concentrations found in scientific literature.

The total concentrations of the analyzed heavy metals rise as the cats get older. In addition, cats above the age of five had statistically significant higher Hg total concentrations compared to cats between 3-5 years old.

Cats eating commercial food had the highest total concentrations of all the analyzed heavy metals compared to cats eating other feed types, however no statistical significance was found.

The findings of this research support the assumption that cats which are raised outdoors, in a polluted environment, accumulate higher total concentrations of some heavy metals.

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RENAL BIOPSY - CONSIDERATIONS ABOUT ITS USEFULNESS IN DOGS WITH KIDNEY DISEASE

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Abstract

This paper underlines the role of kidney biopsy in the diagnosis of various kidney diseases in dogs. Even though it is an invasive method, kidney biopsy is often required to establish a definitive diagnosis and an accurate prognosis. Its use should always be considered after weighing the indications and potential complications. Several techniques are used to obtain kidney samples, such as percutaneous renal biopsy (blind or palpation technique or using ultrasound guidance, keyhole technique, laparoscopic biopsy) and surgical biopsy. Once the method has been chosen, the renal sample should only be obtained from the renal cortex. This procedure requires a patient that is stable to undergo general anesthesia or deep sedation. Kidney biopsy samples may be evaluated using light microscopy and special stains, transmission electron microscopy and immunofluorescence to obtain a diagnosis of certainty and to guide treatment options.

Key words*: kidney biopsy, indications, contraindications, complications, percutaneous biopsy techniques.*

INTRODUCTION

Renal disease is defined as the presence of renal lesions of any size or degree or clinical pathology abnormalities that pertain to renal function (Lattimer, 2011). Kidney disease encompasses a group of disorders that affect kidney function and structure. Even mild abnormalities in kidney function are associated with an increased risk for developing complications in other organ systems and mortality, all of which occur far more frequently than kidney failure (Levey, 2013).

For some decades, kidney biopsy was rarely used as a diagnostic tool. The main reasons for this limitation were the difficulty and complexity of the technique and fear of complications such as renal hemorrhage. Furthermore, the pathological examination of the sample provided scarcely useful information for the clinical and therapeutical management of the patient.

Today, it is essential to obtain an accurate and definitive diagnosis of renal disease, particularly in acute, congenital, or glomerular forms. It is essential to differentiate between immune-mediated glomerulonephritis and non-immune-mediated glomerulonephritis to prescribe an adequate therapy. Furthermore, various renal diseases in dogs, including acute kidney injury (AKI), chronic kidney disease (CKD), kidney tumors, and glomerulopathies require different diagnostic and therapeutical approaches. Kidney biopsy does not replace the current standard diagnostic methods but completes them in selected kidney pathologies.

The objectives of this study are to provide an overview of renal biopsy and to analyze the utility of this procedure according to current scientific data.

MATERIALS AND METHODS

Comprehensive online databases were used to review the literature describing the procedure of renal biopsy in dogs with kidney disease. The following keywords were used: kidney biopsy, indications, contraindications, complications, percutaneous biopsy techniques. Thirty relevant articles on renal biopsy in dogs were identified. Of these articles, in the last decade, research of Cianciolo et al., Crivellenti et al., focused more on renal biopsy as a means to obtain a specific diagnosis, prognosis, or guidelines for further research and were included in this review.

This article will describe the evolution of the renal biopsy from its introduction as a means of diagnosis in dogs with various kidney diseases to its use today. This study will also systemize its indications and contraindications, describe the most common biopsy techniques, including the required tools and possible complications. It will also emphasize the utility of renal biopsy in dogs as described by retrospective studies conducted in veterinary diagnostic renal pathology centers.

RESULTS AND DISCUSSIONS

The Renal biopsy - the beginning

In human medicine, the first renal biopsy of the native kidney was performed more than one century ago. It happened during a surgical procedure for renal decapsulation performed as a treatment of Bright syndrome (Edebohls, 1905). In 1944, Nils Alwall performed the first percutaneous renal biopsy, but his results were not published until 1952 (Alwall, 1952; Iversen et al., 1951). Since then, other biopsy techniques have been introduced to improve the quality of the samples obtained. Use of a cutting needle (the Vim Silverman needle) on a prone patient was proved to be more successful in providing an adequate tissue sample than the previous percutaneous method (Kark et al., 1954).

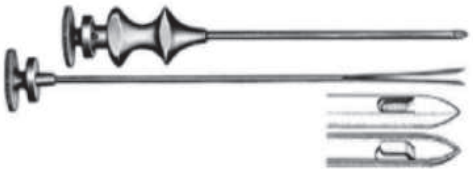

Figure 1. The diagram of a Silverman-needle for percutaneous renal biopsy (Merlini & Pozzi, 2007)

The early 1980s marked by the use of a biopsy 'gun' that employed an automated spring cutting needle and led to more suitable biopsy samples for histopathological evaluation as well as less trauma to the patient (Lindgren, 1982). The initial device evolved into an automatic or semi-automatic spring-loaded biopsy gun, which provides better renal cortex specimens in a safer way (Visconti et al., 2016). Before renal biopsy became common practice, clinicians could only recognize certain clinical syndromes, such as acute nephritis, nephrosis, asymptomatic hematuria, and chronic kidney failure, but they do not ascribe them to distinct pathologic processes. Over the past 50 years, renal pathology has evolved and, by the turn of the last century, the ability to diagnose kidney disease outstripped the knowledge on its pathogenesis.

Figure 2. Automatic disposable biopsy system - Medone® (www.medax.it/soft-tissue-biopsy/automatic-biopsy-system/item/medone?category_id=22)

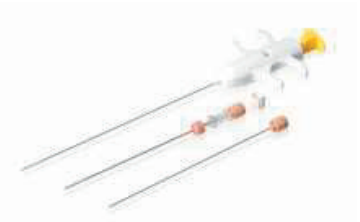

Figure 3. Semi-automated spring-loaded Biopsy system-Medeasy® (www.medax.it/soft-tissue-biopsy/item/medeasy)

In dogs, renal biopsy was reported for the first time in 1967 (Osborne et al., 1967) and it has improved tremendously ever since. Kidney biopsy is now the gold standard technique to determine the type of renal damage, when mildly increased UPC cannot differentiate tubular from glomerular lesions (Vaden et al., 2005; Lees et al., 2011; Salama et al., 2011). Ultrasonography has been proven to have a low diagnostic value due to the poor correlation between renal cortical echogenicity and histopathological lesions (Banzato et al., 2017; Zotti et al., 2015).

According to the International Renal Interest Society (IRIS), staging of chronic kidney disease is based on the concentration of serum creatinine and serum symmetric dimethyl-arginine, SDMA (IRIS Staging of CKD, 2019). Although widely available, creatinine does not increase until renal function declines by approximately 75%. Another disadvantage of

creatinine is that it provides little information about ongoing pathology. Therefore, more specific and sensitive biomarkers are needed to determine risk, establish a diagnosis and prognosis, and evaluate the result of therapeutic interventions (Salama et al., 2011; Yerramilli et al., 2016). SDMA is a more sensitive biomarker; it can detect a reduction of renal function by 25%-40% and is less influenced by lean body mass or other conditions (Nabity et al., 2015; Hall et al., 2014).

Frequently, evaluating kidney disease in dogs based on the history, clinical examination, laboratory tests (hematology, biochemistry, and urinalysis), and ultrasound examination does not identify the cause. In these patients, a renal biopsy may be required for a definitive diagnosis (Jankowski et al., 2008). Renal biopsy plays a key role in defining the disease process, and can also help clarify the pathophysiology of disease to improve therapeutic management. The histopathology result helps devise an adequate prognosis and treatment plan for some conditions, including protein-losing glomerulopathy and AKI (Dhaun et al., 2014).

Indications of renal biopsy

This procedure should only be executed by an operator that is well-trained and experienced in the technique that will be employed and knowledgeable about the potential complications (Walker, 2004).

In canine nephrology, renal biopsy is performed when an assessment of the type of kidney damage and its severity is required. Its use should be considered after careful weighing of the benefits against potential complications. For this purpose, a complete evaluation is required before the biopsy. This prebiopsy checkups should include physical examination, blood pressure measurement, and laboratory tests (complete blood count, biochemistry, urinalysis, urine protein to creatinine ratio, and coagulation tests) (Vaden, 2004).

In dogs, the most common indications for kidney biopsy are protein-losing nephropathy (PLN) or other suspected glomerular proteinuria, AKI, familial renal disease, and renal masses. Its purpose is to establish an accurate diagnosis and prognosis and to guide therapy according to its results (Polzin, 2009; Salama et al., 2011; Lees et al., 2011; Cianciolo et al., 2013; Littman et al., 2013; Vaden et al., 2016).

Contraindications for renal biopsy include severe anemia, coagulopathy, solitary kidney, uncontrolled hypertension, hydronephrosis, perirenal abscess, and bilateral reduction in kidney size (Polzin, 2009; Vaden & Brown, 2017). It is necessary to identify risk factors for bleeding (anemia, severe hypertension, prolonged bleeding time) and to correct them; when not possible, it is recommended to postpone the procedure (Visconti et al., 2016).

According to the IRIS Canine Glomerulonephritis study subgroup, this procedure should not be considered in dogs with stage 4 chronic kidney disease or whenever other medical contraindications cannot be improved. Kidney biopsy is also contraindicated when the results would not modify the treatment, diagnosis, or prognosis. (Pressler et al., 2013)

Current biopsy techniques

In dogs, several methods may be employed to collect renal specimens. They consist of percutaneous biopsy techniques such as blind or palpation biopsy, ultrasound-guided biopsy, keyhole biopsy, laparoscopic-guided biopsy, and surgical biopsy (Cianciolo et al., 2013; Vaden & Brown, 2017).

It should be emphasized that the choice of the kidney biopsy technique depends on the species, the size of the animal, operator experience, available equipment, and the clinical condition of the patient referred for biopsy. This procedure requires that a patient be stable to undergo general anesthesia or deep sedation.

Only cortical tissue should be sampled. The first reason for this reasoning is safety. Large vessels are not located in the renal cortex, so the damage (hemorrhage, infarction, or fibrosis) to the parenchyma will be minimal. The second reason is the structure of the kidney (the cortex contains the glomerulus and convoluted tubules). Special care must be taken when sampling to ensure enough glomeruli have been obtained for evaluation (Brown et al., 2013).

For specific classification of glomerular diseases, renal biopsy samples should be evaluated using light microscopy (LM), transmission electron microscopy (TEM) and immunofluorescence (IF) according to the World Small Animal Veterinary Association-Renal Standardization Study Group (WSAVA-RSSG) (Cianciolo et al., 2016). To be declared suitable for evaluation, a kidney biopsy should contain at least 5-10 glomeruli for LM and additional tissue specimens for TEM and whether to make a definitive diagnosis (Lees et al., 2011; Crivellenti et al., 2018). In a 2008 study, Jankowski et al. reported an average number of 14 glomeruli per sample, even if their cut-off value to ensure histopathological validity was the presence of five glomeruli (Jankowski et al., 2008).
The most common methods to estimate the number of glomeruli in the biopsy sample evolved from eye loupe inspection to light microscopy (Lees et al., 2011). A recent study shows that the most effective method to get an estimate glomerular number is to use a light microscope with lowering of the condenser lens (Costa et al., 2019).

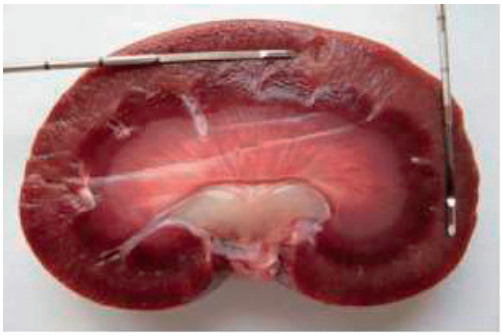

Figure 4. Correct insertion of the biopsy needle (Jankowski et al., 2013)

Whenever possible, the sample should be taken from the caudal or cranial pole of the kidney, as is easier to not accidentally penetrate the medullar tissue. The right kidney is preferred to the left when bilateral pathology is suspected (De Rycke et al., 1999; Brovida, 2003; Nowicki et al., 2005; Vaden, 2004; Rezaie et al., 2008).
The number of core samples required for a valid histopathological examination depends on the type of needle employed and the evaluations that will be performed on the tissue. Using a needle with a short core requires three sampling to ensure an adequate specimen, whereas longer devices (up to several centimeters long) may only need one pass (Yau, 2019). Samples for IF will be frozen or set aside in a special transport solution. The remainder is quickly placed in fixative (formalin or glutaraldehyde) for light LM and TEM and sent to the laboratory. (Vaden, 2004; Walker, 2004).

Required tools and materials vary depending on the technique used. A wide range of dedicated veterinary automated biopsy devices is available. They provide satisfactory renal samples when used properly (Lees et al., 2011). It is recommended to use 14–18 G needles, although no major differences in the quality of the samples were observed between different gauges (Vaden, 2004; Crivellenti et al., 2018).
In human medicine, 14-18 G needles are most commonly employed; 18 G needles are often used in pediatric patients because the internal diameter of the needle is slightly larger than a glomerulus (Tøndel et al., 2012). A recent study from China identified no significant difference in the number of glomeruli obtained or subsequent patient complications between 18 G and 16 G needles (Xie et al., 2020).
The materials, advantages, and disadvantages of each technique will be described below.

The blind or palpation biopsy technique involves sampling after manual localization and immobilization of the kidney through the abdominal wall. In dogs, the right kidney is harder to approach due to its location under the costal arch, but it is more stable puncture is required (Vaden, 2004). The left kidney, even if more mobile can be approached easily due to its anatomical position (Osborne et al., 1971; Vaden, 2004; Jankowski et al., 2013).

The advantage: it is the cheapest biopsy technique because it only requires a biopsy needle.
Disadvantages:
- it is difficult to perform in dogs due to the topography of the internal organs;
- it is challenging to control the biopsy needle in the abdominal cavity, which increases the

risk of complications during the procurement of the specimen (Osborne et al., 1996; Vaden, 2004; Vaden & Brown, 2017).

A keyhole biopsy requires an incision through the abdominal wall, caudal to the right costal arch. Immobilization of the kidney is accomplished with the index finger inserted through the incision. Next, a smaller incision is made in the abdominal wall, through which the biopsy needle is advanced toward the kidney with the help of the operator's fingers (Vaden, 2004; Jankowski et al., 2013).

Advantages of the keyhole biopsy technique:
- it is low-cost as it does not require ultrasound guidance (Stone et al., 1992; Osborne et al., 1996);
- the quality of the tissue samples is good, comparable to that of specimens obtained through laparoscopy (Wise et al., 1989);
- the operator has better control of the biopsy needle compared to a blind biopsy.

Disadvantages:
- this technique can only be used in dogs and only for the right kidney;
- the incision of the abdominal wall is associated with pain (Nowicki & Depta, 2001; Nicpoń et al., 2004; Vaden, 2005).

Ultrasound-guided renal biopsy is currently the least invasive method to obtain cortical tissue specimens in dogs, as well as in humans (Walker, 2004). Ultrasound guidance permits visualization of renal structure and size and enables the correct placement of the biopsy needle (Hager et al., 1985).

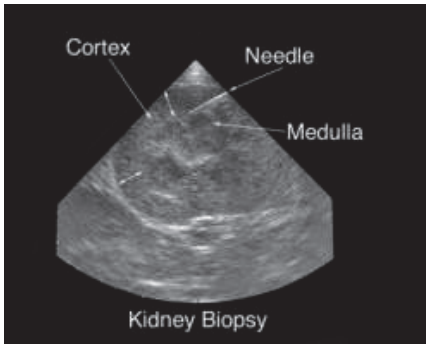

Figure 5. Ultrasound-guided biopsy (Vaden, 2005)

After the kidney is visualized with ultrasonography, the area is clipped and asepticized. A small incision is then made through the skin, through which the needle is advanced toward the renal capsule. The cortical sample is removed using the appropriate technique for each device. It is recommended to apply transabdominal digital pressure to reduce the risk of bleeding (Yamamoto et al., 1991; Bigge et al., 2001; Rawlings et al., 2003; Vaden, 2005; Zatelli et al., 2005).

Advantages:
- it offers high precision in obtaining a suitable specimen with a reduced risk of complications (Lees et al., 2011);
- this technique is relatively economical, rapid and can be performed using most Ultrasound machines (Haaga et al., 1983).

Disadvantage: this method is not recommended in small dogs (adult weight under 5 kg) (Hager et al., 1985).

Laparoscopic biopsy enables the sampling of sterile renal tissue under endoscopic guidance. For better visualization and identification of the organs, it is necessary to introduce a certain amount of gas (usually carbon dioxide) into the abdominal cavity (Grauer et al., 1983; Wise et al., 1989; Nowicki & Lew, 2001; Lew et al., 2003; Vaden, 2005).

Advantages:
- it is a minimally invasive diagnostic technique that provides better visualization of the kidneys and better control of post-biopsy bleeding (Nowicki et al., 2010; Vaden & Brown, 2017);
- it provides excellent tissue samples when 14 gauge double-spring-activated biopsy needles are used (Rawlings et al., 2003). A more recent study showed that the use of biopsy forceps with a 5 mm cup ensures a greater number of glomeruli compared to basic 16-gauge biopsy needles (Park et al., 2017).

Disadvantages:
- it is expensive because it involves special equipment and highly qualified personnel;
- it has additional contraindications: peritonitis, hernia, coagulopathies, obesity, subcutaneous emphysema (Vaden & Brown, 2017; Silvinato et al., 2019).

The open or surgical renal biopsy technique involves performing a laparotomy and

removing a wedge-shaped sample from the renal cortex using a scalpel. In the end, the renal capsule is sutured and digital pressure is applied to control hemorrhage. This method is used only when it is considered that other techniques cannot provide enough cortical tissue (Osborne et al., 1996; Nowicki & Depta, 2001; Vaden et al., 2005; Jankowski et al., 2013).

The advantage: it is the method of choice in dogs under 5 kg, in those with cystic kidney disease or other contraindications (Vaden, 2004).

Disadvantage: being a surgical procedure, it is more invasive than percutaneous techniques; pain associated with the trauma is the most common complication (Vaden & Brown, 2017).

Complications of renal biopsy

From the time of widespread implementation of renal biopsy in dogs until today, the associated complications have been estimated at a rate of 1%-20% (Osborne, 1971; Osborne et al., 1996; Jankowski et al., 2013).

Renal biopsy is a safe procedure and the risk for developing major complications is rare. Minor consequences, nevertheless, are more frequent. They occur due to the chosen technique, patient status, or operator experience and include micro- or macrohematuria, perirenal hematoma, and pain. All these adverse events can be safely managed without further complications for the patient. A study conducted in 2004 in a group of young dogs without renal disease showed that biopsy lesions were minor and the glomerular filtration rate was not affected after biopsy (Groman et al., 2004). Another study proved that ultrasound-guided biopsy had minimal complications in healthy dogs (Rezaie et al., 2008). To minimize the risk of post-biopsy complications, the patient should be hospitalized 24 hours. Isotonic fluids should be given to ensure diuresis and careful monitoring of PCV for possible bleeding should be done (Vaden & Brown, 2017).

However, the reports are radically different when the biopsy is performed on dogs with kidney disease. The most commonly reported complications of renal biopsy are presented in the box below.

Reported complications of renal biopsy (Vaden et al., 2004)
Arteriovenous fistula formation
Biopsy of nonrenal tissue (eg. liver, adrenal gland, fat, muscle, connective tissue, spleen)
Cyst formation
Death
Hemorrhage
Microscopic hematuria
Macroscopic hematuria
Perirenal hematoma
Intrarenal hematoma
Lacerated renal artery or vein
Intra-abdominal hemorrhage caused by laceration of other organs or vessel
Hydronephrosis
Infarction and thrombosis
Infection
Scar formation and fibrosis

Discussion on the utility and limitations of renal biopsy in veterinary medicine

Renal biopsy is indicated in dogs with various kidney diseases when identifying the underlying condition would improve the therapeutic management and patient status or when an accurate prognosis is required. For example, renal interstitial fibrosis is a poor prognostic indicator because it is associated with irreversible renal injury and nephron loss (Lees et al., 2011; Cianciolo et al., 2013; Vaden & Brown, 2017). This procedure is valuable in young dogs who develop chronic kidney disease and in breeds prone to familial (soft-coated Wheaten Terrier, Samoyed) or inherited kidney disease (Samoyed, Bull Terrier, English Cocker Spaniel, Shih-Tzu) (Lees, 1996).

Concerning acute kidney injury, renal biopsies can be evaluated using light microscopy and routine stains to identify tubular causes. This method provides an accurate prognosis based on the appearance of the renal tissue and the integrity of the tubular basement membrane. The most common biopsy findings associated with tubular AKI are acute tubular necrosis and acute tubulointerstitial nephritis. Nonetheless, TEM and IF should be used to identify or rule out a possible glomerular cause of AKI (Vaden, 2004; Vaden & Elliott, 2016; Aresu et al., 2017).

Glomerular disease is clinically suspected when severe and persistent proteinuria is present. In dogs, the most sensitive method to assess glomerular proteinuria is to evaluate the urine protein to creatinine ratio (UPC). A value higher than 2 in a urine sample with inactive sediment is highly suggestive of glomerular disease (Lees et al., 2005; Vaden & Elliott, 2016). The recognition of several forms of glomerular disease was possible by properly performed and analyzed kidney biopsies (Polzin, 2009).

Several clinical trials were conducted in the past years by experienced veterinary nephropathologists (Cianciolo et al., 2013; Schneider et al., 2013; Crivellenti et al., 2018; Vessieres et al., 2019). The results of two extensive retrospective studies will be presented to emphasize the utility of performing renal biopsies in canine nephrology.

One of the most recent studies on this subject was published by Crivellenti et al., in 2018. Their objective was to identify factors affecting the diagnostic quality of renal biopsy samples from dogs with suspected kidney disease. Their team analyzed over 500 renal biopsy specimens from dogs suspected of various kidney diseases that were submitted to the International Veterinary Renal Pathology Service (IVRPS). Out of the 522 samples, 30 were declared non-diagnostic while the remaining 492 were considered histopathologically valid biopsy tissue. They diagnosed immune-mediated glomerulonephritis in 212 dogs, focal segmental to global glomerulosclerosis in 96 dogs, amyloidosis in 64 dogs, tubulointerstitial disease in 43 dogs, non-immune-mediated nephropathy in 38 dogs, glomerulopathy not otherwise specified in 34 dogs, and normal kidney in 5 dogs (Crivellenti et al., 2018). By evaluating these results one can conclude that renal biopsy in dogs is an indispensable tool in the diagnosis of kidney disease and its underlying conditions.

A larger study on the value of the kidney biopsy in dogs was published in 2015 by members of the WSAVA-RSSG. The study identified specific immune complexes or amyloidosis (using LM, IF, and TEM) and to create a guideline for the appropriate evaluation of renal biopsy specimens. The study included 960 canine renal biopsy samples from the IVRPS and Utrecht Veterinary Nephropathology Service. They classified glomerular disease according to biopsy findings into three broad categories based on their significance for prognosis and treatment: amyloidosis, immune complex-mediated glomerulonephritis, and non-immune complex-mediated glomerulonephritis (Cianciolo et al., 2013). The guidelines they provided in this paper optimize the diagnostic workflow in veterinary nephropathology. This study reinforces the utility of kidney biopsy as a key element in the ongoing research on kidney disease in dogs. This finally translates into the necessity of accurate morphological diagnosis to guide the clinical management of dogs with various renal disorders. For example, various diseases and pathophysiological mechanisms can lead to nephrotic syndrome, nephritic syndrome, and AKI, but they can have vastly different prognoses and therapies. These recommendations can guide the decision of renal biopsy in patients with proteinuria as well as the use of immunosuppressive drugs in those patients where renal biopsies were not performed (Cianciolo et al., 2013). Unfortunately, the histopathological findings with light microscopy are not always specific and devising a definitive diagnosis becomes difficult (Dhaun et al., 2014). In these complex cases of kidney disease, complete evaluations (using LM, IF and TEM) by experienced nephropathologists are needed to obtain an accurate diagnosis (Schneider et al., 2013).

CONCLUSIONS

Renal biopsy is an invasive diagnostic technique that is essential for the diagnosis of various kidney disorders in dogs. Accurate identification of the underlying disease can be made through the complete evaluation of the renal tissue; this procedure is a prerequisite for an informed prognosis, decision-making, and specific therapy.

Renal biopsy is a safe procedure and the risk of major complications is low when an appropriate biopsy technique is used. Minor side effects of the procedure occur frequently, but they can be managed and do not usually further harm the patient.

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DYNAMIC OF ANTIBODIES AGAINST CANINE DISTEMPER VIRUS AND CANINE PARVOVIRUS IN ROMANIAN CANINE BLOOD DONORS

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Abstract

The objective of the current study was to determine the immunological changes in canine blood donors in Romania. The data of the study is being collected since 2016 and is still in process of research. The samples are represented by serum antibodies IgG - Canine Parvovirus and IgG - Canine Distemper virus, which were analysed at each blood donation of dogs being part of the donation program. They fulfil the eligibility criteria of being clinically healthy with a completed vaccination schedule. ***The study is based on 17 canine blood donors (5 males/12 females), over one-year-old (2-4 years), with owners and living in similar environments.*** *The ELISA VetLine Canine Distemper Virus (CDV) and VetLine Canine Parvovirus (CPV) kits from NovaTec (Immundiagnostica GMBH, Germany) were used following the kits manufacturer's recommendations. The analysis of the results obtained from the serum samples collected showed that no individual presented negative results (0/17-0%) below the protection standard on the two viral strains, all the serum samples having positive results (17/17-100%). The results indicate that repeated blood donation cannot influence the loss of post-vaccine antibodies.*

Key words*: CDV-CPV, IgG - ELISA, canine blood donors.*

INTRODUCTION

The request for blood components associated with better emergency and critical care treatments has increased in the last decade in the veterinary medicine field, mainly for canine patients, leading to the creation of canine blood banks in several countries, thus consequently leading to an increased number of dogs that provide frequent donations (Ferreira et al., 2014).

More concerns have been raised upon the safety and bioethics for frequent blood donations regarding the donor's well-being because of this arising demand for canine blood all over the world.

Until now, studies have been made on how the frequency of donations can affect the canine donor's iron status that can cause iron-deficient erythropoiesis (like in human donors) because of the act of excessive phlebotomies (Giger, 2005; Lewis & Stone, 2012) and on some hematologic variables such as haemoglobin concentration, platelet count, WBC count and reticulocyte count (Ferreira et al., 2014) but no other research was made on other parameters such as immunological ones, especially regarding serum antibody titres evolution following vaccination from one blood donation to another.

The lack of studies on the immunological status of the canine blood donors recommends future research to establish if there is an immunelogical risk and if a specific vaccination program needs to be developed for this group of animals.

The guideline for the vaccination of dogs compiled by the Vaccination Guidelines Group of the World Small Animal Veterinary Association recommends that Canine Parvovirus-2 (CPV), Canine Distemper Virus (CDV), and Canine Adenovirus-2 as core vaccines (vaccines which all dogs should receive), while rabies where required by statue or in areas where the disease is endemic (Day et al., 2016).

No study was yet performed on canine blood donors and the consequences of regular blood donations upon the capacity of the donor's immune system to maintain a protective IgG level against none of the core vaccines.

In this context, our study aims to investigate how does the immune system of canine blood donors responds from one donation to another and if it is able to maintain protective IgG levels against two of highly pathogenic

microorganisms namely CPV and CDV from one donation to another.

MATERIALS AND METHODS

Serum samples (n = 80) were collected from healthy dog donors (n = 17; 5 males and 12 females) that provided frequent donations within a Romanian blood bank. All dogs were client-owned animals and the owner's consent was provided for the participation of their dogs in this study. All dogs were 2 to 6 years old, weighing between 25 and 60 kg, dewormed and with a complete initial vaccination program and yearly boosters administered. Furthermore, at each donation they have been tested and provided negative results for *Anaplasma phagogytophilum*, *Anaplasma platys*, *Ehrlichia canis*, *Ehrlichia ewingii*, *Borrelia burgdorferi*, *Dirofilaria immitis* using the enzyme immunoassay technology (EIA) - SNAP 4Dx Combo Plus® (Idexx Laboratories, Fremont, CA) and negative blood smears for *Babesia canis*. The register code, breed, gender, last vaccination date and the number of blood donations were noted for each animal (Table 1).

Table 1. Donors' identification by register number, age, gender, and number of donations

No	Donor	Breed	Gender	Age	Number of donations
1	BNA 04MP	American Staffordshire Terrier	M	4	6
2	BNA 05FN	American Staffordshire Terrier	F	4	6
3	AMN 16MN	Cane Corso	M	2	6
4	HER 12FN	American Staffordshire Terrier	F	6	6
5	BRD 16FP	Crossbred	F	3	5
6	CRV 02F-	Cane Corso	F	2	5
7	CRV 06M-	Cane Corso	M	3	5
8	BNA 11FP	German Shepherd	F	6	5
9	DRS 14FP	Golden Retriever	F	5	4
10	CRV 07F-	Cane Corso	F	3	4
11	CRV 15MP	Cane Corso	M	3	4
12	BNA 03FP	American Staffordshire Terrier	F	4	4
13	BNA 13M-	Crossbred	M	4	4
14	BNA 14F-	Crossbred	F	4	4
15	BNA 15F-	Crossbred	F	4	4
16	IMDB13 FN	Doberman	F	4	4
17	IMGR05 FP	Golden Retriever	F	6	4

Study protocol

The dog donors were set up in three groups: the first group providing 6 donations, the second group providing 5 donations and the last one providing 4 donations. All the dogs donated 450 mL of whole blood at an interval of 2 to 4 months apart (starting in October 2016 and ending in December 2018) to fulfil the minimum "resting" time, as described in veterinary literature (Schneider, 1995; Ford & Mazzaferro, 2006; Mathews et al., 2006; Gibson & Abrams-Ogg, 2012), but considering also the maximum period agreed in human medicine standards (Europe Council, 2011).

Blood donations

All blood collections were performed by the same operator. Each donor dog has undergone a complete physical examination before each donation.

Dogs were placed in right lateral recumbency and the puncture area over the left jugular vein was aseptically prepared using 70% alcohol. The hair was not clipped as most of them were show dogs. Jugular venepuncture was then performed, and blood was collected by gravity into the collection bag.

Sample collection

All blood samples (9 ml per sample) were collected on clot activator vacutainers directly from the blood bag's tube (containing whole blood with no anticoagulant) at the end of the blood collection after clamping the tube. After 30 minutes at room temperature, the vacutainers were centrifuged at 3500 rpm for 10 minutes. Serum samples were then separated and stored at – 20°C for 2 years until being analysed.

Qualitative ELISA assay technique

The immunological status of the investigated canine blood donors was evaluated using the qualitative ELISA commercial kits: VetLine Canine Parvovirus (CPV) and VetLine Canine Distemper Virus (CDV) kits from NovaTec (Immundiagnostica GMBH, Germany).

Working protocols were followed as indicated by the manufacturer for both test methods and the interpretation of the results.

Briefly, all reagents and the microtiter strip wells precoated with Canine Parvovirus

/Morbillivirus antigens to bind corresponding antibodies of the specimen were brought to room temperature (20-25ºC). The serum samples, the positive and negative controls were diluted 1:50 in sample diluent, and 100 µl were dispensed into the appropriate wells of the microtiter plate.

The microtiter plate was sealed with adhesive film and incubated for 60 minutes at 37ºC and washed three times with 300 µl of washing solution. Afterwards, 100 µl Vet Line Canine Parvovirus/Morbillivirus Protein A/G Conjugate was dispensed into each well; the microtiter plate was sealed with adhesive film and incubated 30 minutes at room temperature (20-25ºC) and washed four times with 300 µl of washing solution.

Then, 100 µl tetramethylbenzidine substrate (TMB 0.25%) prepared just before use was dispensed into each well and incubated in the dark at room temperature (20-25ºC) for 15 minutes resulting in the immune complex formed by the bound conjugate which gives a blue reaction in the specimen.

In the end, 100 µl of the Stop solution (1N sulphuric acid solution) were added to each well to stop the reaction producing a yellow endpoint colour.

The results were read at dual wavelength mode of 450-620 nm and recorded for statistical analysis.

For the interpretation of the results the following values were considered as a guideline: For Canine Parvovirus: Positive at > 11 NTU (NTU = NovaTec Units calculated like indicated in Table 2); Equivocal at 9-11 NTU; Negative < 9 NTU. For Canine Distemper Virus: Positive at > 7 NTU; Equivocal at 6-7 NTU; Negative at < 6 NTU.

Table 2. Results in units [NTU] – method of calculation

$\frac{\text{Sample (mean)absorbance value x 10}}{Cut-off}$ = [NovaTec Units = NTU]
Example: $\frac{1.591 \times 10}{0.43} = 37$ NTU (units)
Calculated Cut-off for Canine Parvovirus = 10 NTU Calculated Cut-off for Canine Distemper Virus = 6.5 NTU

Statistical analysis

All NTU values for each individual (for both Canine Parvovirus and Canine Distemper Virus) were recorded and analysed in Excel application of Microsoft Office 365 suite and One-Way ANOVA Analysis Tool pack; $p < 0.01$ was considered significant.

RESULTS AND DISCUSSIONS

There are studies for human donors that observed the effect of blood donations on the profile of lymphocytic cells stating that following ordinary blood donations, no change in Ig levels and peripheral lymphocyte populations was found (Ieromnimon et al., 1981; Lewis et al., 1992).

Some more recent studies from human transfusion medicine suggest that there are some transient changes in lymphocyte subsets following a single blood donation in male subjects (Borai et al., 2017). On the other hand, veterinary transfusion studies from the past decades have led to the development of general guidelines for donor's selection to increase their safety (Yagi & Bean, 2016) but there is no data available at the moment on the effects of blood donation neither on the dog's immune system nor on the vaccine-induced antibody levels.

Enrolled donors

Seventeen dogs enrolled in the elected canine blood banks' donation program were included in this study.

The median age of included dogs was 3.9 years. There were 5 (29.41%) male dogs (MC 1/5; MI 4/5) and 12 (70.59%) female dogs (FC 8/12; FI 4/12).

Breeds included were represented by American Staffordshire Terrier (4), Cane Corso (5), German Shepherd (1), Dobermann (1), Golden Retriever (2), Crossbreed (4). From the 17 dogs tested, 4 were crossbreeds and 13 were purebred.

The small number of tested animals did not allow a breed analysis; therefore, the statistical analysis and interpretation of the results were done across the group for both the Canine Parvovirus group and Canine Distemper Virus group.

It is to be mentioned that breed, age, and gender frequency distributions were generally representative of the canine blood banks' donors.

NTU for Canine Parvovirus

For the Canine Parvovirus, the overall picture of NTU results revealed non-significant variation between and within D1-D6 groups of values ($p<0.01$, $F<F$ crit) as showed in Table 3. NTU values were above the minimum value used as a guideline (>9 NTU) in all groups, ranging between 10.03 NTU and 23.55 NTU (Table 4, Table 5, and Table 6), but only one dog (BNA11FP) had equivocal NTU values (9-11 NTU) at 3 of 5 blood donations (10.03; 10.78; 10.55) with a booster of 12.78 NTU following the annual booster vaccine, but the NTU value did not drop under 9 NTU for the dog to be considered not protected by the vaccine-induced antibodies (Table 5 and Figure 2), thus we recommend that the canine blood donors should be vaccinated yearly in order to maintain protecting antibody levels for Parvovirus. In all three groups, the dynamic between donations tends to be the same for each tested donor (Figure 1, Figure 2, and Figure 3)

Table 3. Statistical analysis – ANOVA Single Factor (alpha = 0.01) – NTU values for Canine Parvovirus for all donors

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	14.31435	5	2.86287	0.449675	0.812220825	3.275224
Within Groups	471.1231	74	6.366528			
Total	485.4374	79				

Table 4. Results as NovaTec Units (NTU) for Canine Parvovirus in the first group (dogs with 6 blood donations)

No.	Donor ID	Donation					
		D1	D2	D3	D4	D5	D6
1.	BNA04MP	17.41	16.66	16.98	17.03	15.96	15.41
2.	BNA05FN	17.61	15.71	16.05	16.95	17.31	16.83
3.	AMN16MN	17.66	17.75	21.63	18.16	23.01	20.68
4.	HER12FN	15.18	15.11	19.26	13.96	15.45	11.68

*the values after the yearly vaccine booster are highlighted in blue
*Positive > 11 NTU; Equivocal 9-11 NTU; Negative < 9 NTU

Table 5. Results as NovaTec Units (NTU) for Canine Parvovirus in the second group (dogs with 5 blood donations)

No.	Donor ID	Donation				
		D1	D2	D3	D4	D5
5.	BRD16FP	14.78	16.06	15.68	17.73	16.48
6.	CRV02F-	19.66	14.68	14.23	12.95	12.90
7.	CRV06M-	17.76	17.65	17.41	18.33	16.88
8.	BNA11FP	10.03	12.78	10.78	11.71	10.55

*the values after the yearly vaccine booster are highlighted in blue
*Positive > 11 NTU; Equivocal 9-11 NTU; Negative < 9 NTU

Table 6. Results as NovaTec Units (NTU) for Canine Parvovirus in the third group (dogs with 4 blood donations)

No.	Donor ID	Donation			
		D1	D2	D3	D4
9.	DRS14FP	19.23	18.11	16.58	15.06
10.	CRV07F-	15.60	19.91	18.91	16.98
11.	CRV15MP	17.01	23.55	16.85	16.21
12.	BNA03FP	14.53	17.38	15.70	16.31
13.	BNA13M-	17.48	17.00	16.68	15.83
14.	BNA14F-	20.36	17.83	18.26	18.43
15.	BNA15F-	18.90	18.91	18.46	18.38
16.	IMDB13FN	17.73	17.11	15.33	17.50
17.	IMGR05FP	16.73	16.50	15.55	13.51

*the values after the yearly vaccine booster are highlighted in blue
*Positive > 11 NTU; Equivocal 9-11 NTU; Negative < 9 NTU

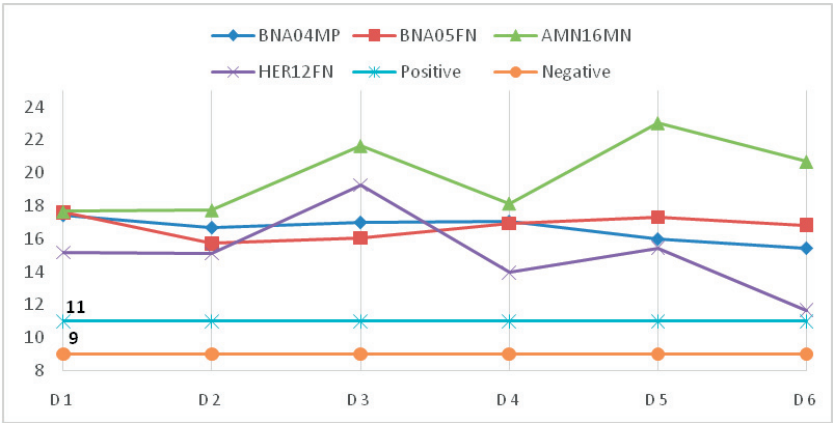

Figure 1. Graphic representation of NTU for Canine Parvovirus in the first group (dogs with 6 blood donations)

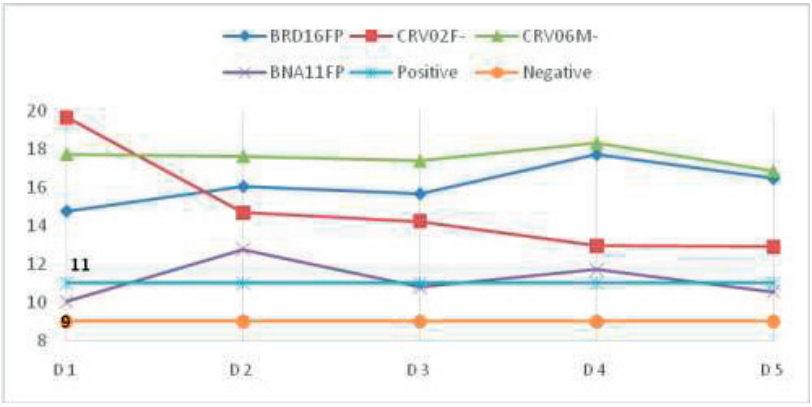

Figure 2. Graphic representation of NTU for Canine Parvovirus in the first group (dogs with 5 blood donations)

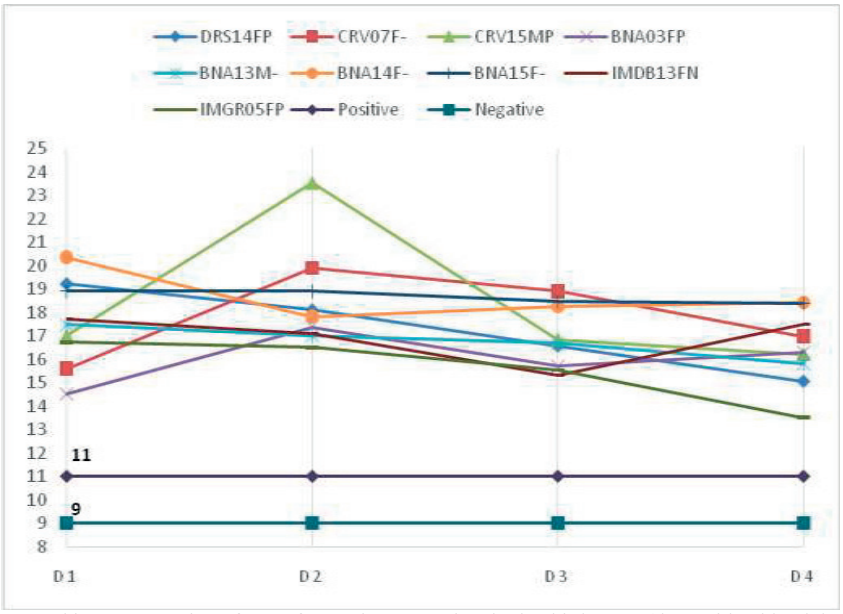

Figure 3. Graphic representation of NTU for Canine Parvovirus in the third group (dogs with 4 blood donations)

NTU for Canine Distemper Virus

As for the Canine Parvovirus, for the Canine Distemper Virus, the overall picture of NTU results revealed also a non-significant variation of values between and within D1-D6 groups ($p<0.01$, $F<F\ crit$) as showed in Table 7. NTU values were above the minimum value used as a guideline (>6 NTU) in all groups, ranging between 8.39 NTU and 44.45 NTU (Table 8, Table 9, and Table 10). No donor dog had equivocal NTU values (6-7 NTU) at any donation in all the three groups analysed, thus suggesting an optimal immunization of canine blood donors even after frequent donations. We can also observe the rhythmicity of antibodies between donors and that the dynamic between donations tends to be the same for each tested donor (Figure 4, Figure 5, and Figure 6).

Because of the low number of donors, a correlation between age/gender and antibody levels could not be determined.

Table 7. Statistical analysis – ANOVA Single Factor (alpha = 0.01) – NTU values for Canine Distemper Virus for all donors

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	88.873	5	17.7746	0.31162	0,904508387	3.27522
Within Groups	4220.9	74	57.0392			
Total	4309.77	79				

Table 8. Results as NovaTec Units (NTU) for Canine Distemper Virus in the first group (dogs with 6 blood donations)

No.	Donor ID	Donation					
		D1	D2	D3	D4	D5	D6
1.	BNA04MP	13.90	12.14	15.08	12.82	15.87	10.27
2.	BNA05FN	26.64	28.09	35.33	32.61	31.69	24.01
3.	AMN16MN	19.38	22.90	44.45	41.33	33.13	36.43
4.	HER12FN	13.06	10.99	18.75	14.41	8.64	13.27

*the values after the yearly vaccine booster are highlighted in blue
*Positive > 7 NTU; Equivocal 6-7 NTU; Negative < 6 NTU

Table 9. Results as NovaTecUnits (NTU) for Canine Distemper Virus in the second group (dogs with 5 blood donations)

No.	Donor ID	Donation				
		D1	D2	D3	D4	D5
5.	BRD16FP	8.39	10.16	11.35	10.27	13.47
6.	CRV02F-	19.60	19.78	24	21	17.33
7.	CRV06M-	27.06	21.35	20.81	21.11	20.37
8.	BNA11FP	12.99	17.29	14.41	14.93	14.55

*the values after the yearly vaccine booster are highlighted in blue
*Positive > 7 NTU; Equivocal 6-7 NTU; Negative < 6 NTU

Table 10. Results as NovaTecUnits (NTU) for Canine Distemper Virus in the third group (dogs with 4 blood donations)

No.	Donor ID	Donation			
		D1	D2	D3	D4
9.	DRS14FP	14.27	13.80	15.80	12.90
10.	CRV07F-	15.31	20	18.68	15.74
11.	CRV15MP	26.25	25.85	26.41	25.40
12.	BNA03FP	16.70	19.33	17	13.67
13.	BNA13M-	21.81	19.83	22.19	16.73
14.	BNA14F-	31.53	29.18	26.70	20.18
15.	BNA15F-	18.70	20.41	19.74	17.89
16.	IMDB13FN	17.58	15.49	17.09	13.29
17.	IMGR05FP	18.93	15.45	14.21	11.44

*the values after the yearly vaccine booster are highlighted in blue
*Positive > 7 NTU; Equivocal 6-7 NTU; Negative < 6 NTU

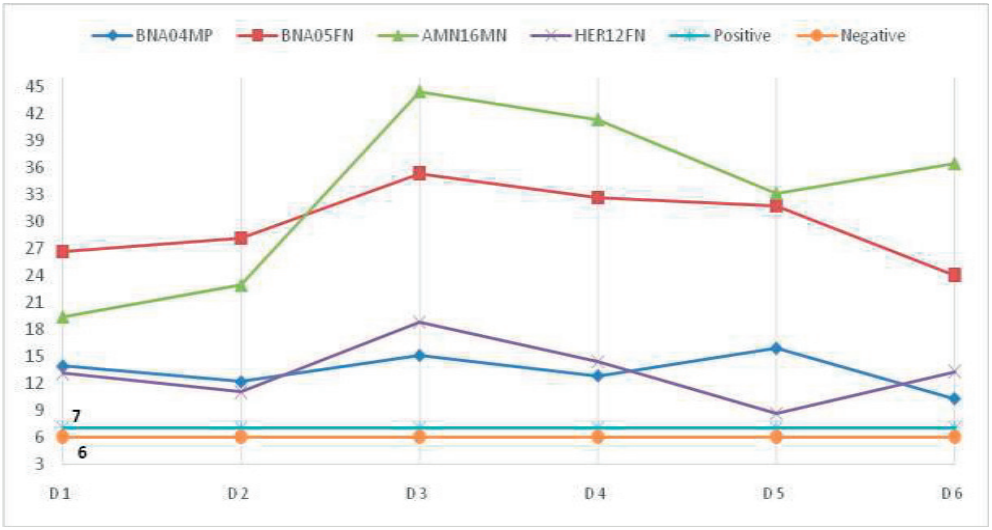

Figure 4. Graphic representation of NTU for Canine Distemper Virus in the first group (dogs with 6 blood donations)

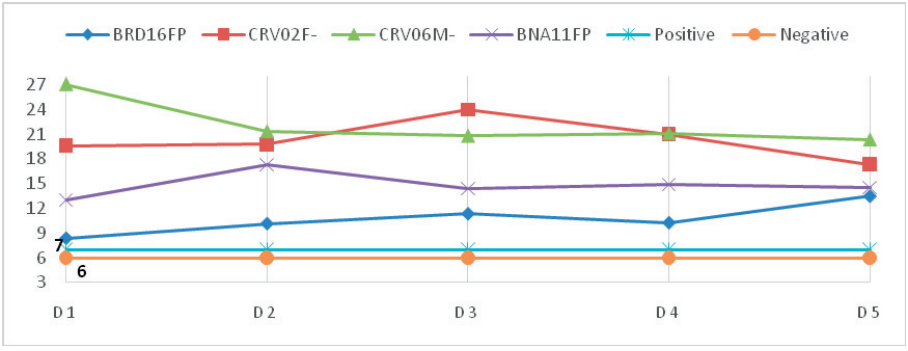

Figure 5. Graphic representation of NTU for Canine Distemper Virus in the third group (dogs with 5 blood donations)

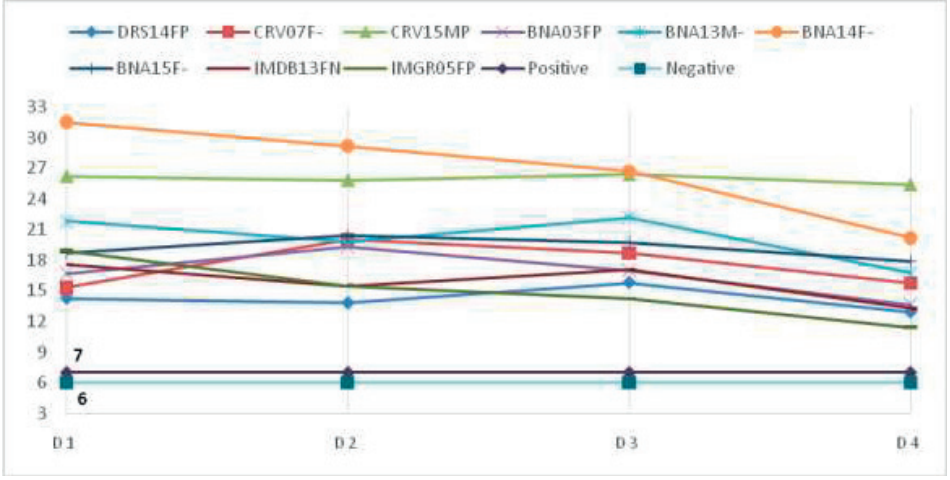

Figure 6. Graphic representation of NTU for Canine Distemper Virus in the third group (dogs with 4 blood donations

Immunology studies show that vaccination stimulates both humoral responses via antibody production and cellular responses via B and T lymphocytes (Day, 2012). How long the post-vaccine immune response is maintained at a protective level is mainly dependent on the immunological memory developed (Day et al., 2016).

However, it is unclear whether a vaccinated dog is fully protected throughout its life or whether revaccination is always necessary (Abdelmagid et al., 2004) and, moreover, if a canine blood donor's immune system can maintain a protective serum antibody titre between blood donations. To clarify these issues, it is important to quantify the rate by which vaccinated canine blood donors become serological-negative again, the so-called seroconversion rate.

There is no data available at this moment for assessing the dynamics of serum antibodies against Parvovirus and Distemper Virus for canine blood donors and if the antibody titre for both CPV and CDV suffers any changes between blood donations, so the present studies results cannot be compared with other studies results.

These preliminary data will be completed with more samples for more than 17 blood donors (different breeds and ages) in order to avoid the individual influence on the results. Also, we will compare in a further study the donors' results with a control group (dogs of the same age and sex, clinically healthy but not enrolled in any blood donation program).

CONCLUSIONS

In conclusion, no significant differences were observed between the average values of serum antibodies against Canine Parvovirus and Canine Distemper Virus, thus the frequency of blood donations does not influence the protective antibody titre against CPV and CDV. The comparison of all three experimental groups with a complete vaccination schedule received by each dog proved a close correlation of immunological status and the time-lapse between annual vaccine boosters.

Further studies are needed in order to assess the real impact of frequent blood donation on the immune system of canine blood donors and its ability to maintain protective antibody levels.

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CORELLATION BETWEEN THE REAL TIME PCR METHOD USED IN CANINE PARVOVIRUS DIAGNOSTIC AND CLINICAL MANIFESTATION

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Abstract

Canine parvovirus infection is one of the most common diseases of puppies. Dogs are affected by two viruses of the Parvoviridae family: CPV-1 (Bocavirus genus), thought to have minimal pathogenic potential, but having being associated with different disorder in all age category dogs, and CPV-2 (Parvovirus genus), known as Canine Parvovirus, the true parvovirus. Despite the extensive use of vaccination, the prevalence of infection registers an oscillating dynamic, the virus is evolving and the requirement for the confirmation diagnosis is being continuous. The aim of the research was to identify the possibly correlations of the clinical manifestations with the results of CPV-2 detection, using the real time PCR. Fourteen dogs with clinical manifestations associated with the suspicion of canine parvovirus, has been tested on feces samples by real time PCR: 13 were positive (92.85 %) to the real-time PCR test. The registered positiveness has been associated with different Ct values ranging from 6.82 to 35. One sample (7.14%) was negative. In this study, despite the variation range of Ct`s, the clinical pattern registered did not directly relate with the virus amount, rather with the age.

Key words*: canine parvovirus, real time PCR.*

INTRODUCTION

Canine parvovirus is one of the most common and yet underestimated diseases of canine youth. The diagnosis of canine parvovirus is a challenge for clinicians in terms of the ubiquity of the virus and rapid diagnostic methods with low sensitivity.

Dogs are affected by two viruses of the Genus Parvovirus: CPV-1, whose pathogenicity is not well known, being associated with "fading puppy" syndrome, causes lethargy, diarrhea, difficulty breathing and sudden death in puppies of 1-3 days. CPV-2 is known as Canine Parvovirus (Barr and Bowman, 2012).

CPV-2 first appeared in 1977 and is related to feline panleukopenia virus and mink viral enteritis virus. In 1980 and 1984, respectively, the variants CPV-2a and CPV-2b appeared, being characterized by different antigenic structures, increased pathogenicity and shorter incubation period compared to CPV-2 (Barr and Bowman, 2012; Carmichael, 2005).

In Italy, a third strain of canine parvovirus, strain CPV-2c, was isolated (Barr and Bowman, 2012).

The CPV-2c strain, known as Glu426, exhibits an amino acid substitution at position 426 from aspartic acid to glutamic acid, which altered the antigenic structure of the capsidal epitope. A (Greene, 2012). These changes in parvoviruses have been associated with genetics adaptation and changes in the B capsid epitope region, allowing the virus to replicate and spread effectively in susceptible dog populations, as well as the ability to infect cats (Greene, 2012).

CPV is a single-stranded, linear, icosahedral, non-enveloped DNA virus with dimensions between 18-26 nm in diameter (Greene, 2012; Gurpreet et al., 2015). The genome consists of 5323 base pairs (bp) encoding two structural proteins, VP-1 and VP-2, and two nonstructural proteins, NS-1 and NS-2. The gene for VP-1 is located between 2285-4537 (2253 bp) and for VP-2 between 2783-4537 (1755 bp) (Gurpreet et al., 2015). The parvoviral capsid contains approximately 10 copies of the VP1 protein and 60-70 copies of the VP2 protein. All the epitopes used to fix neutralizing antibodies are found in the VP2 protein. Mutations of this protein are responsible for the appearance of different antigenic strains (Gurpreet et al.,

2015; Langeveld et al., 1993; Turiso et al., 1991). Different antigenic variants of CPV-2 are prevalent in varying proportions in different countries. CPV-2b has been reported predominantly in Brazil, the USA, Japan, Switzerland and South Africa (Nandi and Kumar, 2010). CPV-2a is considered to be the most common antigenic type in France, Taiwan and Italy. In Spain and the United Kingdom, strains CPV-2a and CPV-2b are eventy distributed (Nandi and Kumar, 2010). CPV-2c was first identified in Italy, and later was reported in Vietnam, Spain, the United Kingdom, South and North America (Nandi and Kumar, 2010). CPV is highly contagious, and most infections occur fallowing the contact with the environment elements, contaminated with the virus shed by feces. In addition, humans, tools (equipment in veterinary units or grooming operations), insects and rodents can serve as vectors. Dogs can keep the virus in the fur for long periods of time. The incubation period for the original CPV-2 strain, under natural conditions was 7-14 days, and the experimental infection was 4-5 days. In the case of new CPV-2 strains (-2a, -2b and -2c), the incubation period in natural infection can be up to 4-6 days (Greene, 2012). Intense elimination of CPV-2 virus begins 3-4 days after infection, usually before obvious clinical signs appear. Following ELISA tests and virus isolation, CPV-2 appears to be massively excreted in the faeces for a maximum of 7-10 days post-inoculation. However, using PCR tests, CPV-2 strains (a, b, c) were detected in faeces for several weeks after infection (Greene, 2012). The age and the immune status of the animal influence the form and severity of the disease. After a short incubation period, the animals with enteric form, suddenly show vomiting and anorexia. Apathy and fever can also be observed. Diarrhea, most often hemorrhagic, occurs after about 48 hours and in severe cases can be expressed as frank hemorrhage. The feces have a characteristic foul odor. The general condition deteriorates rapidly due to dehydration and weight loss (Quinn et al., 2011).

The cardiac form of the disease, whose frequency is quite rare, is found in puppies younger than 6 weeks and manifests itself as acute heart failure. Some animals develop congestive heart failure, weeks or even months after infection (Greene, 2012).

The neurological form, with cerebral damage, and cerebellar hypoplasia are common in cats infected with their specific parvovirus. In contrast, in the case of canines, neurological disorders are most often associated with bleeding in the central nervous system due to disseminated intravascular coagulation, hypoglycemia, sepsis and hydro-electrolyte imbalances. The skin shape is characterized by erythema multiform, ulcers and blisters.

The aim of the present research was to identify the possibly correlations of the clinical pattern with the result of CPV-2 detection, using the real time PCR.

MATERIALS AND METHODS

Fourteen dogs with clinical signs associated with the suspicion of canine parvovirus, has been tested on feces samples by real time PCR. The test samples used were rectal swabs from animals that have shown clinical signs of canine parvovirus infection. The samples were kept at a temperature of 3-4°C and analyzed in no more than 48 hours. A preliminary stage of nucleic acid extraction is required to perform Real-time PCR.

The extraction of the DNA was made with the QIAamp cador Pathogen Mini Kit (Qiagen, Dusseldorf, Germany), according with the insert kit (Table 1). Protein cleavage for the appropriate expression of the genetic material from the biological sample is done using proteinase K, in VXL buffer medium. After mixing by pipetting/vortexing and incubation for 15 minutes at room temperature, the mixture formed is spin-centrifuged to collect the liquid.

The CBA buffer is added to the liquid obtained and after centrifugation, the supernatant obtained is transferred to purification columns. Purification colonies are tubes that contain a single silica membrane that captures genetic material. They contain 2 inner chambers separated by the silica membrane: the upper one contains the liquid to be analyzed, and the lower one contains the solution that crossed the silica membrane. After centrifuge, the solution obtained from crossing the membrane is removed and the AW1 buffer is added to the upper chamber.

The colonies are centrifuged and the previous procedure is repeated with the addition of AW2 buffer. In order to eluate the genetic material, the AVE buffer is introduced in the purification colonies, which has the role of releasing the nucleic acids that were captured at the level of the silica membrane. The colonies are incubated for 1 minute at room temperature, after which there are centrifuged for 1 minute (Table 1). The eluate thus obtained will be used in the amplification step of the specific parvovirus genetic fragment within the Real-time PCR test.

Table 1. Nucleic acid extraction protocol (QIAamp cador Pathogen Mini Kit)

REAGENT	µl/sample	No. samples	Total
Proteinase K	20 µl		
Sample	200 µl		
Buffer VXL	100 µl		
Pipetting/vortex mixing			
Incubate for 15 minutes at room temperature			
Spin centrifuge for liquid collection			
Buffer ACB	350 µl		
Pipetting/vortex mixing			
Spin centrifuge for liquid collection			
Transfer of samples to purification colonitis			
Centrifuge at 8000-10.000 rpm for 1 minute. Replacement manifold tube.			
Buffer AW 1	600 µl		
Centrifuge at 8000-10.000 rpm for 1 minute.			
Eluted remove			
Buffer AW2	600 µl		
Centrifuge at 8000-10.000 rpm for 1 minute. Replacement manifold tube.			
Eluted remove			
Centrifuge at maximum speed for 2 minutes. Introduction of colonitis into the collection tube.			
Buffer AVE	50 µl		
Incubation for 1 minute at room temperature.			
Centrifuge at maximum speed for 1 minute.			
Storage the elute at 1-2°C until the amplification step.			

The PCR amplification temperature protocol for the detection of the canine parvovirus was: 95°C 5 minutes, 40 cycles with 96°C for 5 seconds, 60°C for 5 seconds and 68°C for 3 seconds. The final elongation: 72°C for 1 minute.

The mix was made in a total volume of 25 µl from which 8 µl DNA template, 4.5 µl primers and probes specific for canine Parvovirus, 12.5 µl enzyme mix (Table 2).

Table 2. The reagents and the quantities of the reaction mix for detection of the canine Parvovirus genome

Reaction mix	
Reagents	**µl/sample**
Primeres and probes	4.5 µl
Enzyme mix	12.5µl
DNA template	8 µl
Total	25 µl

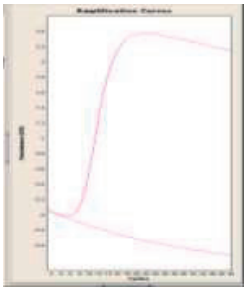
RESULTS AND DISCUSSIONS

From the fourteen tested samples, 13 were positive (92.85%) to the real-time PCR test. The registered positiveness has been associated with different Ct values ranging from 6.82 to 35. One sample (7.14%) was negative. In this study, over 50% of the animals examined were 16 weeks age or younger. Positive Ct value has been registered in samples belonging to dogs aged from 7 to 12 months old (28%), thus proving that canines over 6 months old may develop clinically manifest infection (Table 3).

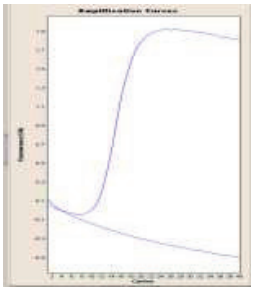
Table 3. The results of the tested samples

Sample No.	Age	Sex	Clinical features	RT-PCR result	Ct value
1	4 m	F	apathy, loss of appetite, diarrhea and vomiting	P	27.88
2	6 m	F	apathy, diarrhea and vomiting	P	32.31
3	2 m	F	apathy, loss of appetite, diarrhea and vomiting	N	-
4	3 m	M	lethargy, loss of appetite	P	18.22
5	3 m	M	apathy, loss of appetite, diarrhea	P	10.22
6	3 m	F	apathy,vomiting,diarrhea, exitus	P	6.82
7	1 y	F	apathy, loss of appetite,vomiting, hypersalivation.	P	32.60
8	8 m	M	apathy, anorexia, vomiting, bloody feces	P	13.09
9	3 m	F	apathy, anorexia, bloody feces	P	22.96
10	4 m	M	apathy, anorexia, vomiting, bloody feces	P	30.93
11	4 m	F	apathy, anorexia, fecal diarrhea	P	33.51
12	9 m	M	apathy, anorexia, vomiting, diarrhea	P	35
13	8 m	M	lethargy, anorexia, repeated vomiting, hemorrhagic diarrhea	P	35
14	4 m	F	apathy, vomiting, abdominal pain	P	30.28

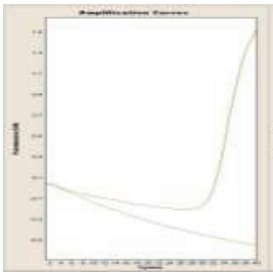
m = month; y = year; P = positive; N = negative; M = male; F = female



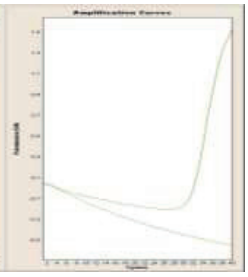
Sample 6-Ct. 6.82



Sample 5-Ct. 10.22



Sample 10-Ct. 30.93



Sample 14-Ct. 30.28

Figure 1. Images with different Ct values from Real-Time PCR; in each image are selected the tested sample with the melting curve register and the negative control with no melting curve

Real-Time PCR analysis of the tested samples revealed the following results: 28.57% of cases had a large amount of parvoviral DNA (Ct range 6.82-18.22) in feces, reflecting severe infection of the digestive tract with massive elimination of viral particles; 14.28% of the cases had an average amount of parvoviral DNA (Ct = 22.96-27.88) in feces reflecting the active infection of the digestive tract; 50% of cases had a small amount of parvoviral DNA (Ct = 30.28-35) in feces reflecting the reduced infection of the digestive tract with low elimination of viral particles; in one case (7.14%) the result was negative, there was no genetic material of parvovirus in the examined fecal swab. The 3-4 months old dogs register the highest share, their samples containing large and medium amounts of virus. However, high concentrations of canine parvoviral DNA can also be seen in individuals older than 6 months in the present study. The increased prevalence of parvovirus in dogs, under 6 months old, described in the literature is in accordance with the results of this study, which shows that over 50% of the tested dogs had canine parvovirus infection at 3-4 months of age. Clinically expressed infection in individuals older than 6 months is increasingly common, especially in the case of CPV-2c infection (Decaro et al., 2009; Decaro and Buonavoglia, 2012). Females are represented in greater numbers compared to males. This is in contradiction with studies from the literature that state either that there is no predisposition to sex, or that males are represented in a higher percentage compared to females (Behera et al., 2015; Khare et al., 2019; Mokhtari et al., 2017). Real-time PCR is a method of diagnosing canine parvovirus that has high specificity and sensitivity compared to other laboratory tests due to the fact that detect a small quantities of virus and has 100% of specificity because of the primers (Decaro et al., 2005; Desario et al., 2005). Low Ct values are mainly registered in the subjects of young age and, so, with an unfavorable survival prognosis. Clinical manifestations cannot be attributed to a category dictated by Ct, being quite uniform regardless of the amount of CPV eliminated in the feces.

CONCLUSIONS

In the studied group, 50% of the positive subjects by Real-Time PCR were less than 6 months old. Positive results by Real-Time PCR were also recorded in 2.28% of subjects over 7 months of age: this demonstrates that the calendar age it self is not a criterion for excluding/including suspicion. Also, regarding the sources of infection, this result supports the observation according to which the parvovirus is carried and eliminated by the subjects that were previously contaminated.

In the studied group, the ratio of females is 14% higher than that of males. The characteristic symptoms of canine parvovirus infection - vomiting/diarrhea, has been manifested in only 4.92% of dogs.

Using the Real-time PCR, we found the highest amount of viral particles (DNA material) in the subjects of 3-4 months aged. Dogs older than 6 months may suffer severe infection of the digestive tract and clinically expressed disease.

Despite the variation range of Ct`s, the clinical pattern registered did not directly relate with the virus amount, rather with the age.

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POTENCY EVALUATION OF TWO COMMERCIAL VACCINES AGAINST CONTAGIOUS AGALACTIA OF SMALL RUMINANTS

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Abstract

Immunoprophylaxis is the most affordable, effective and eco-friendly tool, which recommends it as the first option to control contagious agalactia in small ruminant flocks. The purpose of this study was to evaluate the immune response toward two marketed vaccines. Both products contain Mycoplasma agalactiae inactivated with formalin, on aluminum hydroxide gel. The trial has been carried out on a flock of 700 sheep. Each vaccine was administered to 250 animals according to the manufacturer instructions and 200 animals were in the control group. Serum samples were collected on vaccination days (0 and 21) and post vaccination, at 30, 90, 180 and 360 days. The immune response was assessed using a commercial indirect ELISA kit for antibody detection. Antibody titers increased rapidly after vaccination, reached the highest level between 21 and 30 days and declined after 180 days. No statistically significant differences in titers were identified between the two vaccines.

Key words: *Contagious agalactia, ELISA, Mycoplasma agalactiae, small ruminants, vaccine.*

INTRODUCTION

Contagious agalactia of sheep and goats is a transmissible disease, first described by Metaxa in 1816, in Italy, being called "mal de sito" which means "disease of the place" because of its persistence in the environment and ability to contaminate newly introduced flocks (Jaÿ & Tardy, 2019). Initially confined to the Mediterranean basin, the disease has spread through sheep trading and population migration, nowadays being reported on every continent (Lambert, 1987; Manzat, 2001). In Romania, contagious agalactia was first diagnosed in 1935 by Riegler and Stamatin (Manzat, 2001). The primary etiological agent of contagious agalactia in sheep and goats is *Mycoplasma agalactiae*. In goats, the disease can also be attributed to *Mycoplasma mycoides* subsp. *mycoides*, *Mycoplasma capricolum* subsp. *capricolum*, and *Mycoplasma putrefaciens* (Jaÿ & Tardy, 2019).

M. agalactiae is a small, polymorphic bacterium. The lack of cell was provides resistance to penicillin and its analogues, but the microorganism is susceptible to osmotic shock and the effect of detergents. Diagnosis through classical bacteriology is difficult to establish, as isolated strains adjust very slowly to laboratory conditions and may take over a week to develop colonies (Kumar et al., 2014).

The infection is often enzootic, causing mastitis in lactating female animals, with a consecutive drop or complete loss of milk production. It also affects non-lactating females, males and young animals, causing multiple clinical signs, such as pneumonia, arthritis, keratoconjunctivitis and sepsis (Madanat et al., 2001). Non-specific symptoms such as fever, anorexia and weakness can often be a cause of mortality in young animals, while going unnoticed in adult sheep and goats. Also, joint infections can be more severe in the young, taking the form of polyathritis, while in adults occasionally causing lameness (Jaÿ & Tardy, 2019). Primary sources of infection are diseased animals, which can spread the etiological agent through urine, feces and genital discharge. The disease can also be spread through infected milk. Animals that have overcome the disease can still remain carriers for up to 2 years (Manzat, 2001).

An outbreak of contagious aglactia can cause major economic loss to a herd, therefore, efforts to prevent the onset of the disease relay mainly on immunoprophylaxis. In Europe,

formalin inactivated vaccines against *M. agalactiae* are widely used. The vaccines produced using laboratory strains and usually contain an adjuvant such as aluminum hydroxide or an oil emulsion (OIE Terrestrial Manual, 2018). There have also been reports of phenol or saponin-inactivated *Mycoplasma* vaccines that were effective in experimental challenges (Tola et al., 1999).

The aim of this study was to determine and compare the efficacy of two contagious agalactia vaccines produced and marketed in Romania.

MATERIALS AND METHODS

The trials took place in Braila county, between November of 2019 and December of 2020. The animals included in the study belonged to a flock of over 1000 sheep. The flock included all categories of age and sex (rams, gestating females, lactating females, reformed females and lambs of both sexes). As per the producers' instructions, two categories of animals were omitted from the trial: lambs under the age of three months and female sheep during the last month of gestation. Also, only clinically healthy animals were selected. Also, the animals proving suspicious or positive serological results at day 0 were eliminated from the trial. The final number of animals included in the study was 703 subjects. For the immunization, two commercially available vaccines were selected (Vaccine A and Vaccine B), both Romanian products, by different producers. The composition of the two vaccines, as specified on each product's label, was as follows:

- Vaccine A: *Mycoplasma agalactiae* AG6 strain (≥0,60 ELISA units, according to the manufacturer data), inactivated with formalin (≤0.5 mg) and adsorbed onto aluminum hydroxide gel (2.8-3.4 mg Al_2O_3);
- Vaccine B: *Mycoplasma agalactiae* S/94 strain (minimum 5 ELISA units/dose, according to the manufacturer data), aluminum hydroxide gel (0.2-0.25 ml/1 ml of vaccine), formaldehyde (maximum 0.5 mg/ml).

Both vaccines are advertised to provide immunity against *M. agalactie* infection for 6 months.

The sheep were divided into three groups (Table 1). Group 1 (251 sheep) received two doses of Vaccine A, 21 days apart, via subcutaneous route, 1 ml/animal. Group 2 (249 sheep) was immunized with Vaccine B following the same protocol. Group 3 (203 sheep) represented the control group and was administered sterile saline solution via the same route.

Table 1. Trial design and group composition

Group/specification	Rams	Lambs > 3 months	Lactating females	Gestating females	Reformed females	Total
Group 1/Vaccine A	84	47	91	15	14	251
Group 2/Vaccine B	75	52	103	12	7	249
Group 3/Saline solution	52	38	76	24	13	203

The animals were monitored for 7 days after each inoculation in order to observe and record any systemic or local adverse reactions. Blood samples were collected from the animals on vaccination days (day 0 and day 21), and post vaccination, on days 30, 90, 180 and 360 (after the second dose of vaccine). The samples were collected using vacuum blood collection tubes coated with a clot activator. The tubes were kept at room temperature for four hours and were centrifuged 15 minutes at 2000 rpm, 4°C. The serum samples were collected into sterile Eppendorf tubes, identified with each animal's unique serial number and stored at -20°C until processing. The serological response was assessed by indirect enzyme-linked immunoassay (ELISA). A commercially available ELISA kit (CIVTEST ovis *M. agalactiae,* Hipra) was used according to the manufacturer's instructions. The mean antibody titers were calculated for each group, and also for each age and sex category inside the groups.

RESULTS AND DISCUSSIONS

Post-vaccination side effects included local reactions at the site of inoculation, less than 2 cm diameter, inapetence lasting 1-2 days after

vaccination, mainly in lambs, and a temporary (5-6 days) drop in milk production for the majority of the lactating ewes. The control group showed no general, nor local side effects, following the inoculation of the saline solution.

The calculation of the Rz values [Rz = OD_{450} Sample/2x (Mean OD_{450} Negative Control)] and interpretation of ELISA test results was performed according to the manufacturer's instructions (CIVTEST® ovis *M. agalactiae* Product Manual). All animals included in the study were free of antibodies against *M. agalactiae* at day 0 (the day of the first inoculation). The percentage of positive animals in Group 1 was slightly higher at day 21 then Group 2, with increased antibody titer means. Animals in both groups showed a significant rise in antibody titers 30 days after the second vaccination. At day 90, the serological response of both groups was still positive, with a slight decrease in antibody levels for the animals of Group 1. Testing at 180 days post-vaccination showed a marked decline in mean antibody titers for both groups, with only a small percentage of animals remaining positive. Very few positive results were recorded at the 360 days post-vaccination test.

The animals in the control group remained negative for the duration of the trial (Figure 1). The percentage of positive animals in each group and for each serological test is presented in Table 2.

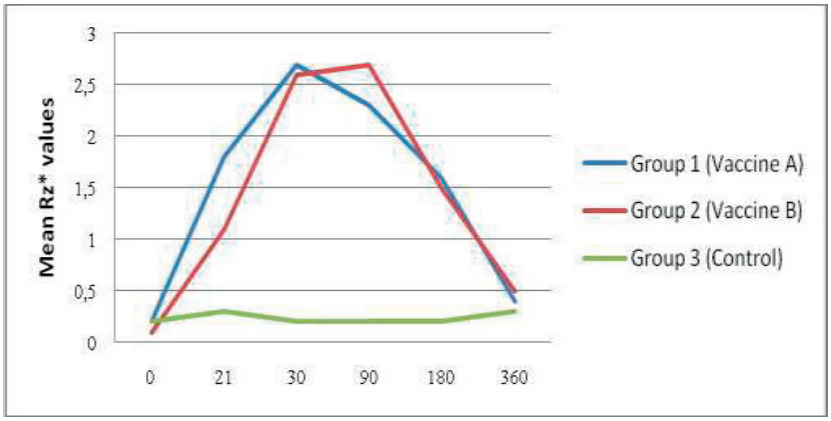

Figure 1. Mean Rz values throughout the trial period
(*Rz <1 - negative; Rz 1 - 1.5 - suspicious; Rz > 1.5 - positive)

It can be observed that even though antibody levels were higher in Group 1 at day 21, similar levels of protection were reached in both vaccinated groups 30 days after the booster. The immune response remained at close values for the next 2 months, with similar results obtained 90 from the booster. As expected, 6 months later, the mean Rz values dropped below the cut-off value of 1.5. It can be deduced from the test results that a repeating the vaccination scheme at a 6 months interval is necessary to ensure a constant level of protection in the flock. Mean antibody titers for each category of animals inside the vaccinated groups are shown in Figure 2 (Group 1 - Vaccine A) and Figure 3 (Group 2 - Vaccine B).

Table 2. Percentage of positive results for each test

Test Group	Positive animals (%)					
	Day 0	Day 21	Day 30	Day 90	Day 180	Day 360
Group 1 - Vaccine A	0	60	96	87	54	4
Group 2 - Vaccine B	0	45	94	96	58	11
Group 3 - Control	0	0	0	0	0	0

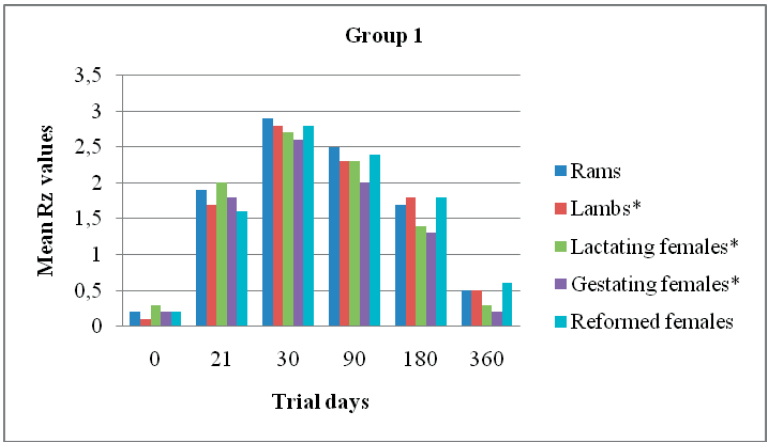

Figure 2. Evolution of antibody titers over the trial period for each category of animals in Group 1 (*the same animals were tested, even though their status changed over time)

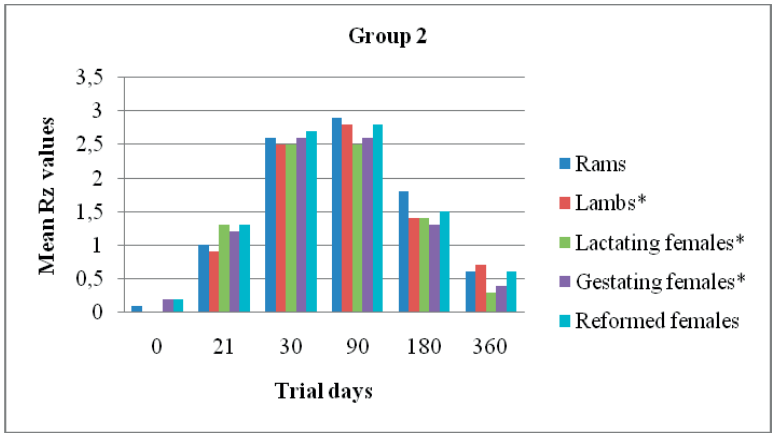

Figure 3. Evolution of antibody titers over the trial period for each category of animals in Group 2 (*the same animals were tested, even though their status changed over time)

The results presented in the study prove that both vaccines induced sero-conversion in the vaccinated animals of all ages and physiological status. For vaccine A, positive reactions were recorded after day 21. Antibody titers reached a maximum level at day 30 after the booster, and had started to decrease at day 90. At day 180, three of the five categories of sheep in Group 1 (rams, lambs and reformed females) still had positive reactions on the ELISA test.

For the animals in Group 2, the serological response of the vaccinated animals was below the cut-off value of 1.5 at day 21. At day 30, antibody titers had reached a similar level to those of Group 1, however, at day 90, the mean Rz values were higher. At day 180, antibody titers had decreased significantly, with only two categories of animals remaining positive on the ELISA test (rams and reformed females).

The efficacy of formalin inactivated vaccines against *M. agalactiae* has been investigated and disputed intensely for the last decades. In 2018, El-Yazid et al. studied the efficacy of four types of inactivated *M. agalactiae* vaccines, using formalin, phenol, saponin and sodium hypochlorite. The tests were carried out on mice and goats, and proved that the formalin inactivated vaccine provided only moderate protection in both serological and challenge trials. Saponin and phenol inactivated vaccines gave the highest level of protection, while the sodium hydroxide inactivated vaccine induced the lowest protective efficacy. Similar results

had been obtained before by researchers who demonstrated that phenol and saponin inactivated *Mycoplasma* vaccines induced the highest level of serological response in vaccinated sheep, compared to formalin, sodium hypochlorite and heat inactivated formulas (Tola et al., 1999). The level of immune response can also vary depending on the adjuvant used for vaccine preparation. A study carried out in Brazil reported superior results using Montanide IMS 2215 VG as adjuvant for a inactivated *Mycoplasma agalactiae* vaccine, compared to aluminum hydroxide and a Montanide Gel 01 adjuvanted vaccines (Campos et al., 2013). Other researchers have demonstrated that live attenuated vaccines provide superior clinical protection in sheep, despite the lack of serological response (Agnone et al., 2013; Ozdemir et al., 2019). However, live attenuated vaccines for contagious agalactia are not permitted in the European Union (OIE Terrestrial Manual, 2018).

CONCLUSSIONS

Both vaccines tested in the current study provided adequate levels of immune response in the vaccinated animals.

Results of the ELISA tests demonstrate that antibody levels decrease 6 months after vaccination, two vaccinations per year are necessary in order to provide a constant level of protection in the flock.

Further studies are necessary to assess the correlation between the serological response and the clinical protection provided by these vaccines.

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PERIANAESTHETIC MANAGEMENT OF CANINE PATIENTS THAT UNDERWENT HEMILAMINECTOMY FOR MEDULLAR COMPRESSION

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Abstract

This study presents the perianaesthetic management for eight dogs, aged between 3 and 9 years old that underwent a hemilaminectomy surgical procedure for medullar decompression. The patients were presented at the Faculty of Veterinary Medicine in Bucharest for acute onset of posterior unilateral paresis. Following the examination through magnetic resonance imaging, medullar compression was diagnosed at different thoraco-lumbar levels and surgery was recommended (hemilaminectomy). Four of the patients were premedicated with Fentanyl 3 µg/kg and Midazolam 0.2 mg/kg administered intravenously (IV) and the other four received Dexmedetomidine 2 µg/kg, Butorphanol 0.2 mg/kg and Ketamine 2 mg/kg intramuscularly (IM). For induction Propofol was administered IV at a dose of 3 mg/kg and patients were intubated. All patients had an epidural anaesthesia with Lidocaine (2 mg/kg). Maintenance consists in the administration of a constant rate infusion (CRI) of Fentanyl (3 µg/kg per hour) and Ketamine (1 mg/kg per hour) for the first four patients and a CRI of Lidocaine (3 mg/kg per hour) and Ketamine (1 mg/kg per hour) for the other four patients. Post-surgery multimodal analgesia protocols ensured pain control during recovery.

Key words*: analgesia, perianaesthetic management, hemilaminectomy.*

INTRODUCTION

Intervertebral disc disease is a condition where the nucleus pulposus of the intervertebral disc extrudes into the spinal canal causing compression of the spinal cord (Jeffery et al., 2013). Clinical signs depend on the location of the injury and are always associated with manifestations of acute pain. Part of the perianaesthetic management is represented by a good pain management during and also after surgery (Buvanendran et al., 2009).

Sedative agents may be given by intravenous (IV), intramuscular (IM), subcutaneous (SC) and oral routes based on their pharmacokinetic profiles. An IV catheter will be put in place, facilitating IV administration of sedative agents, which will aid in using lowered doses of these drugs. Some sedatives (alpha 2 agonists) will be more beneficial in aggressive animals until IV access is established.

Dexmedetomidine is the active dextro-isomer of the previous medetomidine formulation. Higher doses are indicated to achieve sedation in smaller dogs and lower doses for larger dogs. Doses can be given IM or IV, but the patient should be left undisturbed for 15 minutes for sedation until effect. Decreased doses of dexmedetomidine can be considered in combination with opioid analgesics (Tang et al., 2011).

Due to the widespread location of alpha2 receptors, their effects could promote analgesia at many levels of the pain pathway (Murrell, 2005).

Diazepam and midazolam are most commonly used as sedatives or as co-induction agents in small animal practice. Benzodiazepines are commonly administered with an opioid in sick patients to reduce the risk of excitement. The main reasons for using a co-induction agent with an injectable anesthetic agent are to smooth the overall induction process enabling endotracheal intubation without swallowing or coughing, minimize the negative side effects of propofol and also providing analgesia for the beginning of the procedure (Skelding, 2021).

Fentanyl is a synthetic mu-agonist of rapid onset and short duration. Low doses of fentanyl (1-5 µg/kg IV) result in short duration of analgesic effects (20-30 minutes) because of rapid lowering of the plasma therapeutic

analgesic concentrations, but higher doses can prolong the analgesia to more than 1 hour with a single bolus (Vanderah, 2010).
Bradycardia or associated bradyarrhythmia can be noticed in patients after IV bolus administration and is likely with higher doses. To avoid these effects, you can titrate the initial dose of fentanyl to effect and continue with this dose as a constant rate infusion (3-6 μg/kg/h) (Vanderah, 2010).
Butorphanol is a mixed kappa agonist-μ antagonist opioid. It offers analgesic and sedative effects, but it antagonizes the actions of mu-agonists if administered simultaneous. A pure mu-agonist can be administered for supplemental analgesia an hour after butorphanol administration (Feng et al., 2012).
Lidocaine is primarily used for loco-regional anesthesia, but it is also used as an infusion during anaesthesia to reduce the inhalant required to maintain anaesthesia. Lidocaine has been shown to alleviate neuropathic pain and hyperalgesia and to reduce opioid requirements following surgery when administered as a constant rate infusion (Gutierrez-Blanco et al., 2015). A constant rate infusion (1-3 mg/kg per hour) can be used intraoperatively to reduce the inhalant requirements or post-operatively in combination with opioid and ketamine for the severely painful patient (Gutierrez-Blanco et al., 2015). Ketamine is an adjunctive analgesic recommended for use in a multi-modal regimen for treatment of severe pain (Pozzi et al. 2006).
The analgesic dose is much lower compared with dose that is used for anaesthesia. Ketamine is an NMDA receptor antagonist with anti-hyperalgesic component and as part of a multimodal analgesia protocol reduces the need for opioids post-operatively and the potential adverse effects associated with higher dosages of this class of analgesics when managing severe pain (Costea, 2016).
The aim of this study was to evaluate the perianaesthetic management of eight canine patients that underwent hemilaminectomy and also, the postoperative analgesic effect of fentanyl and meloxicam drug combinations.

MATERIALS AND METHODS

Eight canine patients aged between 3 and 9 years old were presented at the Faculty of Veterinary Medicine in Bucharest for acute onset of posterior bilateral paresis. Breeds presented in the study were represented by crossbreed (3 patients), French Bulldog (3 patients) and Shih Tzu (3 patients) (Figure 1).

Figure 1. A 8-year-old dog, male, cross-breed with medullar compression at L1-L2 level

We excluded from the study aggressive dogs and those that had cardiac, renal or hepatic disease.
Complete clinical evaluation and preanesthetic blood tests were performed along with echocardiology. After complete clinical and neurological assessment of the patients, MRI examination was performed under general anaesthesia and continuous monitoring (Tudor, 2018) at thoraco-lumbar levels (Figure 2).

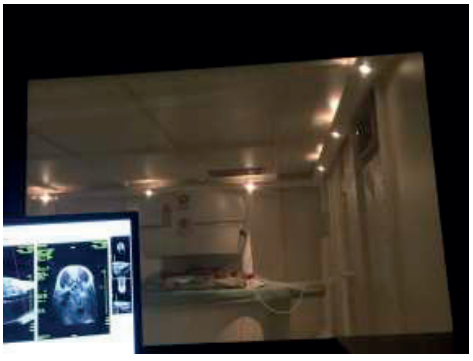

Figure 2. Magnetic Resonance Imaging scan of a 9-year-old dog for medullar compression at thoraco-lumbar level

Following MRI, medullar compression was diagnosed at different thoraco-lumbar levels: T11-T12, T12-T13, T13-L1 and L1-L2 and

surgery was recommended (Neagu et al., 2018) (Figures 3-5).

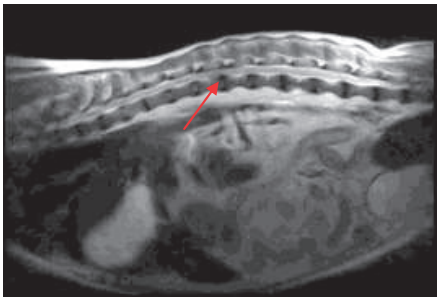

Figure 3. T2 sequence in sagittal plain, medullar compression at T13-L1 level

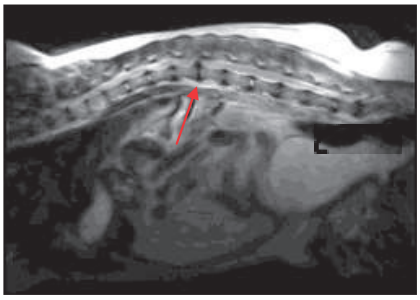

Figure 4. T2 sequence in sagittal plain, medullar compression at L1-L2 level

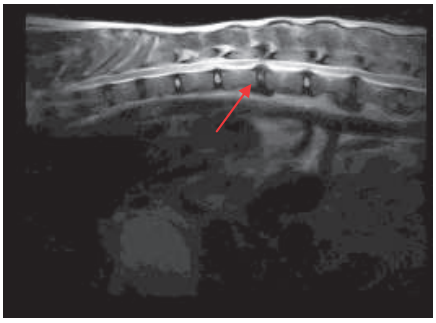

Figure 5. T2 sequence in sagittal plain, medullar compression at T13-L1 level

Prior to surgery intervention, all patients were fasted for 12 hours but had free access to water one hour before premedication. All patients had a peripheral catheter in the cephalic vein.

Five minutes before premedication, baseline values for heart rate (HR), respiratory rate (RR) and rectal temperature were recorded.

Following preanesthetic evaluation two groups were created depending on the anesthetic and analgesic drugs that we intended to use.

American Society of Anesthesiologists (ASA) risk scale scores were recorded for each patient and were included in this study dogs with an ASA score of II.

For the first group (FM) with a total number of 4 patients, age between 6-9 years old, belonging to different breeds (French Bulldog - 2 patients who had medullar compression at T11-T12 and T12-T13; Crossbreed - 2 patients (Figure 6) with medullar compression at T13-L1 and L1-L2 level), premedication with Fentanyl 3 µg/kg and Midazolam 0.2 mg/kg was administered intravenously (IV).

Figure 6. A 9 year old dog, cross-breed male with medullar compression at T13-L1 level

For the second group (DBK) with a total number of 4 patients with age between 3-5 years old belonging to different breeds (French Bulldog - 1 patient with medullar compression at T11-T12 level, Crossbreed - 1 patient with medullar compression at L1-L2 and Shih Tzu - 2 patients with medullar compression at T12-T13 and T13-L1 levels) premedication was made with Dexmedetomidine 2 µg/kg, Butorphanol 0.2 mg/kg and Ketamine 2 mg/kg administered intravenously (IV).

Induction was made with Propofol 3-5 mg/kg intravenously. Patients were intubated and anaesthesia was maintained with Isoflurane and 100% Oxygen.

Spontaneous or intermittent positive-pressure ventilation (IPPV) were maintained by the use of a volume-cycled ventilator delivering 12-15 breaths/minute to achieve a target end-tidal CO_2 of 35-45 mm/Hg. Oxygen flow was initially delivered at 2 L/min with the vaporizer set to achieve an end-tidal concentration C% of 2.0% isoflurane within 10 minutes of induction. After the target concentration was achieved,

oxygen flow was decreased to (500 + 10/kg) L/min, and isoflurane was constantly maintained at 1.5 vol. % in all cases.
For both groups anesthesia protocols were completed with a regional epidural block with Lidocaine (2 mg/kg).
The first group of patients (FM) received for anaesthesia maintenance a constant rate infusion (CRI) of Fentanyl (3 μg/kg/h) and Ketamine (1 mg/kg/h) and for the second group (DBK) a CRI of Lidocaine 3 mg/kg/h and Ketamine 1 mg/kg/h (Tudor R., 2019). Vital signs of the patients were recorded every 5 minutes after induction and until the extubation of the patients. We recorded EKG, heart rate, $EtCO_2$, SpO_2, pulse rate, mean arterial pressure and esophageal temperature (Figure 7).

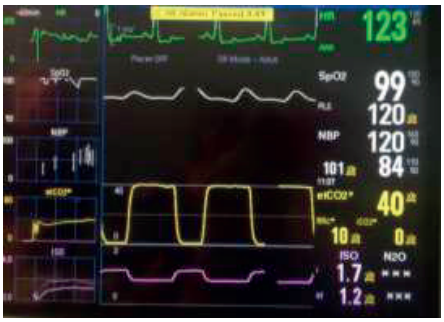

Figure 7. Patient monitoring

An electric blanket was used to maintain the temperature between 38-39°C.
At the beginning of the surgery (Figure 8), during skin incision if the patient reacted to surgical stimulation by a rapid increase of the heart rate, mean arterial pressure or signs of tachypnea additional analgesic and anaesthetic drugs boluses were given: Fentanyl 3 μg/kg IV, Ketamine 1 mg/kg IV.

Figure 8. Medullar decompression in a 9 year old male at T13-L1 level, intraoperatory hemilaminectomy aspect

At the end of the surgery the Isoflurane was turned off. Dolichocephalic dogs were extubated when they began to breathe spontaneously and had palpebral reflex. Brachycephalic dogs were extubated when they had signs of awareness, chewing on the endotracheal tube (Figure 9).

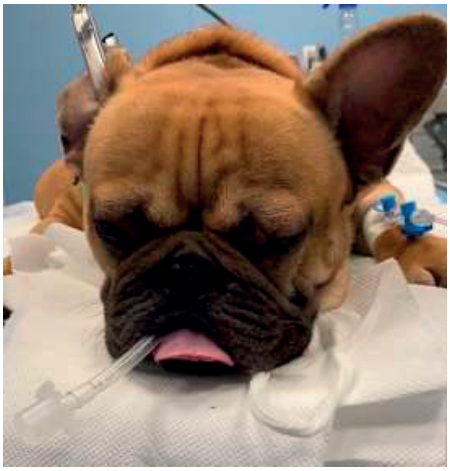

Figure 9. Patient awakening

The patients were moved into the intensive care unit (ICU) where they received as analgesia a bolus of Fentanyl at 3μg/kg and a CRI of Fentanyl (3 μg/kg per hour IV) and Meloxicam 0.2 mg/kg SC every 24 h.

RESULTS AND DISCUSSIONS

Two treatment groups were created with a total number of eight patients. Breed of dogs included mixed breed dogs (n = 3), French Bulldog (n = 3) and Shih Tzu (n = 2) (Figure 10).

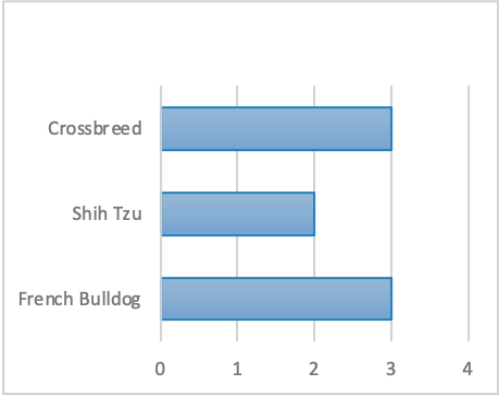

Figure 10. Data about animals included in the study

The perianaesthetic protocols were efficient for pain control throughout the study period. Two dogs from Group 2 (DBK) received additional analgesia during surgery represented by a bolus of Ketamine (1 mg/kg IV) because of a more than 20% increase in the mean arterial pressure and also heart rate.

The anesthesia time for group 1 (FM) had a mean time of 59 minutes (from the time we intubated the patients till the extubation). For group 2 (DBK) mean anesthesia time was of 58 minutes (Figures 11 and 12).

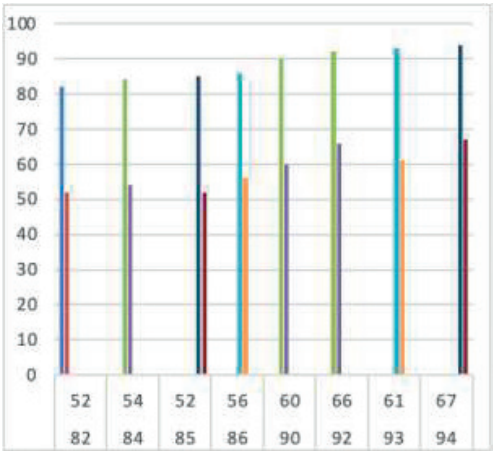

Figure 12. Anesthesia and surgery time for each animal

Figure 13. Patient with no pain manifestation 1 hour after surgery

The results for the groups were compared and analyzed at 12 hours after the first administration.

All dogs were observed for adverse reactions following pain medication therapy. Records of pain manifestation for each patient were assessed at 15 min, 30 min, 45 min, 1, 2, 6 and 12 hours after the analgesic drug was given. All patients were evaluated using the Glasgow Composite Pain Scale (GCPS). Behavior categories used to assess pain included vocalization, attention to wound area, mobility, response to touch, demeanor and posture/ activity. A categorical score was assigned within each behavior category based on the severity of the behavior or the response observed (Table 1). Potential cumulative pain scores ranged from 0 (least painful) to 23 (most painful). To ensure interpretative consistency, a single person was trained in evaluating the dogs for pain. The person first observed the dog`s behavior from a distance so as not to disturb the dog, then the assessor increased his interaction with the dog, including manipulation of the surgical site and removing the dog from the cage.

Table 1. Glasgow Composite Pain Scale (GCPS)

Behavior Category	Score	Definition
Vocalization	0	Quiet
	1	Whimpering or crying
	2	Groaning
	3	Screaming
Attention	0	Ignoring
	1	Looking
	2	Rubbing
	3	Chewing
Mobility	0	Normal
	1	Lame
	2	Slow or reluctant
	3	Stiff
	4	Refuses to move
Response to touch	0	Do nothing
	1	Looks around
	2	Flinch
	3	Growl or guard area
	4	Snap
	5	Cry
Demeanor	0	Happy and content and bouncy
	1	Quiet
	2	Indifferent or nonresponsive to surroundings
	3	Nervous, anxious or fearful
	4	Depressed or nonresponsive to stimulation
Posture/activity	0	Comfortable
	1	Unsettled

In the intensive care unit (ICU) patients were in a steady plane with no further analgesic requirements (Figure 14).

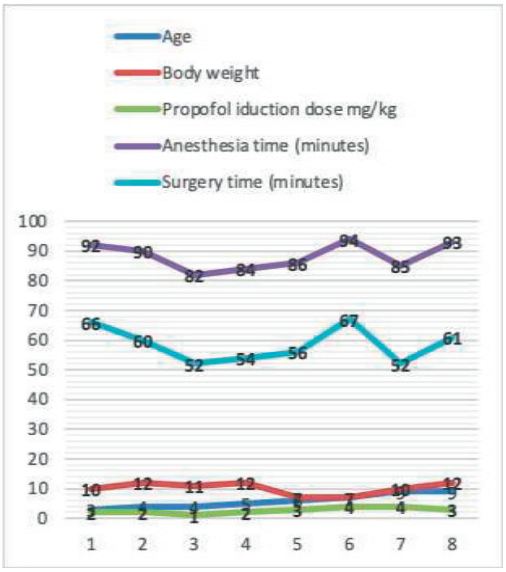

Figure 14. Description of patients anesthetized for hemilaminectomy at thoraco-lumbar level

At discharge all patients received Meloxicam 0.2 mg/kg per os for 5 days along with Tramadol 3 mg/kg every 12 h for 3 more days. A multimodal approach is recommended as this helps minimize the side effects that may occur (Costea, 2016).

CONCLUSIONS

Premedication with Fentanyl and Midazolam, induction with Propofol and maintenance with Isoflurane and a CRI of Fentanyl and Ketamine represents a good multimodal analgesic pain for patients that underwent hemilaminectomy.
Very important in order to achieve a good level of analgesia and a predictable recovery of the patient is represented by a good and well documented perianaesthetic management during hemilaminectomy for medullar decompression.

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MORPHOLOGY AND EPIDEMIOLOGICAL ASPECTS OF SPLENOMEGALY IN DOGS – RETROSPECTIVE STUDY

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Abstract

Splenomegaly in dogs is frequently diagnosed in veterinary practice. Establishing its morphological substrate is of major importance in choosing the therapeutic course and establishing the prognosis. The current study analyzed 194 dog spleens (190 surgically removed), submitted to the Pathology department, between August 2005 and October 2020. Macroscopic, cytological and histopathological examinations were performed. According to our results, localized/asymmetric splenomegaly represents 78.35% of the total cases examined. Splenomegaly was diagnosed mainly in mixed-breed dogs (22.68%), among the purebreed dogs the most affected being those of medium and large size, such as German Shepherd (11.86%) and Rottweiler (10.3%). Old age is a risk factor, 51% of the subjects being over 10 years old; 53% were males and 47% females. In 55% of all cases the splenomegaly had a non-neoplastic substrate, in 45% being represented by neoplastic processes. The most frequently diagnosed tumor was hemangiosarcoma (50.57%); the most common non-neoplastic diseases were hematoma (40.19%) and splenic congestion (26.17%).

Key words: *dog; epidemiology; splenomegaly; tumoral and non-tumoral lesions.*

INTRODUCTION

The spleen is a secondary lymphoid organ that performs multiple functions in the body, the most important being hematopoiesis, blood filtration, immune response, blood storage and iron metabolism.

The diagnosis of splenic lesions in canine patients is increasingly required in current veterinary practice, mainly in old dogs. Imaging exams represent the main toll to identify the changes in the splenic parenchyma, while the histopathological examination is used to establish the definite diagnosis (Vulpe et al., 2015).

Splenic lesions most often cause splenomegaly, which can be localized/asymmetrical or diffuse/uniform. Localized splenomegaly is most often diagnosed in dogs, while in cats the diffuse form is more common (Zachary, 2017).

Localized splenomegaly is characterized by the presence of one or more nodules, grouped into two types - bloody nodules and firm nodules. Bloody nodules appear in case of splenic hematomas, acute infarcts or hemangiosarcoma, while firm nodules occur most frequently in nodular lymphoid hyperplasia and in some primary and secondary neoplastic processes (lymphoma, fibrosarcoma, histiocytic sarcoma, lipoma, liposarcoma, leiomyosarcoma etc.) (Spangler & Kass, 1998; Șahînduran et al., 2016). Congested splenomegaly may be mainly the consequence of acute hyperemia and stasis. Noncongested splenomegaly occurs in case of metaplasia, generalized lymphoid hyperplasia, inflammatory and neoplastic processes (lymphoma, visceral mast cell tumors, histiocytic sarcoma) and splenic amyloidosis (Jubb et al., 2017; Zachary, 2017).

Studies have described multiple aspects which could possibly indicate an underlying malignant tumor in case of splenomegaly, in an attempt to develop a long-term prognosis. The presence of hemoperitoneum and the size of the splenic masses may indicate a malignant process and decrease the post-splenectomy survival rate. According to Mallinckrodt & Gottfried (2011), compared to the dogs with splenic hemangiosarcoma, those with benign splenic masses had a higher mean mass-to-splenic volume ratio and also a higher mean splenic weight as a percentage of body weight. Cleveland & Casale (2016) showed that the

median life expectancy of dogs with benign splenic lesions is 436 days and 110 days in the case of malignant splenic lesions. In Spangler and Kass's study (1997) on the survival rate of post-splenectomy in dogs, only 7% of patients diagnosed with hemangiosarcoma were still alive one year after the surgery.

The aim of this retrospective study is to review the main types of lesions that cause splenomegaly in dogs, with the identification of a possible susceptibility regarding breed, age and sex. Secondly, we emphasized the value of cytological examination in establishing and confirming a diagnosis of the underlying cause of splenomegaly.

MATERIALS AND METHODS

For this study we reviewed records of 194 spleens surgically removed or after necropsic examinations between August 2005 and October 2020, from canine patients diagnosed with splenomegaly. The spleens were examined and processed within the Pathological Department of the Faculty of Veterinary Medicine Bucharest. Splenomegaly was mainly diagnosed by imaging examination. After excision, the spleens were grossly examined. In some cases, cytologic smears were made preoperatively using the fine-needle aspiration technique and by impression and scraping in case of surgically-removed spleens. The smears were stained using the May-Grünwald Giemsa (M-G.G.) method. Subsequently, the samples collected from representative areas were fixed 10% neutral buffered formalin, dehydrated in alcohol and paraffin-embedded. Histopathology slides were stained with hematoxylin and eosin (HE) and also with Perls staining, which was used to highlight the siderophages.

RESULTS AND DISCUSSIONS

Between August 2005 and October 2020, at the Pathological Department of the Faculty of Veterinary Medicine Bucharest, 194 dog spleens were examined, of which 98% (n = 190) were surgically removed and 2% (n = 4) were collected during necropsies.

From the 194 spleens analyzed, 78.35% (n = 152) had localized splenomegaly and 21.65% (n = 42) had diffuse splenomegaly (Table 1). Malignant tumors represent 41% of the total cases, the benign tumoral and non-neoplastic lesions being the most common findings in diffuse (73.8%) and localized splenomegaly (55.26%). Similar results were obtained in a retrospective study conducted at the University of Londrina, Brazil (Olegário da Silva et al., 2016), where out of a total of 71 spleens examined, 67.8% (n = 59) had localized splenomegaly and the remaining 16.9% (n = 12) had diffuse splenomegaly. In this study, non-neoplastic lesions were also more common in the mentioned subcategories. Cleveland & Casale (2016) also noted that localized splenomegaly is most frequently associated with benign and non-tumoral masses.

Table 1. Type of splenomegaly according to gross examination and distribution of malignant, non-tumoral and benign tumoral lesions

Type of splenomegaly	**Number of cases (%/total cases)**	**Number of cases with malignant lesions (%/cases)**	**Number of cases with non-tumoral and benign tumoral lesions (%/cases)**
Localized	152 (78.35%)	68 (44.74%)	84 (55.26%)
Diffuse	42 (21.65%)	11 (26.2%)	31 (73.8%)
Total	**194 (100%)**	**79 (41%)**	**115 (59%)**

Regarding to the breed predisposition of splenomegaly, our data shows that most cases corresponded to mixed-breed dogs (22.68%), followed by German Shepherd (11.86%), Rottweiler (10.31%), Bichon (5.67%), Cocker Spaniel (5.67%), Poodle (5.15%), German Shorthaired Pointer (4.64%), Labrador Retriever (3.09%), Pekingese (3.09%), Golden Retriever (2.58%), Husky (2.58%), Boxer (2.06%), Romanian Mioritic Shepherd Dog (1.55%), Fox Terrier (1.55%) and Shih Tzu (1.55%); medium-sized and large-sized dogs are thus the majority. Similar results have been described in other articles (Bandinelli et al., 2011; Olegário da Silva et al., 2016). Given the fact that splenomegaly occurs mainly in large-breed dogs, Corbin et al. (2017) decided to study the splenomegaly in small-breed dogs. In their study, the most affected breeds were

Wheaton Terrier, Bichon Frize, Cocker Spaniel and Pembroke Welsh Corgi.

In our study, 53% of the spleens were from males and 47% from females. Therefore splenomegaly has no sex predisposition. The distribution of cases according to age reveals a preponderance of splenomegaly in the category of dogs aged between 6 and 10 (43%) and especially those over 10 (51%). Dogs under 6 years of age represented 6% of the cases.

The identified splenic lesions in the current study were classified into non-neoplastic lesions and neoplastic lesions (Tables 2 and 3). Out of the total cases, 55% (n = 107) represented non-neoplastic lesions, while 45% (n = 87) were neoplastic, of which 9.2% (n = 8) were benign and 90.8% (n = 79) were malignant. The diagnosis was established by histopathological examination in 87% of cases (n = 168) and by cytological examination in 13% of cases (n = 26). In 51% (n = 99) of the cases the cytological examination was followed by the histopathological one. Of these 99 cases, in 82.83% (n = 82) the cytological diagnosis corresponded with the histopathological one. Also, the cytologic examination could easily identify the malignant processes. In case of 9 patients (9/82), the cytological examination indicated a non-tumoral lesion, without being able to establish its origin, and a histopathological examination was needed to establish the definitive diagnosis. We can conclude that in case of splenomegaly, the cytological examination is useful in differentiating the malignant processes from the benign ones. All 99 cytopathological examinations were performed on surgically excised spleens, and none of the fine-needle aspirates (n = 26) of the spleen were followed by a histological examination. Thus, we could not determine the accuracy of the cytological examination performed by ultrasound-guided fine-needle aspiration. Yankin et al. (2019) analyzed 125 smears from samples collected through ultrasound-guided aspiration from splenic nodules and identified a clinically relevant diagnosis in only 20% of cases. However, in O'Keefe and Couto's study (1987) about the utility of cytological examination by fine-needle aspiration in the diagnosis of splenomegaly, in all 14 cases in which both cytological and histopathological examinations were performed, the diagnoses corresponded completely. Also, Ballegeer et al. (2007) obtained a correlation between the cytological and histopathological examinations in 61.3% of the 31 cases.

Of the total splenic lesions diagnosed in the present study, 55% were non-neoplastic. The most common lesions were hematoma (40.19%), splenic congestion (26.17%) and reactive hyperplasia (24.3%). These types of lesions were also the most common in Lee et al.'s study (2018), in which non-neoplastic lesions represented 68.8% (n = 32) of total cases, with 18 reactive hyperplasias, 4 hematomas and 4 splenic congestions. In our study, 5 cases consisted of non-specific changes, including hemorrhage, extramedullary hematopoiesis and hemosiderosis, along with 2 cases of splenitis, 2 cases of splenic infarction and one case of accessory spleen (Table 2). The diagnoses were classified according to the predominant lesion, as there were cases in which several types of lesions coexisted in the same histopathological sample. Being a long-term retrospective study, no information could be collected on the post-splenectomy survival rate of the canine patients.

Table 2. Type and distribution of non-neoplastic lesions

Diagnosis	Number of cases (%)
Hematoma	43 (40.19%)
Congestion	28 (26.17%)
Reactive hyperplasia	26 (24.30%)
Non-specific changes	5 (4.67%)
Splenitis	2 (1.87%)
Splenic infarction	2 (1.87%)
Accessory spleen	1 (0.93%)
Total non-neoplastic lesions	**107 (100%)**

We diagnosed only 2 types of benign tumors: hemangioma (n = 7) and myelolipoma (n = 1).

The most common malignant tumor was hemangiosarcoma (50.57%), followed by histiocytic sarcoma (13.78%), splenic lymphoma (9.2%), splenic fibrosarcoma (6.9%) and malignant fibrous histiocytoma (4.6%). Although it is a rare malignant tumor (Soare et al., 2012), our cases included 12 dogs diagnosed with splenic histiocytic sarcoma. Hemangiosarcoma is identified in many studies as the most common malignant splenic tumor (Bettini et al., 2001; Cleveland & Casale, 2016; Day et al., 1995; Leyva et al., 2018), the data of

our study being in accordance with these studies. Other malignant tumors from our cases were splenic mmast cell tumor (2.3%) and metastases (3.45%) (Table 3).

Table 3. Type and distribution of neoplastic lesions

Diagnosis	Number of cases (%)
Hemangioma	7 (8.05%)
Myelolipoma	1 (1.15%)
Total benign lesions	**8 (9.20%)**
Hemangiosarcoma	44 (50.57%)
Histiocytic sarcoma	12 (13.78%)
Splenic lymphoma	8 (9.20%)
Splenic fibrosarcoma	6 (6.90%)
Malignant fibrous histiocytoma	4 (4.60%)
Splenic mast cell tumor	2 (2.30%)
Splenic metastasis of adenocarcinoma	2 (2.30%)
Splenic metastasis of mesothelioma	1 (1.15%)
Total malignant lesions	**79 (90.80%)**
Total neoplastic lesions	**87 (100%)**

Hemangiosarcoma was the most common splenic tumor in the examined cases (n = 44). The highest incidence was registered among mixed-breed dogs (27.27%), and among the pure breeds, the most affected were German Shepherd (20.45%), Bichon (9.09%) and Siberian Husky (6.82%).

Out of a total of 44 cases of hemangiosarcoma, 54.55% of the patients studied were females, and the remaining 45.45% were males. The dogs' age ranged from 6 to 14 years, the average age being 10.68 years. In another retrospective study conducted at the Faculty of Veterinary Medicine Cluj-Napoca (Biriş et al., 2019), hemangiosarcoma was mainly diagnosed in German Shepherds (23%), mixed-breed dogs (22%) and Rottweillers (11%); the average age of the animals was 10.91 years, and in terms of sex distribution, 67% were males and 33% females. Another study, made between 2007 and 2010 (Tăbăran et al., 2010), found that the main cause of neoplastic splenomegaly in dogs was the hemangiosarcoma. It mainly affected the older animals, over 9 years, while the most affected breeds were German Shepherd and Rottweiller; there was no sex predilection. The results of our study are similar to those of the studies mentioned above, the differences between the results regarding gender predilection emphasizing the need for further research on this subjects.

Regarding the spleens in our study, on gross examination, the hemangiosarcoma appeared predominantly as a single nodular mass, of variable size, with the characteristic „bloody nodule" appearance (Figures 1 and 2).

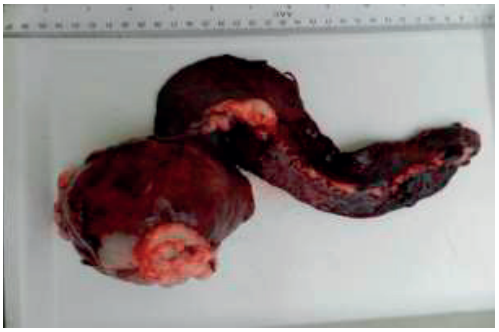

Figure 1. Localized splenomegaly - hemangiosarcoma, German Shepherd male

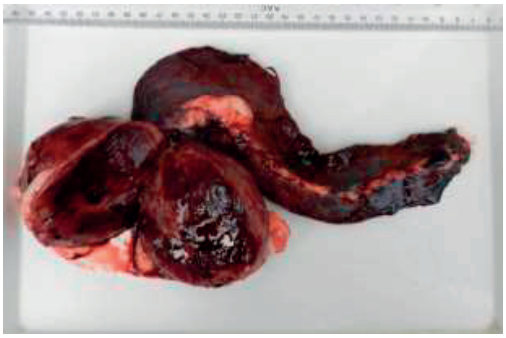

Figure 2. Splenic hemangiosarcoma, German Shepherd male - cut section surface

Cytological examination of splenic hemangiosarcomas (Figures 3 and 4) reveals a cellularity of mesenchymal type, with cell enlargement, pleomorphism and features of malignancy. The nuclei are round or oval, with coarse chromatin, and the nucleoli are proeminent and numerous. The cytoplasm of neoplastic cells is basophilic, occasionally with punctate vacuolation (Christopher, 2003). Siderophages may be present in large numbers, this feature also being observed in the smears examined by us.

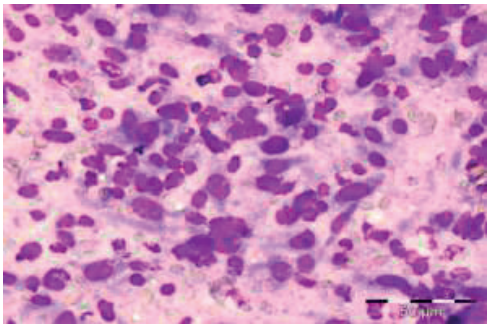

Figure 3. Cytological examination of splenic hemangiosarcoma - spindle cells, with elongated nucleus, basophilic cytoplasm, evident nucleoli, M-G.G., x 400

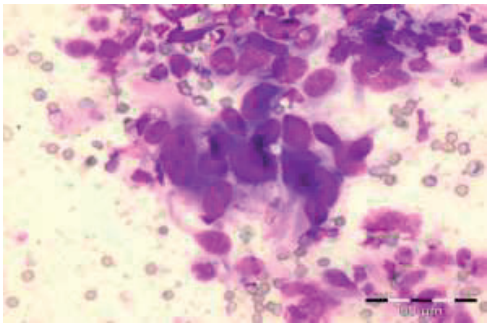

Figure 4. Cytological examination of splenic hemangiosarcoma - mesenchymal spindle-shaped cells with anisocytosis and anisocaryosis, M-G.G., x 400

Histopathological examination revealed the splenic parenchyma replaced by a tumoral proliferation, not encapsulated, represented by tissue of mesenchymal origin, respectively angioblasts, conjunctival stroma and sclerosis.
The vascular spaces are lined by tumoral cells, are anastomosed and filled with numerous erythrocytes.
Tumoral angioblasts are medium sized, polygonal or spindle-shaped, have a moderately abundant basophilic cytoplasm and a round or oval nucleus, centrally positioned.
Cellular atypia is generally moderates and represented by anisocytosis, anisokaryosis, cariomegaly and evident nucleoli (Figures 5 and 6).

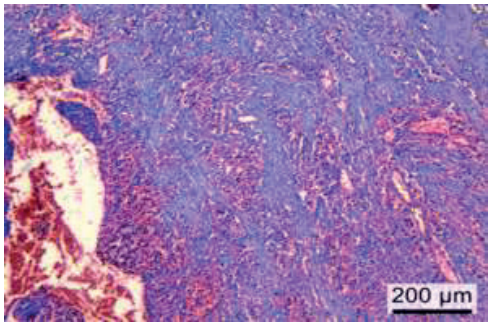

Figure 5. Splenic hemangiosarcoma - Vascular spaces of variable size and shape, anastomosed and filled with numerous erythrocytes, HE, x 100

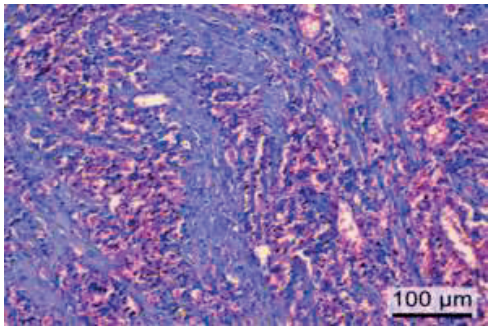

Figure 6. Well-differentiated splenic hemangiosarcoma - Cellular atypia, anisocytosis, anisokaryosis, caryomegaly, evident nucleoli, HE, x 200

Splenic hematoma is the most common non-neoplastic lesion underlying the splenomegaly (n = 43) in our study (Figures 7 and 8). On gross examination, the hematoma appears as a nodular mass, well delimited, of different sizes.

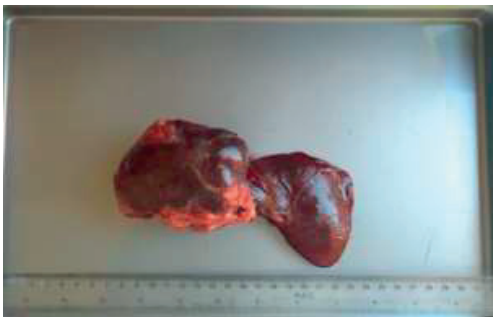

Figure 7. Splenic hematoma, male Jack Russel Terrier- Prominent large nodular mass (10/9 cm), with well-defined borders

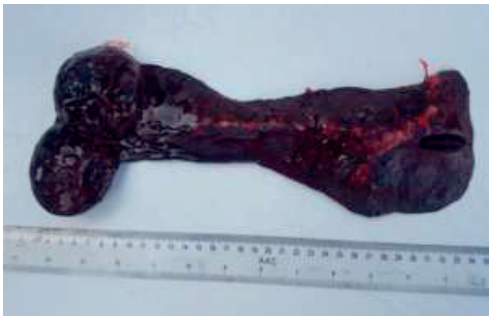

Figure 8. Splenic hematoma, male German Shorthaired Pointer - cut section surface of a compact, homogeneous blackish red mass

Cytologically, the splenic hematoma is characterised by the presence of numerous erythrocytes within the background, most of them being altered and lysed, numerous siderophages and magakaryocytes (Figure 9 and 10). In old hematomas inflammatory cells are present (neutrophils, lymphocytes, plasma cells), as well as fibroblasts who contribute to the formation of the capsule.

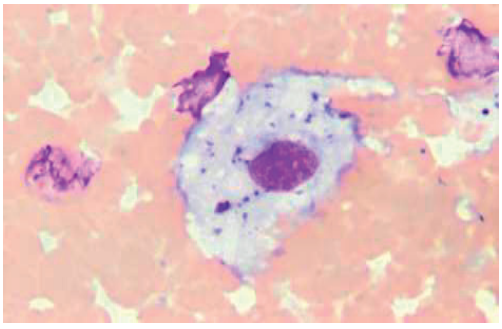

Figure 9. Cytological examination of splenic hematoma - many lysed erythrocytes in the background and a siderophage, M-G.G., x 1000

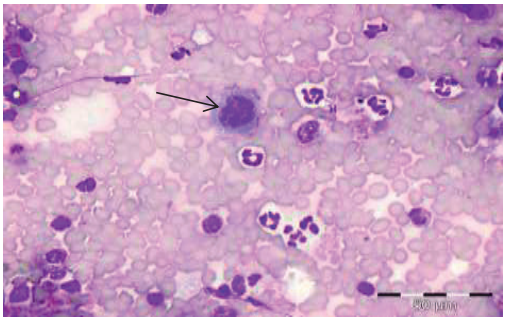

Figure 10. Cytological examination of splenic hematoma - Numerous erythrocytes, lymphocytes, neutrophils; megakaryocyte (arrow), M-G.G., x 400

On histopathological examination, vascular changes are predominant, with the accumulation of excess erythrocytes in the splenic pulp and the presence of many siderophages, the latter being an important indicator of a chronic pathological process, focused on erythrophagocytosis. The hematoma contains multiple fibrin networks. We also observed many megakaryocytes in the histological sections of splenic hematomas. Their presence in large numbers in splenic hematomas is also described in the study of Zamokas et al. (2016).

In order to highlight the siderophages, Perls staining was used, in which the hemosiderin granules from the splenic macrophages acquire a bluish-black color, corresponding to the iron deposits (Figures 11 and 12).

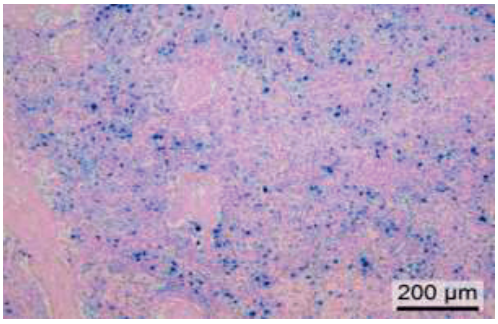

Figure 11. Splenic hematoma - Congestion and hemorrhage, numerous siderophages and fibrin network, PERLS, ob. x100

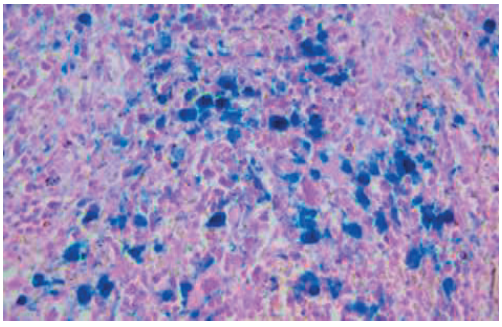

Figure 12. Splenic hematoma - Siderophages - Blackish blue hemosiderin granules, PERLS, ob. x400

Analyzing our datas, we observed that mixed-breed dogs (23.26%), followed by Rottweilers (9.3%), Boxers (6.98%), Cockers (6.98%) and Labrador Retrievers (6.98%) were most commonly diagnosed with splenomegaly due to

hematoma. Females diagnosed with splenic hematoma represented 51.16% of the cases, while males constituted 48.84%. The mean age of dogs with splenic hematoma was 11.27 years, higher than those with hemangiosarcoma (10.68 years). These results do not match to those in another study (Bettini et al., 2001), where the mean age of dogs with splenic hemangiosarcoma (10.3 years) is higher than that of those with splenic hematoma (8.2 years), proving the need for more detailed studies on this subject.

CONCLUSIONS

Splenomegaly in dogs appeared in 78.35% of the 194 cases examined as asymmetric/ localized splenomegaly.

Splenomegaly occurred in 51% of the examined cases in dogs over 10 years of age, older dogs being more predisposed to this condition. Most cases were registered in mixed-breed dogs (22.68%), German Shepherd (11.86%), Rottweiller (10.3%) and Bichon (5.67%), with no sex predilection.

In 55% of cases the splenomegaly had a non-neoplastic substrate and in 45% it was caused by a neoplastic process. Regarding neoplastic processes, 90.8% were malignant and 9.2% were benign. The most common neoplastic diseases were hemangiosarcoma (50.57%), histiocytic sarcoma (13.78%) and splenic lymphoma (9.2%); the most common non-neoplastic diseases were hematoma (40.19%), splenic congestion (26.17%) and reactive hyperplasia (24.3%).

In case of splenomegaly in dogs, cytological examination is useful in differentiating malignant from benign tumors, with confirmation of diagnosis by histopathological examination.

Hemangiosarcoma is the most common splenic tumor and it affects mixed-breed dogs (27.27%), German Shepherds (20.45%) and Bichons (9.09%); the average age of the subjects is 10.68 years.

Hematoma is the most common non-neoplastic splenic lesion. It is frequently found in mixed-breed dogs (23.26%), Rottweilers (9.3%) and Boxers (6.98%). The average age of the dogs with splenic hematoma is 11.27 years.

ACKNOWLEDGEMENTS

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STUDIES ON THE DIAGNOSIS OF BEE ASCOSPHEROSIS ON LIVE BEES SAMPLES AND BROOD COMB THROUGH MORPHO-CLINICAL TESTING AND LABORATORY EXAMINATION

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Abstract

The purpose of this work is to monitor bee health through the morpho-clinical testing and laboratory examination of live bee samples and brood comb for the prophylaxis and control of bee ascospherosis. We investigated in the active year 2020 a number of 68 samples (34 samples of live bees and 34 samples of brood combs). Samples were collected in the beginning of the inactive season and examined morpho-clinically and in the laboratory. The laboratory method employed was in accordance with OIE regulations (2008) and adapted to an original methodology in the Pathology Laboratory of ICDA. Laboratory test results emphasized the presence of Ascosphera apis fructification bodies and hyphae in 20 live bee intestines (58.82%) the remaining 14 samples being negative (41.18%). The morpho-clinical testing of the 34 brood comb samples proved the existence of chalk brood in 10 samples (29.41%), the respective samples being correlated to the existence of Ascosphaera in the live bee intestines sample, and the remaining 24 samples were negative (70.59%).

Key words: *bees, chalkbrood, Ascosphera apis, fructification bodies.*

INTRODUCTION

Ascospherosis is the most important mycotic disease that affects the health of bees in apiaries (*Apis mellifera carpathica*) throughout the year, it evolves in both beekeeping seasons (active and inactive), it weakens the bee colony and is critical in the development of other contagious diseases. (Savu & Sapcaliu, 2013; Ansari et al., 2016). Ascospherosis affects the larvae of the *Apis* melliferous bee (Liang et al., 2000), as well as the larvae of solitary wild bees, like *Megachile* (Castagnino et al., 2020). In *Apis mellifera*, the disease is caused by the *Ascosphera apis* fungus, in the *Fungi* genus, the *Ascomycota* family, the *Eurotiomycetes* class, *Ascospherales* order; being characterized by dead larvae coverage, as a mantle, in the fungal mycelium, and by dehydration and dead larvae transformation into white rugous mummies (Gilliam et al., 1978), looking like chalk-brood or black in color (Savu & Sapcaliu, 2013; Asiminei et al., 2016). There are also significant losses as result of diminished number of bees as well as decreased bee colony productivity, and lower honey yield by 5-37% (Ansari et al., 2016). The damages caused by mycoses in bee colonies and in the hive economy, respectively, (Aronstein et al., 2010), are the more important as they evolve together with other parasitoses (varroasis, nosemosis) and major bacterial infections (American loca, European loca) (Ansari et al., 2016). There is data that proves a rise in incidence in the recent years. In Europe, in 1913, Maassen described the ascospherosis for the first time, and in the second half of the 20th century it was diagnosed in Germany (Aronstein et al., 2010), Russia and Great Britain. By 1977, ascospherosis was recognized as the most serious infectious disease in bees in Norway (Heath, 1985). In 1957, ascospherosis started evolving beyond Europe, being identified in New Zeeland (Reid M., 1988),

Central America, Japan (Yoshiyama et al., 2011), North America and Canada (Aronstein & Murray, 2010), Australia (Sheba et al., 2020) and China (Zhi et al., 2018).

MATERIALS AND METHODS

Bee colonies' health monitoring through morpho-clinical and laboratory tests on samples of bees (Milea, 2017; 2019) and brood comb was performed with purposes of prophylaxis and control of ascospherosis in bees (Radoi, 2018).

In 2020, investigations were carried out on 68 bee colonies in 4 apiaries (17 bee colonies per each apiary), through morpho-clinical examinations, a number of 68 samples being collected at the beginning of the inactive season for laboratory tests, consisting in 34 live bee samples and 34 brood comb samples (Table 1).

Table 1. Number of samples collected per bee colony

Examined apiary	Number of bee colonies (experimental lot)	Samples of collected live bees	Samples of collected brood comb
Apiary 1	17	8	8
Apiary 2	17	8	8
Apiary 3	17	9	9
Apiary 4	17	9	9
Total	68	34	34

The morpho-clinical examination of the collected samples was followed by the laboratory test which was performed through an original method in the Pathology Laboratory of ICDA Bucharest, adapted for intestine samples collected from live bees according to OIE regulations (OIE, 2018) to identify bee diseases (Jensen et. al., 2013). Some descriptions of the ascosphaera and observations on the ascospores were also made by use of a NIKON ELIPSE E_{400} microscope and a morphometrics software.

RESULTS AND DISCUSSIONS

As result of the morpho-clinical examination and laboratory tests in the 4 apiaries, samples from 30 bee colonies were found positive for *Ascosphera apis*, out of which 20 samples of live bees (58.82%) and 10 samples of brood comb (29.41%), the remaining samples being negative (Table 2, Figure 1).

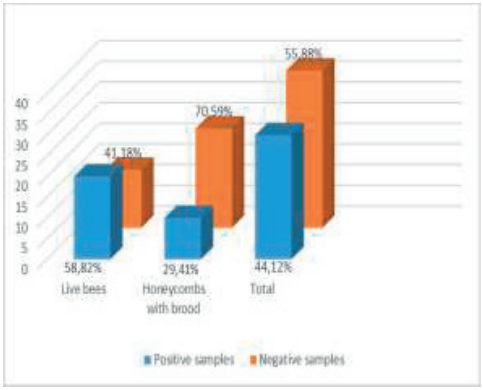

Figure 1. Proportion of positive and negative samples for ascospherosis in bee colonies examined in the 2020 season

Table 2. Samples diagnosed positive and negative for *Ascosphera apis* in live bees and brood comb

Bee colonies examined	Live bees	Brood combs	Total samples
Positive	20 (58.82%)	10 (29.41%)	30 (44.12%)
Negative	14 (41.18%)	24 (70.59%)	38 (55.88%)
Total	34	34	68

As noticed in Table 2 and Figure 1, the morpho-clinical examinations of hive brood samples highlighted the existence of chalk-brood in 10 samples (29.41%). Interestingly, some brood larvae that seemed unaffected presented initially only small white spots under the dermis and later (3-5 days) these larvae turned into chalk-brood (Figures 2, 3). This sign (small white spots under dermis in bee brood larvae), noticed while examining the larvae in the brood comb samples, may constitute a presumptive diagnosis of chalk-brood.

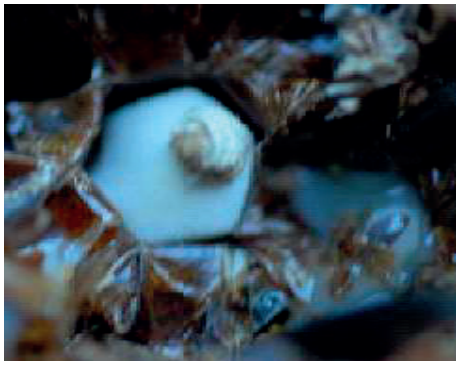

Figure 2. Comb with larvae affected by chalk-brood

Figure 3. Larva affected by chalk-brood (left) and larva with white spots susceptible of developing chalk-brood

The laboratory examination of intestine samples from live bees showed the existence of fructification bodies and hyphae of *Ascosphera apis* in 20 samples (58.82%), the remaining samples being negative (41.18%). The 20 samples of intestines from live bees that were found positive through microscopic examination can be deemed suspicion of ascospherosis in the examined bee colony (Figures 4, 5, 6, 7). To confirm this funding, all intestine samples from the live bees found positive for fructification bodies and hyphae of *Ascosphera apis* (Chorbinski et al., 2003) were correlated with samples of positive brood combs for ascospherosis that had been collected from the same bee colony.

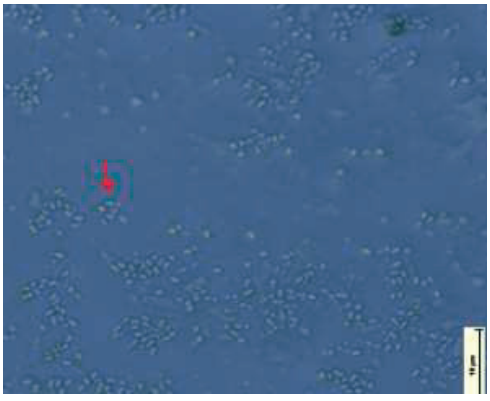

Figure 4. Spores of *Ascosphera apis* during examination of intestine in live bees. Prepared directly x 400

Figure 5. Hyphae and fructification bodies (ascospheres) of *A. apis*. Prepared directly from live bee intestine x 400

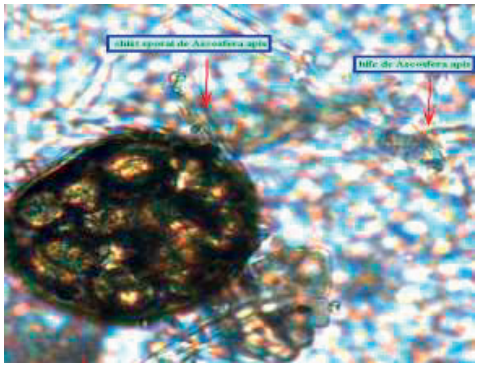

Figure 6 Ascosphaera with ascospores and hyphae of *A. apis* from live bees. Prepared directly, x 400

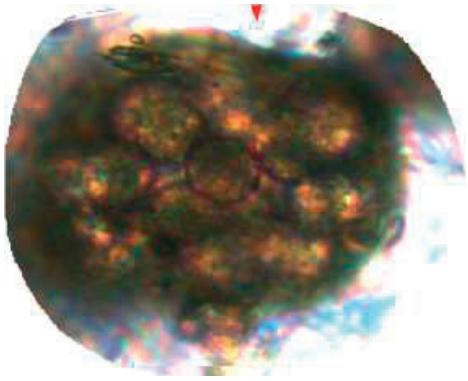

Figure 7. Ascosphaera with ascospores of *A. apis* from live bee intestine. Prepared directly, x 400

This demonstrates that the examination of live bee intestines may be introduced as an simple laboratory examination for the suspicion of ascospherosis in the bee colony, without samples of brood comb. Testing bees before the inactive season for *A. apis* in samples of intestines, correlated with the morpho-clinical examination of combs, represents an important

prophylactic method to diagnose chalkbrood in bees (Savu, 2017; Sapcaliu, 2017; Radoi, 2018; 2019).

The laboratory examination of live bee intestines also included measuring dimensions of ascosphaera, puffballs and ascospores with the morphometric microscope. The results we have obtained show a dimension of the ascosphaera' diameters of 110-190 µm (average 155 µm), the dimension of puffballs being 16-41 µm (average 32 µm), and the dimension of ascospores (length/width) was 2.7-4/1.3-1.9 µm (Figure 8). Our results concur with the results obtained by Wynns et al., 2013; Aronstein et al., 2009; Anderson et al., 1998.

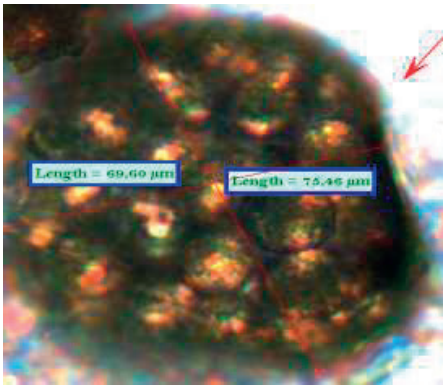

Figure 8. Morphometry of ascosphaera and puffballs in the intestine of live bees

Correlating the results of laboratory tests on live bee intestines with the morpho-clinical examination of larvae in the brood combs (Heath, 1982a), even in the visible absence of chalk-brood larvae, we may suspicion an ascospherosis diagnosis. Thus, the examination of live bee intestine, when we notice the existence of ascospheres, puffballs and ascospores, may become an instrument for early diagnosis of ascospherosis in bee colonies.

CONCLUSIONS

The morpho-clinical and the laboratory examinations of live bee intestines in 2020 on 68 bee colonies revealed ascopherosis in 30 bee colonies (40.12%).

Of the 30 bee colonies diagnosed with ascopherosis, in 20 samples the ascopherosis was suspicioned in the microscopic examination of live bee intestines (58.82%), while in 10 samples the ascopherosis was shown by morpho-clinical examination of brood combs (29.41%).

All live bee intestine samples found positive for the existence of fructification bodies and hyphae of *Ascosphera apis* were correlated with samples of brood combs found positive for ascospherosis collected from the same bee colonies.

The morphometrics of ascosphaera, puffballs and ascospores showed dimensions of 110-190 µm, 16-41 µm and 2.7-4/1.3-1.9 µm, respectively, for ascospores, values that concur with studies by other authors.

Testing bees before the inactive season for *A. apis* on intestine samples correlated with the morpho-clinical examination of combs represents an important prophylactic method to diagnose chalk-brood in bees.

The examination of live bee intestines, where we notice the existence of ascosphaera, puffballs and ascospores, may suspicion early diagnosis of ascospherosis in bee colonies.

Compliance with ethical standards. The research does not involve human and/or animal experimentation.

Conflict of interest. The authors declare that they have no conflict of interest. We mention that the research conducted has no connection with the activity of official territorial or central laboratories nominated for the monitoring and control of bee diseases.

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A RAPID ANTIGEN TEST SCREENING FOR *Giardia duodenalis* INFECTION IN DOGS AND CATS WITH DIGESTIVE DISORDERS

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Abstract

Giardia duodenalis, a flagellate protozoan with potential zoonotic risk, is one of the frequent causes of diarrhea in animals and humans. The aim of the present study was to investigate the prevalence of G. duodenalis infections and associated risk factors in carnivores. For this, a total of 107 client-owned animals living in Bucharest area (Southeast Romania), including 89 dogs and 18 cats with a history of digestive disorders, were included in the study. Animals were of different breeds and different ages; dogs aged between 2 months and 13 years (average 3.08 years; standard deviation - SD = 3.06) whereas cats aged between 5 months and 16 years (average 3.12 years; SD = 3.07). Fresh fecal samples were collected and tested for the presence of coproantigen (Ag) of G. duodenalis using a commercially available rapid immuno-chromatographic test. Additionally, a subset of 55 fecal samples (44 from dogs and 11 from cats) were subjected for a copro-parasitological examination for detection of Giardia cysts and other parasitic elements (protozoan oocysts, helminth eggs), using zinc sulphate flotation method. Overall, 21.4% and 5.6% of dogs and cats, respectively, were positive for G. duodenalis copro-Ag. Furthermore, 31.8% of the 44 copro-parasitologically tested dogs were positive for parasitic infections, of which 20.5% (9/44) were positive for G. duodenalis cysts, as single 11.4% (5/44) or mixed 9.1% (4/44) infections with other intestinal parasites, such as Isospora spp., Toxocara canis, Ancylostoma caninum and Trichuris vulpis. All 11 cats tested negative by coproscopy. A good correlation between the Ag rapid test and microscopic identification of cysts for Giardia infection was registered. These findings confirm G. duodenalis and other intestinal parasites as causative agents of enteric disorders in client-owned dogs and cats and emphasize on potential zoonotic risks.

Key words: Giardia duodenalis, copro-antigen, cysts, digestive disorders, carnivores.

INTRODUCTION

Giardia duodenalis is a protozoan parasite that represents one of the common causative agents of digestive symptoms (such as diarrhea) in animals and humans (Feng and Xiao, 2011). Its zoonotic potential is widely known in relation to companion animals, as pets live in the proximity of the owner (Thompson, 2004). *Giardia* infections in dogs, especially in puppies, are clinically important due to their high prevalence, serious symptoms in some occasions, and its potential zoonotic risk (Liu et al., 2014).

In cats, however, *Giardia* infections can occur in healthy specimens, as well as some with acute or chronic small bowel diarrhea, with or without weight loss (Scorza and Lappin, 2004). Symptomatic giardiosis is frequently addressed to decrease symptom length, eliminate complications and reduce parasite spread to other hosts (Dixon, 2020).

The management of giardiosis implies an accurate diagnosis, as in the case of other parasites. Laboratory tests focus on detection of microscopic cysts in stool samples, as well on immunological-based testing and molecular methods. In all diagnostic procedures, different sensitivities and specifics may be encountered. This condition depends on the testing methodology, competences and performances of the assays used (Hooshyar et al., 2019).

The aim of the present study is to investigate the prevalence of *G. duodenalis* infections and associated risk factors in carnivores with digestive disorders.

MATERIALS AND METHODS

Between August 2014 and October 2018, a number of 107 client-owned animals, 89 dogs and 18 cats, which exhibited digestive symptoms, were included in the study. The dogs and the cats were of different or mixed

breeds, with ages ranging from 2 months to 13 years, and from 5 months to 15 years, respectively, all living within the metropolitan area of Bucharest, Romania. Animals were grouped in three different age categories, as presented in Table 1.

Table 1. Animals (dogs and cats) included in the study, categorized by age and gender

The animal species	Number of animals included in the study							
	Animal age and gender categories							
	≤ 1 year		1 - ≤ 8 years		> 8 years		Animal's average age (years) [standard deviation:SD]	Total number of animals
	Male	Female	Male	Female	Male	Female		
Dogs	19	7	35	23	4	1	3.08 [3.06]	89
Total	26		58		5			
Cats	2	4	6	3	2	1	3.12 [3.07]	18
Total	6		9		3			

Fresh fecal samples collected from the animals (89 dogs and 18 cats) were examined within 24-48 hours after sampling, using a rapid immunochromatographic *Giardia* copro-antigen test (Bionote Anigen Rapid Giardia Ag Test Kit.), in accordance with the manufacturer' recommendations.

Additionally, a subset of 55 fecal samples (44 from dogs and 11 from cats) were subjected for copro-parasitological examination for detection of *Giardia* cysts and other parasitic elements (protozoan oocysts, helminth eggs), using a flotation method (33% zinc sulphate solution, and Lügol staining) (Ioniță and Mitrea, 2013).

Moreover, for 7 samples (4 of dogs and 3 cats), a bacteriological exam was also performed.

RESULTS

In order to assess the prevalence of *Giardia* infection among owned dogs and cats showing digestive disorders, we conducted an investigation using rapid immunochromatographic test and copro-parasitological exams. In some cases, a bacteriological investigation was also carried out.

Animals were of different breeds and different ages: dogs' age varied between 2 months and 13 years (average 3.08 years; standard deviation - SD = 3.06 years), while for cats, the age varied between 5 months and 16 years (average 3.12 years; SD = 3.07 years).

Results of the Giardia - copro Antigen test

Out of the 107 animals tested, 20 were positive for the *Giardia* copro-Ag test, 19 dogs (19/89; 21.4%) and 1 cat (1/18; 5.6%). By age groups, the test was positive for 30.8% of the dogs up to 1-year-old; 17.2% of adult dogs and 20% dog over 8 years old. The positive cat was 10 year-old, belonging to the age group of over 8 years (Table 2).

Table 2. Data on positive animals (dogs and cats) for the *Giardia* - coproantigen test (stratification by age and gender)

Animal species		Positive animals/total number examined animals [number and percentage]						Total positive animals
		≤ 1 year		<1 - ≤ 8 years		>8 years		
		Male	Female	Male	Female	Male	Female	
Dogs		5/19 (26.3%)	3/7 (42.9%)	5/35 (14.3%)	5/23 (21.7%)	1/4 (25%)	0/1 (0%)	19/89; (21.4%)
	total	8/26 (30.8%)		10/58 (17.2%)		1/5 (20%)		*p = 0.331
Cats		0/2	0/4	0/6	0/3	1/2	0/1	1/18 (5.6%)
	total	0/6 (0%)		0/9 (0%)		1/3 (33%)		*p = 0.167

*p>0.05 with no statistic significant regarding positive *Giardia* Ag tests among age groups.

Results of the flotation test

The coproparasitological examination of the 44 dog samples revealed that 31.8% (14/44) were infested with at least one parasitic species as follows: *Giardia* sp. (20.45%; 9/44), *Isospora* spp. (6.8%; 3/44), *Toxocara canis* (11.4%; 5/44), *Ancylostoma caninum* (4.5%; 2/44) and *Trichuris vulpis* (2.3%; 1/44) (n = 44) (Table 3).

Additionally, mixed infections were identified in the case of the 9 dogs positive by flotation for *Giardia*: *Isospora* spp. (n = 3), *T. canis* (n = 1), *A. caninum* (n = 1) and *T. vulpis* (n = 1). It is worth noting the case of a dog identified with 4 parasitic species: *T. canis, A. caninum, Isospora* spp., *G.duodenalis*, but also the case of a positive antigen result and microscopic examination illustrating just the presence of *T. canis* (Table 3).

None of the cats (11) which were the subject of the coproparasitological examination was positive (the cat that was positive for the *Giardia* Ag test was not tested by flotation).

Table 3. Data on positive dogs for the flotation test, *Giardia* infection prevalence and other intestinal parasites, including co-infections (stratified by age category)

Parasite species and/or associations	Dog age category (number and percentage)						Total	
	Puppies (≤1 year)		Adult (>1-≤8 yrs.)		Old (>8 years)			
	No.	%	No.	%	No.	%	No.	%
Giardia duodenalis total	6	37.5(6/16)	2	8.3(2/24)	1	25.0(1/4)	9	20.5 (9/44)
G. duodenalis as single parasite	4	25.0(4/16)	1	4.2(1/24)	0	0 (0/4)	5	11.4 (5/44)
G. duodenalis and *Isospora* spp.	1	6.3(1/16)	1	4.2 (1/24)	0	0/4	2	4.5 (2/44)
G. duodenalis and *T. vulpis*	0	0/16	0	0/24	1	25.0(1/4)	1	2.3 (1/44)
G. duodenalis, Isospora spp., *A. caninum* and *T. canis*	1	6.3(1/16)	0	0/24	0	0/4	1	2.3 (1/44)
Ancylostoma caninum	0	0/16	1	4.2(1/24)	0	0/4	1	2.3 (1/44)
Toxocara canis (one sample was positive for *Giardia* Ag)	3	18.8(3/16)	1	4.2(1/24)	0	0/4	4	9.1 (4/44)

Results of the bacteriological test

Some of the bacteriological tested dogs that tested negative for both *Giardia* Ag test and coproscopical exam, were diagnosed with bacterial infections that consisted of: *Escherichia coli* (4/4), *Enterococcus* spp. serological group D (4/4), *Proteus mirabilis* (1/4), *Citrobacter youngae* (1/4), *Pseudomonas aeruginosa* (1/4), *Streptococcus canis* serologic group G (1/4). Bacterial infections were mixed, as 4 to 5 bacterial species were found in all of the dogs' sample: 1 with *Citrobacter youngae*, *Escherichia coli*, *Enterococcus* spp. serologic group D, *Pseudomonas aeruginosa*; another 1 with *Escherichia coli*, *Enterococcus* spp. serologic group D; an additional 1 with *Escherichia coli*, *Enterococcus* spp. serologic group D, *Streptococcus* serologic group G; 1, with *Escherichia coli*, *Proteus mirabilis*, *Enterococcus* spp. serologic group D (n = 4).

The cat which tested positive for *Giardia*-Ag was also positive at the bacterial exam, showing positive results for the following species: *Escherichia coli; Enterococcus* spp. *Serological group D; Proteus mirabilis.*

In the two case of cats that tested negative for both *Giardia* Ag test and coproscopical exam, they were positive for bacterial infections as following: *Escherichia coli* (2/2), *Enterococcus* spp. serological group D (1/2), *Enterobacter cloacae* (1/2). Similarly, bacterial infections were mixed in some cases, either with *Escherichia coli*, *Enterococcus* spp. serologic group D, *Proteus mirabilis* (1/3); or with *Escherichia coli*, *Enterobacter cloacae*, *Enterococcus* spp. serologic group D (1/3).

Thus, the clinical signs of the examined dogs (*Giardia* Ag test and coproscopical exam) were associated with the following parasite infections: *G. duodenalis,* single infection (22.7%; 10/44), co-infestations of *G. duodenalis* and other species, 11.4% (5/44), infestations with other parasitic species (other than *Giardia*) (9.1%; 4/44).

Bacterial infections represented a factor of symptomatology for 4 dogs and 2 cats (having negative results for the rapid test and coproparasitological examination). For cats it was found one co-infection of *G. duodenalis* and bacteria.

DISCUSSIONS

The aim of the present study was to investigate the prevalence of *G. duodenalis* infections and associated risk factors in carnivores, owned dogs and cats with digestive disorders. Our study has showed that the ascertained *Giardia* prevalence varies according to the animal species included in the study and the method used. The prevalence of *G. duodenalis* was higher in the case of dogs (21.4%) than cats (5.6%), even if the cats investigated were in lower number.

In Romania, screening studies were carried out, most of them in dogs, by means of coproscopic methods. *Giardia* prevalence in these studies ranged from 8.5% (Mircean et al., 2012), 21.3% (Soran et al., 2017) to 42.62% (Sorescu et al., 2014). Our study has shown a *Giardia* prevalence of 20.5% by coproparasitological exam.

Higher percentages were obtained by using ELISA tests: 34.6% (Mircean et al., 2012), 36.1% (29.5% in client-owned dogs) (Sommer et al., 2015), 42.1% (Sorescu et al., 2014) and 51.1% (Jarca et al., 2008). This can be explained by the better sensitivity of the tests, although in our study a good correlation was found between the microscopy and the tests. Furthermore, similar percentages (42.6%, 42.1%) have been observed in dogs from Timis county (Sorescu et al., 2014).

In cats, the prevalence observed (5.56%) is lower than reported in previous studies, of 27.9% (51/183), increasing up to 32% (16/50) in symptomatic cats (Mircean et al, 2011). By using flotation and Lügol staining, a 22.8% (18/79) prevalence was reported in owned cats in the urban area of Bucharest (Soran et al., 2017).

In comparison with similar studies in Europe, *Giardia* prevalence in dogs obtained by using microscopy ranged from 7.8% (Italy; Scaramozzino et al. 2018) to 25% (France; Osman et al, 2015). In other studies, by using rapid tests, a prevalence of 11.4% in dogs and 6.8% in cats were registered, all animals without owner, from foster homes (Germany; Becker et al., 2012), while other studies showed a prevalence of 18.6% in dogs and 12.6% in cats, from privately-owned pets (Germany; Barutzki and Schaper, 2011).

Using similar study designs, animals with digestive disorders and rapid copro-Ag testing, the reported prevalence was of 16% for dogs (Italy; Symeonidou et al., 2020) and 15.3% for cats (France; Epe et al., 2010). The prevalence of *Giardia* infection in dogs in our study (21.4%) is closer to the one from Italy, with cats having a smaller percentage (5.6%).

It is clear that the observed prevalences differ greatly depending on the diagnostic method used. In our study, the results of the fecal Ag rapid test and microscopic identification of cysts for detecting *Giardia* infections were consistent, confirming the copro-Ag test as a useful diagnostic tool for *Giardia* infections. Similarly, Giardia cysts were demonstrated in the majority of the ELISA-positive samples in the IFA, also for samples from Romania (82.4 %) (Sommer et al., 2015).

Recently, some research (Symeonidou et al., 2020) has shown that fecal examination with the Speed™ *Giardia* test was more sensitive than the parasitological method and results differed consequently.

Similarly, a recent study on comparing diagnostic tests for *G. duodenalis* in dogs showed that the copro-Ag-test had the highest specificity, followed by coproscopy (centrifugation sedimentation flotation microscopy), while qPCR showed the highest sensitivity. Therefore, depending on the purposes, qPCR is recognized as valuable screening tool, due of its high sensitivity, whereas for studies in which high specificity is required microscopy-based methods or Ag-test are recommended (Uiterwijk, 2018).

However, fecal flotation has the ability to detect also mixed infections with other parasites, as reported also for other parasite infections (Mitrea et al., 2013).

Thus, a combination of both immunoassay and microscopic techniques would provide more sensitive approach for detection of *Giardia* infection and other internal parasites (Saleh et al., 2019). Therefore, in the case of negative results but with reasonable suspicions, complementary tests should be carried out in order to have a certain diagnosis, as a base for an appropriate therapy and management protocol to be applied.

To be noted, 34 animals from our study (25 dogs and 9 cats) that showed digestive

symptoms had negative *Giardia* Ag tests and coproparasitological examinations. In these cases, a bacteriological examination would have been recommended, however this is largely a matter of availability.

Various studies have shown that the prevalence of *G. duodenalis* could be underestimated (Adell-Aledón et al., 2018; Epe et al., 2010), therefore additional studies are always necessary to shed more light into the real depth of such parasitoses.

CONCLUSIONS

These findings of the present study confirm *G. duodenalis* and other intestinal parasites as causative agents of enteric disorders in client-owned dogs and cats and emphasize on potential zoonotic risks.

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VESTIBULAR SYNDROME IN DOGS AND CATS - CLINICAL APPROACH TO DIAGNOSIS AND A RETROSPECTIVE CASE SERIES REPORT

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Abstract

The vestibular system is a sensory system comprised of several structures and tracts that is responsible for providing the brain information about spatial orientation and balance. Clinical signs like head tilt, nystagmus, positional strabismus or leaning are cardinal symptoms of a vestibular syndrome in small animal neurology. To establish an appropriate therapeutic plan and to provide owners accurate information regarding the prognosis, a differential diagnosis between a central or a peripheral vestibular disease is essential. In 2019, more than 310 cases of dogs and cats with neurological symptomatology were diagnosed in the Clinic of the Faculty of Veterinary Medicine in Bucharest, in agreement with the protocol already implemented in our practice. Full history, physical examination followed by a complete neurological examination were mandatory steps required to localise the lesion within the four main regions of the brain: forebrain, brain stem, cerebellum or vestibular apparatus. Among the total number of cases, approximately 15% showed clinical signs consistent with a vestibular syndrome. This article aims to present the predisposition factors, clinical features and neurological findings of the 32 dogs and 16 cats diagnosed with vestibular pathology.

Key words*: central vestibular syndrome, peripheral vestibular syndrome, head tilt, nystagmus, neurology.*

INTRODUCTION

In human medicine, vestibular syndrome has been studied for more than a century, attracting the interest of several researchers. In 1914, Barany Robert won the Nobel Prize in Medicine and Physiology for his study of vestibular mechanisms. He managed to differentiate the central vestibular syndrome from peripheral vestibular syndrome and he also studied the interdependence between vertigo and nystagmus in affected patients. (Lopez & Blanke, 2014).

The vestibular syndrome is quite common in dogs and cats and it represents a real challenge for the veterinarian in terms of diagnosis and treatment (Fluehmann et al., 2006; Rădulescu et al., 2020).

Neurological examination is essential for differentiation between central and peripheral vestibular syndrome and requires knowledge of the anatomy and physiology of the central nervous system. Clinical signs like imbalance and postural disturbance along with vestibular ataxia may be a result of dysfunction of both peripheral and central components of the vestibular apparatus, as shown in Table 1. (Rossmeisl, 2010).

The peripheral vestibular disease does not affect the strength or general proprioception. Spontaneous or positional horizontal or rotatory nystagmus can occur, and the fast phase will be away from the side of the lesion. Peripheral vestibular lesions can also affect the facial nerve and postganglionic sympathetic innervation to the head (Horner syndrome). Vestibular signs associated with a depressed level of consciousness, spastic hemiparesis, cranial nerve V-XII deficits, or general proprioceptive deficits on the same side as the vestibular deficits should be considered to indicate a central vestibular disorder (de Lahunta & Glass, 2010; Rossmeisl, 2010).

For a correct diagnosis and an accurate prognosis, the interaction between doctor, patient and owner is particularly important in terms of evolution and prognosis of the disease (Lorenz et al., 2011).

In current veterinary practice in our country, the number of patients with vestibular disorders represents a significant percentage of the total number of animals that are presenting for

consultation with neurological deficits. According to a study conducted in 2018 in the Clinic of the Faculty of Veterinary Medicine in Bucharest, out of 209 cases, 22 cases showed a lesion localised in the vestibular apparatus (13%), a higher number compared to the number of cases with cerebellar disease (n = 8), or brainstem lesions (n = 3) (Turbatu et al., 2019).

Table 1. Differentiating clinical features of the peripheral and central vestibular disease (Rossmeisl, 2010)

Clinical sign	Peripheral vestibular lesion	Central vestibular lesion
Head tilt	Towards lesion	To either side
Pathologic nystagmus	Direction unaltered by head position Horizontal or rotatory Fast phase away from the lesion	Direction may change with head position Horizontal, rotatory or vertical
Postural reactions	Normal	Deficits ipsilateral to the lesion
Conscious proprioception	Normal	Deficits ipsilateral to the lesion
Cranial nerve deficits	±ipsilateral cranial nerve (CN) VII	± CNN V-XII ipsilateral to the lesion
Horner syndrome	±postganglionic	± preganglionic (rare)
Consciousness	Normal Disoriented if acute	Normal to comatose

Given the increased frequency of vestibular syndrome in veterinary practice, the current study presents a detailed analysis of the cases diagnosed with the vestibular syndrome (both central and peripheral) during 2019, emphasising important information such as incidence, risk factors, clinical signs or neurological deficits of these patients.

MATERIALS AND METHODS

The analysis of cases (dogs and cats) diagnosed with vestibular syndrome during 2019 implied a mandatory follow-up of the consultation protocol already implemented in the Internal Medicine Department of the Faculty of Veterinary Medicine in Bucharest. Consequently, before establishing an etiological diagnosis, a series of preliminary stages were followed for each patient. Elements like signalment, anamnesis, complete physical and neurological examination, localisation of the lesion within the central nervous system, a list of differential diagnosis and recommendations of paraclinical investigations were part of the consultation sheet of each case. The study was conducted during the year 2019 (1/01-31/12/2019) and the collected data from the Consultation Register have been statistically analysed in order to emphasize the most important factors that were corelated with the vestibular syndrome for the 48 animals (dogs and cats) discussed in this article.
Case inclusion criteria required a final diagnosis of vestibular syndrome. The entire database was manually revised to extract relevant information regarding symptomatology, clinical and neurological examination findings of each case.

RESULTS AND DISCUSSIONS

During the year 2019, more than 2200 small animals were examined in the Clinic of the Faculty of Veterinary Medicine in Bucharest, at Internal Medicine Department. Of the total number of cases, 310 domestic carnivores showed neurological signs specific to one region of the brain - forebrain, brain stem, cerebellum or vestibular apparatus - 14.09 % from the total number of cases.
In total, 32 dogs and 16 cats (15.48% out of the 310 neurological patients) were presented for further investigation due to signs compatible with a vestibular syndrome, during the investigated period.
For a proper analysis of the results, every stage of the diagnosis protocol will be outlined, starting with signalment, who included elements like species, breed, age and gender of the patient (as it is shown in Table 2).
Regarding the species, an important difference between dogs and cats was observed. The number of canine patients with vestibular lesions was almost double compared to the number of feline patients (66.66% dogs and 33.33% cats). Previous studies have also stated that vestibular syndrome is less reported in feline pathology than in canine pathology (Grapes et al., 2020).

Table 2. The distribution of cases by species, breed, age and gender

CRITERIA	NUMBER OF ANIMALS		
SPECIES	Canine 32		Feline 16
DOG BREEDS	Purebred dogs 23		Crossbred 9
AGE	Under 1 year 8	2 to 7 years 12	8 to 18 years 28
GENDER	Male 23		Female 25

The number of affected purebred dogs (n = 23) was higher than the number of crossbred dogs (n = 9). The breeds included in purebred dogs were Beagle (n = 5), Bichon (n = 5), French Bulldog (n = 4), English Bulldog (n = 4), Yorkshire Terrier (n = 2), German Shephard (n = 1), Mops (n = 1), Labrador (n = 1). According to these results, brachycephalic-type breeds are more likely to develop vestibular syndrome compared to crossbreed dogs (39% of the total number of purebred dogs were brachycephalic). Other studies that were following breed predisposition have also shown that the incidence of vestibular syndrome in bulldogs is higher than in other dog breeds, due to the predisposition of this breed in developing brain disorders (Hayes et al., 2010; Mayousse et al., 2017).

According to the age, older patients were more likely to develop a vestibular syndrome compared to juvenile patients (58.33% of patients were within the limit of 8 to 18-year-olds), as shown in Figure 1.

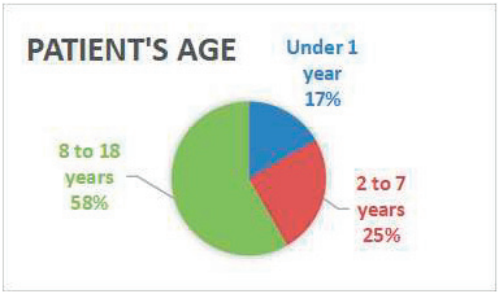

Figure 1. Distribution of patients according to their age

Regarding gender, the number of cases was almost equal - 52% of the investigated carnivores were females and 48% were males.

Another important stage of the diagnosis protocol was anamnesis. Every owner was kindly asked to fill out a template. Important details were obtained, using a series of essential questions concerning:

Patient's signalment - age, bred, sex and hormonal status of the animal;

The reason for the visit: What is the reason for the visit? When did the problem start? (peracute, acute and chronic); The duration and/or frequency of the current problem? Are there any factors that could have triggered the problem? How did the problem evolve?

The owner's perspective: What did the owner notice? What does the owner think is the problem? What worries him the worst and why?

The patient's behavioural history: Did the patient had pre-existing behavioural problems?

The patient's medical and surgical history: Has the patient been diagnosed with one or more medical conditions? If so, what are these conditions, how they were diagnosed and what treatment did he follow? Has he ever been anaesthetized? Has he undergone any surgery? Did he experience side effects from medications, including anaesthetics?

The patient's environment: Does the animal live alone inside or outside? Does he live in the house and outside? What other animals live in the same house?

Physical activity of the patient;

Travel history;

Diet: How often is the animal fed? What does it eat? What rewards does he receive? Does it have the appropriate weight for its size and breed?

Vaccine history: Is he vaccinated? Is the vaccination schedule complete and correct? Did he experience side effects from vaccines?

Pharmacological history – Is he currently receiving any treatment? Is he currently taking vitamins or supplements? Is it internally dewormed? Is it externally dewormed?

Anamnesis revealed a series of common points for the patients with vestibular lesions, like a sudden onset of disease, progressive clinical signs (from days to weeks), including head tilt, circling, nystagmus, asymmetrical ataxia and multiple episodes of vomiting. Also, for 10 cases a history of recurrent otitis was registered. Previous studies have shown that in

cats, a history of otitis externa was significantly associated with otitis media/interna and a diagnosis of a peripheral vestibular syndrome (Grapes et al., 2020).
After recording the data from the anamnesis, a complete physical assessment using inspection, palpation, percussion, and auscultation was performed for each case.
In order to rule out a systemic or a metabolic disorder, physical examination was always used before neurological examination.
Stages of the neurological evaluation included assessment of the mental status and behaviour, posture, cranial nerves, proprioception, gait and other abnormal movements, spinal reflexes, panniculus and perianal reflex (Dewey & da Costa, 2016). As a result, the lesion was localised within the central or peripheral vestibular system.

Figure 2. Dog with a right head tilt (A) and dog with a left head tilt (B)

Clinical signs like a depressed level of consciousness, spastic hemiparesis, cranial nerve V-XII deficits, or general proprioceptive deficits on the same side as the vestibular deficits were compatible with the diagnosis of the central vestibular syndrome. A head tilt and balance loss will occasionally be appreciated in a patient who simultaneously has postural reaction deficits contralateral to the direction of the head tilt. When these specific clinical signs are noticed, the lesion must involve the caudal cerebellar peduncle or the flocculonodular lobe of the cerebellum on the side of the body opposite that of the head tilt. This condition is called paradoxical vestibular disease, and it is always indicative of central vestibular dysfunction (de Lahunta & Glass, 2010).

However, patients with ataxia, head tilt, normal proprioception and absence of cranial nerve deficits other than facial or vestibulocochlear nerve were suggestive of a diagnosis of a peripheral vestibular syndrome (Figure 2). Pathologic nystagmus is present in both types of vestibular syndrome, but vertical nystagmus whose direction changes depending on the position of the head is specific for a lesion localised within the central vestibular apparatus.
In some patients, the lesions were diffuse, so clinical signs of vestibular dysfunction were associated with other clinical manifestations. Consequently, seizures, hypermetria or tremor were recorded in patients in whom lesions were extended within the forebrain or cerebellum.
From the total number of investigated patients, 64% of the patients were diagnosed with central vestibular syndrome (n = 32) and 36% with peripheral vestibular syndrome (n = 18), as it is shown in Figure 3. Other recent reports showed similar results (Boudreanu et al., 2018; Bongartz et al., 2020)
For each patient, after the localisation of the lesion, a list of differential diagnoses has been established using the acronym VITAMIND (Vascular, Inflammatory, Trauma, Anomaly, Metabolic, Idiopathic, Neoplasia, and Degenerative) (Dewey & da Costa, 2015).

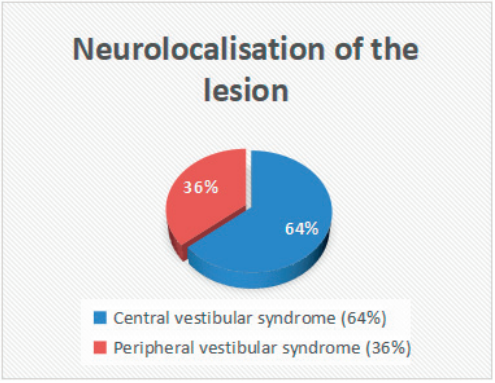

Figure 3. Distribution of patients according to the neurolocalisation of the lesion

For vascular pathology, ischemic infarctions or transient ischemic attacks were taken into consideration for patients who showed acute, focal and nonprogressive signs. From the total number of patients, 8 cases manifested signs

compatible with a stroke. All of them were aged above 10 years old. Additionally, for these patients, the cardiologic examination showed major dysfunctions that could be related to the neurological signs. This result is in concordance with previous studies that have shown that neoplasia and vascular disease were among the common causes of a central vestibular syndrome (Negrin et al., 2010; Bongartz et al., 2020).

The inflammatory pathology was associated with cases with subacute-chronic and progressive onset, asymmetric or multifocal signs and often pain (vocalisations). Meningoencephalitis was one of the causes for central vestibular dysfunction, being in general associated with small breeds of dogs (Schrauwen et al., 2014). In our study, 5 dogs (2 Bichon Frise, 2 French Bulldogs and 1 Yorkshire terrier) showed signs compatible with encephalitis. However, otitis media/interna was incriminated for producing a considerable number of cases of a peripheral vestibular syndrome (n = 10). The diagnostic of otitis was based on a complete otoscopic examination.

For all 48 patients, traumatic lesions were excluded, as owners stated that they did not witness any episodes of trauma involving their pets.

As an anomaly, during the study, one case of hydrocephalus was confirmed by MRI investigation in a 7-month-old crossbreed dog, in which clinical signs were compatible with a diffuse lesion (forebrain and central vestibular system).

For metabolic aetiology, differential diagnosis implied hypothyroid associated neurologic dysfunction and metronidazole toxicity. In this study, 1 case (Labrador, 1-year old) showed clinical signs compatible with a central vestibular syndrome (right head tilt, leaning on the right side, ataxia and loss of balance on thoracic limbs, horizontal nystagmus and modified proprioception on the specific tests) after receiving continuous high doses of Metronidazole.

The idiopathic vestibular syndrome is well known in cats and dogs and is responsible for many cases of peripheral vestibular syndrome (Bongartz, 2020). Considering the unknown pathology, diagnosis is always performed by the exclusion of other causes of peripheral vestibular disease. In this study, 8 cases were showing signs compatible with the idiopathic vestibular syndrome.

The neoplasia was always on the differential list for patients with asymmetric and progressive evolution of neurological sign. For confirmation, an advanced imagining technique is required. During the study period, we confirmed 5 cases of intracranial tumours associated with signs specific for a central vestibular syndrome.

Degenerative processes rarely affect the central vestibular system and their confirmation is difficult. Therefore, no case was diagnosed with a degenerative disease.

For all patients included in the study, after establishing the lesion localisation and the list of differential diagnoses, the next step was to perform a series of paraclinical tests to confirm the initial suspicion.

Depending on the situation, the recommended investigations were blood tests that included haematology, serum biochemistry and thyroid hormone dosage, cardiologic, ophthalmologic and otoscopic examination, radiographs, MRI, or cerebrospinal fluid analysis.

For each patient, additional investigations were performed in correlation with the owners' financial resources.

CONCLUSIONS

To establish the diagnosis of vestibular syndrome, all stages of the examination protocol, including the signalment, anamnesis, clinical and neurological evaluation, must be analysed.

The results of neurological examination will be correlated to differentiate between central and peripheral vestibular syndrome.

The brachycephalic-type breeds showed a predisposition for developing vestibular syndrome compared to other breeds dogs - 39% (n = 9) of the total number of purebred dogs were brachycephalic.

Regarding the age, older patients were more likely to develop a vestibular pathology than young patients - 58.33% (n = 28) were within the limit of 8 to 18-years-old.

The distribution of cases according to gender was almost equal: 52% females (n = 23) and 48% males (n = 25).

The acronym "VITAMIND" was always used to establish a list of differential diagnoses for each case of symptomatology compatible with a central or a peripheral vestibular syndrome.

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PHOSPHORUS CONTENT, NATIVE AND ADDED TO PIKE-PERCH (*Sander lucioperca*) FILLETS, SOLD ON THE EUROPEAN MARKET AND ITS EFFECTS ON TOTAL PRODUCTS' QUALITY

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Abstract

The paper presents the phosphorus content of pike-perch (Sander lucioperca) reared in Germany, in natural ponds and water recirculating systems respectively, compared with the same element measured from fillets imported from Kazakhstan and Russia and marketed in EU retail units from Hamburg Hanseatic City. It is based on the research data obtained in Max Rubner Institute, the Department of Safety and Quality of Milk and Fish Products among federal research institutes within the remit of the German Federal Ministry of Food and Agriculture (BMEL). The data have been processed into the proximate analysis parameters: pH, TVB-N, humidity and dry matter, ash percent, total phosphate, fat, protein and salt content. During the 10 months period of study (October 2013 - August 2014), were analyzed different sample types of pike-perch, refrigerated inland whole fish and frozen imported fillets. The phosphates content of fresh German pike-perch was situated approximately between 4.36 - 4.56 g P_2O_5/ kg of fish muscle, whereas the same parameter in frozen imported fish fillets analyzed were between 2.63 - 3.59 g P_2O_5/ kg. In conclusion, the use of added phosphates is usually suitable for fish fillet production. However, due to their water-binding capacity which also could determine improved juiciness of pike-perch fillets, the added phosphates negatively influenced the fillets' quality by forming an important amount of glaze, which after thawing led to unwanted weight loss, without bringing any threats to fish safety or consumers' health. The water loss during thawing caused a significant depletion of previously added phosphates.

Key words*: pike-perch, native phosphates, added phosphates, marketed frozen fillets, unprocessed pike-perch.*

INTRODUCTION

Fish is an important food both for humans and animals. Fish farming is an important branch of aquaculture in many EU and non-EU countries.
Protein - rich foods, such as seafood and meat products contain phosphorous compounds such as nucleotides, phospholipids, together with naturally occurring orthophosphates. The large range of natural orthophosphate contents (0.11-4.8% - 0.026-1.1% in terms of phosphorous content), makes it hard to detect added phosphates by quantitative analysis alone (Lawrie, 1998; Lee et al., 1998; Ünal et al., 2004).
The addition of phosphates is allowed in frozen products but not in salted or fresh products. The maximum quantity of added phosphates allowed in frozen products is 5 g P_2O_5/kg of the product (phosphorus pentoxide $P_2O_5 \approx 2.29 \times P$; orthophosphate $PO_4^{3-} \approx 3.06 \times P$). Of the list of additives authorized, only a few are a source of phosphorus and they are displayed on labels with a letter and number format: phosphoric acid (E338), phosphates (E339, E340, E341, E343), diphosphates (E450), triphosphates (E451) and polyphosphates (E452) (EU, 2011; Lou-Arnal et al., 2014).
Polyphosphates have been used by some produces in the production of salted fish in the Nordic countries (Lindkvist et al., 2008) in the belief that they are processing aid. The use depends on the export country, product but also on producer / customer.
High levels of phosphorus are related to the development of arteriosclerosis and bone disease in patients with chronic kidney disease (CKD) (Tentori et al., 2008). Phosphorus intake is also a public health problem given its impact on cardiovascular risk in the general population

("new cholesterol") (Dhingra et al., 2007; Sax, 2001; Calvo & Uribarri, 2013).
Hyperphosphatemia is a common disorder in patients with CKD, and may result in hyperparathyroidism and renal osteodystrophy. Hyperphosphatemia also may contribute to deterioration vascular calcification and increase mortality. Hence, correction and prevention of hyperphosphatemia is a main component of the management of CKD. This goal is usually approached both by administering phosphorus binders and by restricting dietary phosphorus (P) intake. Dietary intake of phosphorus (P) is derived largely from foods with high protein content or food additives and is an important determinant of P balance in patient with CKD. Food additives (PO_4) can dramatically increase the amount of P consumed in the daily diet, especially because P is more readily absorbed in its inorganic form. In addition, information about the P content and type in prepared foods is often unavailable or misleading. Therefore, during dietary counselling of patients with CKD, we recommended that they consider both the absolute dietary P content and the P-to-protein ratio of foods and meals including food additives (Kido et al., 2012).
Intake of natural foods that are not pre-cooked and the phosphorus content of some soft drinks (particularly cola) are important aspects that we should consider (Kalantar-Zadeh, 2010).
Currently, the maximum amount allowed in Switzerland and the EU is 5 g P_2O_5/kg for frozen fishery products (Manthey-Karl et al., 2014). This value does not include the natural phosphorus content which is on average 2.2 g/kg (≙5.7 g P_2O_5/kg) in the range of 1.0 to 4.0 mg P/kg. This variability may be related to biological factors and may also be caused by bone fragments (Wheeler & Hebard, 1981).
In this context, the paper presents a comparative analysis of the content of phosphorus pentoxide P_2O_5 in the fresh fish and frozen *Sander lucioperca* fillets produced in Germany, Kazakhstan and Russia, in order to highlight the influence of added phosphates, on the total products' quality.

MATERIALS AND METHODS

Quantification of total phosphate content is usually carried out by spectroscopic analysis. The sampling preparation is based on a decomposition of polyphosphates to orthophosphate in the presence of sulfuric or trichloroacetic acid (Jastrzębska et al., 2008) (Figure 1).

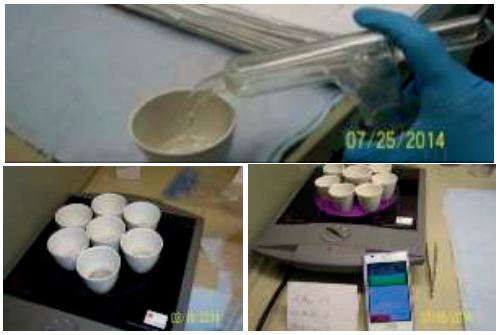

Figure 1. Ashed sample preparation for phosphates extraction

The orthophosphates react with ammonium molybdate and ammonium vanadate in nitric acid (HNO_3) and a yellow precipitate is formed. The concentration of phosphovanadomolybdate is used to calculate the content of phosphate or phosphorus.
In this study, the total phosphorus content was measured by the spectrophotometric reference method, adapted to the German official rules (§ 64 LFGB 06.00-9). The homogenized fish sample was dried and calcined, then the ash was hydrolyzed with nitric acid and filtered (Figure 2).

Figure 2. The ash was dissolved in 10 ml of dilute nitric acid. The filtrate was transferred quantitatively to a 100 ml volumetric flask and made up to the mark with distilled water

Briefly summarized, the ash from homogenized fish samples (5 g) were dissolved in 20% HNO_3 (v/v) by heating in a boiling water bath for 30min. After addition of 0.25% aqueous

ammonium monovanadate (w/v) and 5% ammonium heptamolybdate (w/v) to an aliquot of the nitric acid solution (~ 20%), the mixture formed a yellow-colored complex, whose extinction was photometrically measured at 430 nm, using a Varian Cary 50 UV-Vis Spectrophotometer Agilent (Figure 3).

Figure 3. Measurement of the extinction 15 minutes after mixing the reagents (λ = 430 nm)

Extinction measured was directly proportional to phosphorus concentration.

By using this method were analyzed 25 fillet samples of *Sander lucioperca* imported from Kazakhstan and Russia and 10 samples of raw refrigerated *Sander lucioperca* from Germany, respectively.

Quality assurance of the chemical analysis was performed by analyzing a reference material (muva-Referenzmaterial Nahrungsergänzungsmittel 752; http://www.muva.de/). Results showed an excellent agreement with the certified value (Boiţeanu et al., 2014).

The data, obtained during the research, have been statistically processed and expressed calculating the average and the standard deviation. We carried out a phosphorus content repeatability study in fish, with five repetitions for each sample (Figure 4).

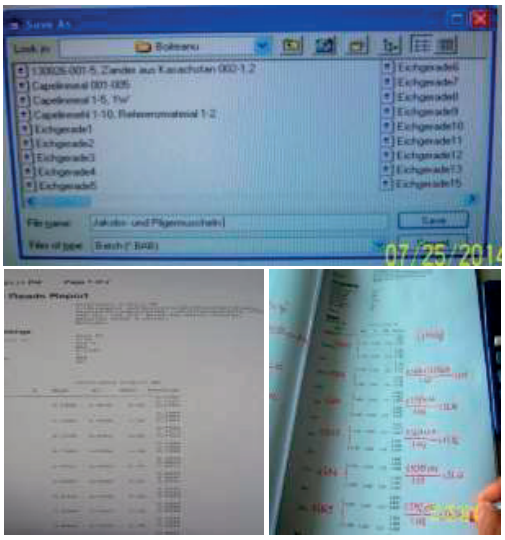

Figure 4. Results' reads and calculations

RESULTS AND DISCUSSIONS

Water is the most abundant component in its muscle, considering weight as well as volume (70-80%). As a main component, it influences the seafood sensory attributes, its shelf life and quality. However, a part of this water is lost during transportation, from its capture to its processing and posterior commercialization, through drip, evaporation and/or cooking (Toldrá, 2003; Gonçalves, 2004a; Gonçalves, 2004b).

The seafood processing companies have a great concern in retaining this water, first for economic reasons (seafood is sold by weight) and secondly, for the quality of the final product (Toldrá, 2003). On the other hand, an excessive loss of water can generate a great dissatisfaction on the part of the consumers for the following reasons: (a) the drip of the fish generates an undesirable appearance; (b) cooking reduces the size of the fish; (c) and mainly, the loss of the sensory attributes (juice, texture and colour) make the seafood less attractive (Gonçalves & Ribeiro, 2008).

The addition of polyphosphates improves the water retention during processing and may lead to an unjustified water uptake and increase in weight. Various phosphates have been widely accepted as additives in frozen fish and seafood (Manthey-Karl, 2015).

The phosphates soluble in the muscle liquid diffuse out of the muscle as the muscle is dehydrated at high salt contents (Þórarinsdóttir et al., 2010).

Polyphosphate solutions act inside the muscle fibres, causing the pH to increase, which ensures a more efficient absorption of water by the muscles, while reducing the loss of exudate during storage and improving the various properties of meat (Lemos et al., 1999).

By adding phosphates to raw muscle there is not only an increase in water retention capacity, but water remains in the muscles even after thawing and heat treatment. The condensed types of phosphates work effectively in this regard, as they promote protein dissociation and water retention. The addition of phosphates is strictly regulated and supervised in fish, crustaceans and molluscs, requiring, in any case, appropriate labelling. Addition of phosphates should perhaps only be carried out by brining. The presence of phosphate in brine used for injection does not improve the weight yields compared to use of salt only. Therefore, the main benefit is related with color and appearance of the fillets, i.e., the retarding effects of phosphate on oxidation and the maintenance of the natural color of the fish muscle. Brining could be an effective way to improve these parameters and at the same time led to lower increases in phosphate content (Þórarinsdóttir et al., 2010).

The Kidney Disease Outcomes Quality Initiative guidelines recommend a dietary ratio of 10-12 mg P/g protein (Lou-Arnal et al., 2013). Fish products represent serious sources of phosphorus intake. The regulatory framework does not bring any help in the effort of reducing phosphorus additives, since it considers them safe for public consumption and public health. There are categories of consumers (e.g., patients with CKD) that should carefully monitor their phosphorus intake.

Overcoming current obstacles and successfully decreasing the phosphorus intake suppose a collaborative effort to demonstrate that these additives possess harmful effects not only to CKD patients but also on the rest of the general population and they should be more strictly regulated.

Taking into account the imposed legal limits of P in fish and fishery products, the values of phosphorus pentoxide (g/kg) has been determined and recorded a decreasing trend in frozen fillets in comparison with refrigerated *S. lucioperca* (Table 1).

Table 1. Phosphorus pentoxide P_2O_5 in filleted pike-perch (*Sander lucioperca*), fish feed, and fish homogenate

Samples' type	P_2O_5 (g/kg) ± SD (n = 5)	Glaze content %
German pike-perch reared in natural ponds (Figure 7)	**4.4 ± 0.3**	-
German pike-perch reared in water recirculation systems	**4.6 ± 0.2**	-
Pike-perch fillets from inland waters of Kazakhstan (Figure 8)	**3.2 ± 0.6**	**11.6 ± 4.3**
	3.5 ± 0.3	**8.8 ± 2**
	3.6 ± 0.3	**12.9 ± 1**
Pike-perch fillets from Volga River, Russia (Figure 8)	**2.6 ± 0.4**	**11.7 ± 2**
Fish feed A	**21.7**	-
Fish feed B	**26.1**	-
Fish homogenate A	**18 ± 5**	-
Fish homogenate B	**19 ± 3.3**	-

This positive aspect was also determined by the reduced quantity of phosphorus pentoxide found in both imported and EU indigenous pike-perch, that did not exceed the limit of 5 g/kg P_2O_5. The fish farmers from Germany have selected the best diet based on the phosphorus content of reared fish in order to register a lower yield of P in the final products' composition.

In the North Eastern part of Germany, the average phosphorus yield in pike-perch homogenate from fish reared in water recirculation systems was 18.5 ± 3 g/kg P_2O_5 (Figure 5).

Figure 5. Pike-perch obtained from aquaculture in water recirculation systems

The fish was homogenized whole with head, skin, bones and intestines (Figure 6).

Figure 6. Whole homogenized pike-perch

Figure 7. Pike-perch obtained from aquaculture in German natural ponds

Figure 8. Frozen pike-perch fillets imported from Russia and Kazakhstan

The phosphorus pentoxide content was higher in German fish muscle compared with imported pike-perch fillet samples from Russia and Kazakhstan (Figure 9). The glaze content in pike-perch fillets exceeded 10% in most of analyzed samples (Table 1).

Adequate glazing (6-10%) of fish fillets prior to frozen storage protects the final product from dehydration, oxidation and quality loss. Excessive glazing (>12%) on the other hand may significantly affect the economic value and end user satisfaction of frozen fish fillets (Vanhaecke et al., 2010).

Our results shown an excess of glaze in both frozen fillets (Table 1) from Russia (with an average of 11.7 ± 2%) and Kazakhstan (with values between 8.8 ± 2% and 12.9 ± 1%) which led to a significant depreciation of the total products' quality.

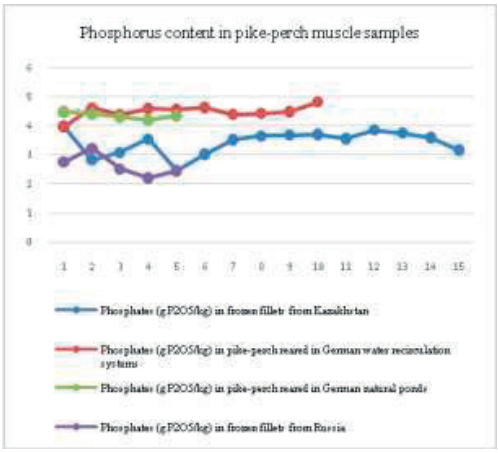

Figure 9. Fluctuation of phosphorus pentoxide (g/kg) in Pike-perch (*Sander lucioperca*) samples reared in four different sweet water sources

CONCLUSIONS

Fish products represent serious sources of phosphorus intake. Phosphorus based additives possess harmful effects not only to CKD patients but also on the rest of the general population and they should be more strictly regulated.

The regulatory framework does not bring any help in the effort of reducing phosphorus additives, since it considers them safe for public consumption and public health. There are categories of consumers (e.g., patients with CKD) that should carefully monitor their phosphorus intake.

The benefit of their use is a better water-binding and retention capacity which improves the sensory attributes of fish fillets.

In this study the observed side effect of phosphates consisted only in excessive weight gain of fish fillets with a medium value of glaze content situated in the range of 8.8 to 12.9%.

All samples analyzed here did nor overpass the legal limit imposed by the EU legislation (5 g added P_2O_4 /kg fish muscle). This value does not include the natural phosphorus content which is on average 5.7 g P_2O_5/kg.

The natural phosphorus content found in pike-perch samples from fish reared in two types of

aquaculture systems in the North Eastern part of Germany was situated between normal limits (4.4-4.6 g P_2O_5/kg) and lower than the previously reported average.

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ASSESSMENT OF THE MICROSCOPIC STRUCTURE - COMPLEMENTARY METHOD OF QUALITY CONTROL OF SAUSAGES

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Abstract

Information concerning the sources of raw material used in order to obtain meat products is of utmost importance, as many technologies permit the replacement of raw material with additives comprising proteins of various animal or plant origin. The purpose of this paper was to assess the quality of preserved sausages (by smoking, boiling, etc.) by using the routine histological examination. In this paper, a total of 22 sausages produced in various processing units were randomly acquired from supermarkets in Bucharest.

The samples were performed according to the routine histological procedure by embedding in paraffin, using a histoprocessor, sectioned and stained by HE (hematoxylin-eosin), and by Tricomic Masson staining. The results of the current study indicate that the studied products contain several types of tissues in varying proportions (muscle, connective and adipose tissues, blood vessels) and amorphous, anhistous structures. The morphological wholeness of the muscle tissue was assessed by the specific structural elements (sarcolemma, sarcoplasm, nuclei). The study supports the introduction of routine histological examination as an additional method for assessing the quality of sausages.

Key words*: sausages, quality, raw materials, integrity, microscopic structure.*

INTRODUCTION

The consumed products 'quality has a major impact on the general health of the population. In this regard, information on the sources of raw materials for the processing of meat products is of utmost importance, as many technologies permit the replacement of raw materials (for instance: muscle tissue) with various additives comprising proteins of different animal or plant origin. Moreover, cases of adulteration of meat products using animal species that are not covered by technology are becoming more widespread. (Natalya L. Vostrikova et al., 2019). Falsification of raw materials by altering the composition of the species modifies the properties of the final product and constitutes a danger for the consumer's health. Severe risks are associated with the replacement of raw materials with animal meat. The use of animal meat as replacement is prohibited or restricted due to the possibility of it being infected with prions or viruses. In some situations, the use of undeclared components like soy, mustard milk proteins, may trigger allergic reactions, a risk about which the consumer remains uninformed. Moreover, adulterating raw materials may infringe on the moral code of consumers whose local or religious views do not allow the consumption of meat obtained from certain animal species. (Natalya L. Vostrikova et al., 2019).

Many studies have shown the potential of histological techniques to evaluate the composition of sausages. This method can accurately assess the quality parameters of meat products, detecting (and quantifying) specific tissues of animal organs, extracellular connective tissue, fat content, bone tissue. (Ghisleni et al., 2010; Latorre et al., 2015; Malaskiene et al. 2016; Moghtaderi et al.,2019; Sadeghi et al., 2003; Sezer et al., 2013).

It is extremely important to verify the composition of meat products (namely: raw materials, intermediate products, and finished products), at all stages of production.

To guarantee the effectiveness of such verification, unfailing and productive analytical methods are needed, so as to grant researchers the detection of individual ingredients and molecular identifiers that depict the content of

different types of raw materials in the finished product (Natal'ya L. Vostrikova et al., 2019).
At present, in the Czech Republic, the microscopic analysis of food is monitored by experts from the Department of Plant-based Food and Plant Production, at the Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, in Moscow, Russia, at the V. M. Gorbatov Federal Research Center for Food Systems. RAS put in place a concept to ensure the quality and complete safety of meat products, The concept includes the use of barrier technologies, of predictive microbiology, the critical control points, the production management principles, the safety and product quality monitoring throughout the production, the transport, and the sales chain (Kalinova Yu., 2007; Pospiech et al., 2009).
Multi-stage technological treatments (e.g., fine grinding, salting, heat treatment,) can make it difficult to identify the structure of the muscle tissue, as recommended by traditional methods.
There are many methods to process and prepare specimens for microscopic examinations today, along with a variety of investigative techniques. These procedures include both conventional methods and methods that use the most recent equipment, but they all show advantages and disadvantages.
Nevertheless, histological analysis may point out a more comprehensive view of the composition of meat products. (Tremlová et al, 2002; Pospiech et al., 2009; Ghisleni et al., 2010; Doaa M. Mokhtar et al., 2018).
The purpose of this study is to establish the quality of preserved sausages (smoked, boiled etc) by using the histological analysis method.

MATERIALS AND METHODS

Throughout this paper, a total of 22 sausages produced in different processing units were randomly acquired from supermarkets in Bucharest. The sausages were selected from the following product categories: cold-smoked, boiled and smoked and smoked and boiled and double-smoked and each sample is a commercial type. The parameters of the samples were set down, including the type of sample and the date of sampling, then the samples were immediately transported and processed.
Samples fixed in 10% formaldehyde solution were subjected to the routine paraffin inclusion method, using a histoprocessor, initially sectioned and stained by the HE (hematoxylin-eosin) technique, and for better differentiation of the connective tissue from sausages was used Tricomic Masson staining. The sections were examined with the Olympus BX 41 Microscope with an integrated computer shooting system.

RESULTS AND DISCUSSIONS

The outcomes of our study indicate that the studied products contain several types of tissues. This diversity of observed tissue types is not completely different from that found by the american researchers in the analysis of meat products (Prayson et al., 2008a; Prayson et al., 2008b; Richard et al., 2013).
Sausages from the category of boiled and double-smoked products and from the category of boiled and smoked products present fragments of striated muscle tissue with moderately preserved cell morphology, with partial homogenization of the sarcoplasm and frequently with destruction of the sarcolemma (Figure1). These tissue fragments are bordered by an amorphous mass, sometimes with an oxyphilic fibrillar character that frequently includes vegetal tissue fragments (spices). Also, in the entire thickness of the product, there are large areas of adipose tissue with a partially preserved morphology with intact cell membranes, but without nuclei (Figure 2).
Other sections of the same category, present masses of connective tissue in the thickness of the muscle, the connective tissue has lost its normal morphology; it is homogeneous blue - light blue (Figure 3) in which rare shadows of nuclei are observed.
Sausages in the category of cold-smoked products have longitudinal and cross sections through striated muscle tissue with moderate preservation of cell morphology, the sarcolemma is occasionally detached, the sarcoplasm is homogeneous. Muscle tissue is frequently embedded in connective-adipose tissue, where fat cells have preserved only their cellular contour. In some sections there are

many granular areas of intense basophilic colour (salt), the plant fragments are more discreet but distributed throughout the product (Figure 4).

The histological method occasionally detected glandular tissues, (Figure 6), lymphoid tissue (Figure 7), nerve threads and blood vessels (Figure 8) in the examined sausage samples. In addition, the present study showed, in addition to muscle tissue, adipose, fibrous, and serous glandular tissue, the presence of parasitic structures *Sarcocystis* spp. (Figure 5),

To compare the morphological structure of the product categories analysed by microscopic examination, we resorted to the quantification of different aspects, shown in Tables 1, 2, and 3. According to the records, muscle tissue is intact in cold-smoked sausages and boiled and double-smoked products at least the architecture of the muscle tissue is preserved.

We note that no conclusion can be drawn on this issue due to the small number of samples analysed, but the method of quantifying tissue integrity can provide important data on the quality of the sausages analysed.

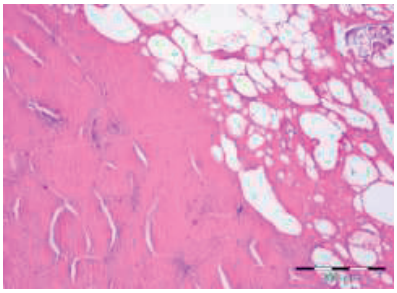

Figure 1. Sausage - boiled and double smoked product. Sections through muscle and adipose tissue with partially preserved morphology. Inaccurate delimitation of muscle fibers by sarcolemma HE stains (ob. 10x)

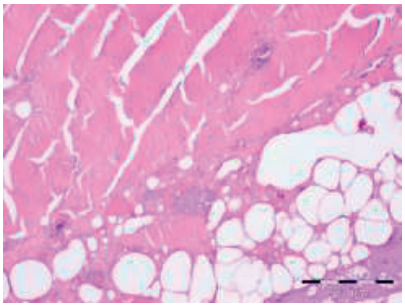

Figure 2. Sausages, boiled and double smoked product, adipose tissue with the preservation of cell membranes, but most of them without nuclei HE stains (ob. 10x)

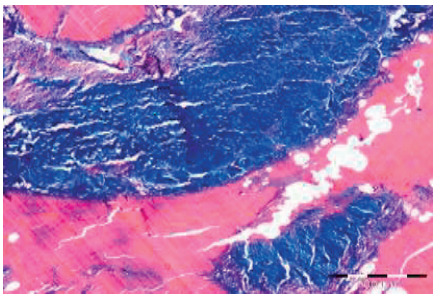

Figure 3. Sausage - boiled and double smoked product. Muscle tissue and adipose tissue with preserved structure and amorphous connective tissue. Tricomic Masson stains (ob. 10x)

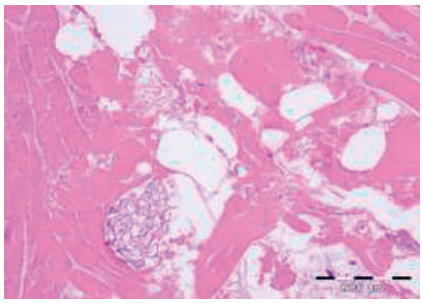

Figure 4. Sausage cold smoked product. Homogenized and fragmented sarcoplasm, plant fragments HE stains (ob. 10x)

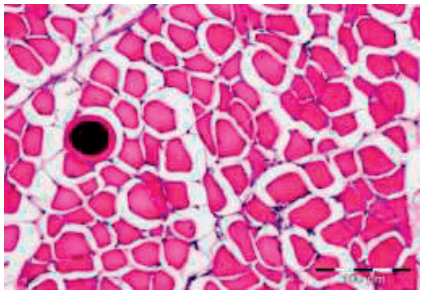

Figure 5. Sausage - cold smoked product. *Sarcocystis* spp. In cross-sectioned muscle fibers; preservation of muscle tissue structure HE stains (ob. 20x)

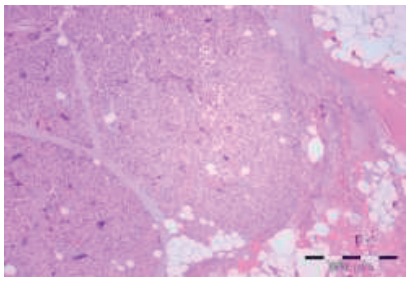

Figure 6. Sausage - boiled and smoked product. Glandular tissue integrated in the product HE stains (ob. 4x)

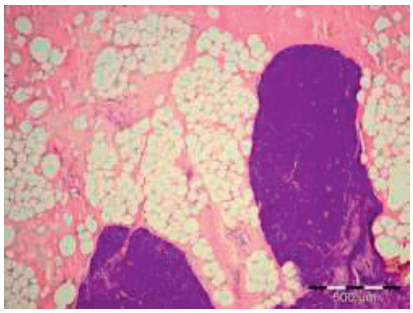

Figure 7. Sausage - cold smoked product. Fragment of lymphoid tissue in the composition of the product. HE stains (ob. 4x)

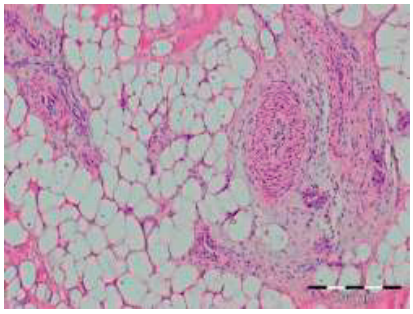

Figure 8. Sausage - cold smoked product. Preserved blood vessels in a mass of adipose tissue. HE stains (ob. 10x)

The utilization of unapproved animal tissue in meat products is due to the economic value of meat itself. The problem of authentication in meat products could include the replacement of meat species, tissues, vegetable proteins, organic compounds, as well as the replacement of vegetable fats with animal fats. (Ballin N.Z., 2010).

Some studies attest to the importance of introducing histological examination, which could be a useful tool for government authorities in fraudulent and quality control of meat products. (Ballin NZ., 2010).

Non-compliant tissues, such as glandular tissue and lymphoid cells, were also highlighted in the current study.

Cetin et al., stated that 21 of the 127 ready-for - sale meat samples held a large quantity of calcium, suggesting the addition of non-meat materials like: scrapings of bone and mechanically deboned meat. (Cetin O., 2016)

Rokni et al., presented salivary gland tissue in boiled sausages, which reflects the use of meat obtained from the heads of slaughtered animals, in meat products. (Rokni et al.,1997)

Sepehri Erayi observed additive tissue consisting of chicken skin, peritoneal fat, hyaline cartilage, and kidney in 30 samples of three different types of sausages. (Sepehri Eraei et al., 2008). Similar aspects were identified in the samples analysed in this study.

Moghtaderi, et al. examined 20 samples of sausages and revealed unauthorized tissues, including conective tissue (6.66%), cartilage (28.30%), bone (8.30%), skin (51,60%), and blood vessel (11.66%), in addition, plant tissues were recognized in 97,70% of the samples. (Moghtaderi, et al., 2019)

Sadeghinezhad and his team focused on the qualitative and quantitative accuracy of the histological examination for the detection of unauthorized plant and animal tissue content in minced beef where the minced meat composition contained between 5 and 20% soybeans and chicken organs (Sadeghinezhad et al., 2015).

The results obtained by Pospiech (2009) indicated the addition of plant additives to meat products, which reduces the quality of meat and affects food safety as allergens (Pospiech et al., 2009).

What is certain is that only the standard physicochemical determinations usually applied to meat products cannot provide sufficient information on the quality and integrity of muscle tissue or the presence of non-compliant tissues.

From this point of view, we consider the microscopic investigation in this paper useful, and we advocate for its introduction as an additional method of surveilling the quality of foodstuff of animal origin.

Table 1 Morphological Characteristics of the different tissue types detected in the category of boiled and double-smoked sausages, by histological methods

Product type	Integral muscle fibers, delimited by the sarcolemma	Presence of muscle fiber interruptions	The presence of striations	The presence of nuclei	Connective tissue	Presence of anhistic material	The presence of spices
Sample A1	inconstant	+	+	inconstant	+	+	+
Sample A2	–	–	–	–	+	+	+
Sample A3	–	+	–	–	+	+	+
Sample A4	–	+	–	–	+	+	+
Sample A5	–	+	–	–	+	+	+
Sample A6	inconstant	–	–	+	+	+	+
Sample A7	inconstant	+	–	–	+	+	+

- not observed; + present

Table 2. Morphological Characteristics of the different tissue types detected in the category of boiled and smoked sausages, by histological methods

Product type	Integral muscle fibers, delimited by the sarco-lemma	Presence of muscle fiber interrup-tions	The presence of striations	The presence of nuclei	Connect ive tissue	Presence of anhistic material	The presence of spices
Sample A8	+	+	-	-	+	+	+
Sample A9	+	+			+	+	+
Sample A10	+	+	+	+	+	+	+
Sample A 11	+	+			+	+	+
Sample A 12	-	+		inconstant	+	+	+
Sample A 13	Incon-stant	+	_	+	+	+	+

- not observed; + present

Table 3. Morphological Characteristics of the different tissue types detected in the category of cold smoked sausages, by histological methods

Product type	Integral muscle fibers, delimited by the sarcolemma	Presence of muscle fiber interrup-tions	The presence of striations	The presence of nuclei	Conn ective tissue	Presenc e of anhistic material	The presence of spices
Sample A 14	+				+	+	+
Sample A 15	+	+		+	+	+	+
Sample A 16	+	+	+	+	+	+	+
Sample A 17	+	-			+	+	+
Sample A18	+	+			+	+	+
Sample A 19	+	inconstant			+	+	+
Sample A 20	+	+			+	+	+
Sample A 21	inconstant	+	+	+	+	+	+
Sample A 22	-	+	_	Incon-stant	+	+	+

- not observed; + present

CONCLUSIONS

Histological evaluation of sausages subjected to various preservation techniques revealed some non-compliant tissues (glandular tissue, lymphoid tissue, vessels, and nerves) and intracellular parasitic forms (*Sarcocystis* spp.).

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IN VIVO EFFECTS OF TITANIUM IMPLANTS TREATED WITH BIOMATERIALS IN THE BONE REGENERATION PROCESS

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Abstract

The biological interface between the host tissue and the medical device used may influence clinical outcomes. Expected effects include osteoinduction, osseointegration, increased vascularity, and mechanical stability. The aim of the study was to evaluate the process of bone regeneration and potential local effects as results of insert implants treated with innovative biomaterials in the intercondylar rat femur. Two groups of rats, Wistar, were created to which titanium implants were applied to the femoral bone. In one of the groups, the titanium implant coated with Poly (3-hydroxybutyric acid-3-hydroxyvaleric acid) polymer microspheres enriched with fibroblast growth factor 2, vascular endothelial growth factor and bone morphogenetic protein 4 (VGF2) was inserted and in the other group, the implant was untreated. After 6 weeks of clinical monitoring, rats were euthanized, and the implanted femur was harvested for histological analysis. In this study the data obtained showed that VGF2-treated implants contributed to faster bone regeneration, with favorable local effects, compared to untreated implants in which signs of persistent inflammation were.

Key words: animal model, implant, osteoinduction, osseointegration.

INTRODUCTION

Regenerative treatment applied to bone trauma depends on the nature of the medical implants used. Large bone defects, osteomyelitis, tumor resection, or skeletal abnormalities can affect normal bone healing. Numerous studies on the mechanisms of skeletal development, pathophysiology and fracture correction have provided important information for establishing methods for regulating the proliferation and differentiation of osteoblasts in the process of bone regeneration (Charoenlarp et al., 2017).

Biomaterials covering medical implants are in a wide range and represent a promising approach that offers a favorable effect in case of bone damage requiring their use. The basic support of implants is Titanium, which is treated with growth factors to ensure better integration into the body.

Our study involved testing Titan implants treated with Poly (3-hydroxybutyric acid-3-hydroxyvaleric acid) polymer microspheres enriched with fibroblast growth factor 2, vascular endothelial growth factor and bone morphogenetic protein 4 (VGF2) of rats, compared to untreated implants. Fibroblast growth factor 2 (FGF2) is the most common ligand of fibroblast growth factors used in regenerative medicine being an endogenous, positive regulator of bone mass (Fei et al., 2011), which stimulates the proliferation of mesenchymal stem cells (Boteanu et al., 2020). Vascular endothelial growth factor together with bone morphogenetic protein also contributes to stimulating the differentiation of

osteoblasts and osteoclasts. (Böhm et al., 2019; Hu, 2016).

MATERIALS AND METHODS

Ethics statement: The animal study was approved by the Ethics Commission of the "Cantacuzino" National Medico - Military Institute for Research and Development (IC) and authorized by the competent authority. The procedures were carried out in accordance with the provisions of EU Directive 63/2010 on compliance with the rules for the care, use and protection of animals used for scientific purposes.

For this study, 20 rats, Wistar, males and females, aged 20 weeks, were used in experiment. The rats were from the laboratory animal farm of IC, Băneasa Animal Facility (SB). They were acclimatized for 5 days, the accommodation being provided in Euro standard Type III cages (425 x 276 x 153 mm) labeled, for each group of 5 rats, arranged in the SB experiment space. Water and food administered ad libitum as well as microclimate conditions (temperature 22-24%, relative humidity 45-65%, cycles of 12 h of light and 12 h of darkness) remained constant throughout the experiment, according to "Guide for the Care and Use of Laboratory Animals".

Only healthy animals were selected, which were not used in previous tests and two groups were formed (Table 1).

Table 1. Allotment of animals

Group	Sub-group	Number of animals	Sex	Tested implant type
1	1a	5	m	Titanium + VGF2
	1b	5	f	
2	2a	5	m	Titanium
	2b	5	f	

Tested implants: Titanium metal bars coated with growth factors and osteogenesis inducers VGF2 and untreated titanium metal bars (1.5 mm length, 1mm diameter).

Procedure: On day 0 of the experiment all animals were weighed and deeply anesthetized with a mixture of Ketamine (75 mg/kg) and Medetomidine (0.5 mg/kg). Preoperative preparation consisted of clipping the hair at the left tibiofemoral and patellofemoral joint and disinfecting the skin with Betadine. After restrain in dorsoventral recumbency on the operating table, periarticular infiltrations with 10% Lidocaine were performed. The actual surgery involved the induction a bone defect at the distal end of the femur. A skin incision was made on the medial parapatellar side, followed by lateral dislocation of the patella and exposure of the femoral condyles. With the limb held in a flexed position, an intercondylar cavity (into trochlear groove) was created by drilling, using a drill with a diameter of 1 mm, under continuous cooling with saline. The test implant (Table 1.) was inserted into the resulting cavity by pressing it.

The patella was repositioned, and the joint capsule was sutured with 4-0 resorbable suture and the skin with 2-0 non-absorbable. After the suture of the surgical wound, Atipamezole i.p. postoperatively, the animals were treated with antibiotics (Enrofloxacin 5 mg/kg, 5 days) and analgesics (Ketofen 5 mg/kg, 3 days).

The monitoring period of the animals was 6 weeks, during it, local and general clinical signs were observed. Hematological examinations were performed at the beginning and end of the study, biochemical, necropsy and histological examinations were performed on day 42.

RESULTS AND DISCUSSIONS

Clinical examination: The groups in which the VGF2-treated implant was tested showed from the first day postoperatively loss of appetite, adynamic and lameness on the operated limb, and in the first 9 days postoperatively, 5 animals (4 males and one female) died, without presenting a direct connection with the implanted device.

The groups with untreated implant had a moderate clinical evolution. In the first seven days after surgery, the rats were adynamic, showed lameness and 2 males who showed prostration, loss of appetite and dehydration, succumbed on day 3.

The survivors had an unfavorable clinical evolution in the first 28 days (prostration, adynamic, limping, swelling of the joint capsule) and in the last 14 days the general condition improved, the limping disappeared, and the animals regained weight loss.

Body weight was monitored by weighing the animals on a RADWANG scale every two weeks, respectively D0, D14, D28, D42. The

animals from sublots 1b and 2b had a weight loss in the first 14 days after implantation, then a constant increase (Figure 1).

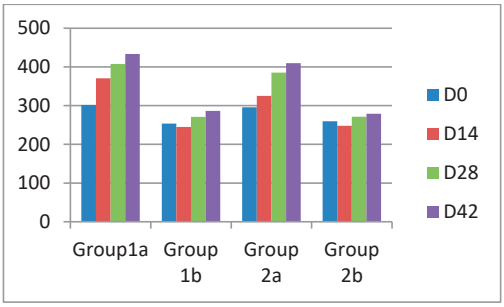

Figure 1. The evolution of body weight

Hematological tests were performed on an Idexx ProCyte 5Diff device, the blood being collected on tubes with EDTA on day 0 and the final day. The mean hematological values obtained on each group are shown in Table 2.
Sub-groups 1b, 2b and 2a bis showed increased values of heterocytes and reticulocytes, double that of Z0. The rest of the analyzed parameters did not provide relevant data for our study.

The biochemical analysis was performed on a VetTest 8008 device, and the blood was collected in Li-heparin vacutainers on day 42. The values shown in Table 3 represent the average of the biochemical parameters analyzed in the group where the VGF2-treated implant was mounted and in the group in which the untreated implant was tested.
The values obtained showed marked increases in creatinine and urea, but the correlation with the implanted devices is uncertain because both animals in group 1 and those from group 2 had increased results of the same parameters.
The necropsy was performed for all euthanized animals according to the study protocol and for animals found dead during the experiment. The animals found dead a few days after the operation, did not show changes in the internal organs or in the operated limb, but the poor state of maintenance, severe dehydration could guide the cause of death. Animals that were euthanized at the end of the experiment were in good condition, with no changes in internal organs, and the operated limb was completely healed at both the skin and capsular level.

Table 2. Average hematological parameters

Analyzed Parameter	Group1a		Group 1b		Group 2a		Group 2b	
	Day 0	Final day	Day 0	Final day	Day 0	Final day	Day 0	Final day
RBCx10^12/L	6.706	7.31	7.58	5.49	6.08	6.38	6.53	6.41
HCT %	33.48	39.08	36.8	29.15	30.02	31.99	31.78	31.94
HGB g/dL	12	13.54	13.34	10.45	10.76	11.51	11.52	11.51
MCV fL	49.78	53.46	48.6	52.8	49.34	50.24	48.34	49.86
MCH, pg	17.94	18.56	17.62	19	17.72	18.11	17.78	18.04
MCHC g/dL	36.08	34.72	36.28	36.02	35.94	36.08	36.8	36.22
RDW %	20.78	24.54	19.18	17.27	20.38	18.94	17.82	18.72
%RETIC	7.3	7.42	2.94	4.22	7.16	4.77	2.8	4.38
RETIC K/µL	498.96	546.44	221.08	228.05	430.56	293.23	180.58	270.7
WBCx10^9/L	8.176	8.28	6.16	6.04	7.2	6.46	4.92	6.16
%HETERO	36.28	26.44	15.24	35.6	38.84	29.89	18.7	27.65
%LYM	53.58	65.32	79.32	57.25	53.98	63.51	73.18	65.44
%MONO	6.16	7.5	4.06	6.12	5.32	5.16	6	5.33
%EOS	3.1	0.36	1.08	1.02	1.46	1.18	1.9	1.33
Analyzed Parameter	Group1a		Group 1b		Group 2a		Group 2b	
	Day 0	Final day	Day 0	Final day	Day 0	Final day	Day 0	Final day
%BASO	0.88	0.38	0.3	0	0.4	0.23	0.22	0.23
HETEROx10^9/L	3.058	2.18	0.95	2.57	2.81	2.11	0.91	1.87
LYMx10^9/L	4.304	5.42	4.87	2.87	3.88	3.87	3.60	3.82
MONOx10^9/L	0.528	0.60	0.25	0.50	0.37	0.37	0.30	0.36
EOSx10^9/L	0.212	0.03	0.07	0.09	0.10	0.08	0.09	0.09
BASOx10^9/L	0.074	0.03	0.01	0	0.03	0.01	0.01	0.01
PLT,K/µL	454.4	228	576.6	1002	272	616.86	229.2	539.33
MPV,fL	9.42	10.02	8.3	10.62	8.85	9.25	8.98	9.20
PDW,fL	8.15	7.4	8.4	8.15	7.3	7.95	7.9	7.94
PCT%	0.4	0.20	0.45	0.43	0.28	0.39	0.18	0.34

Table 3. Average of biochemical parameters

Analyzed Parameters	MU	Group with implant treated with VGF2	Reference interval	Group with untreated implant
GLU	mmol/L	9.2	2.78-7.51	7.53
CREA	µmol/L	108.25	9.00-53	115
UREA	mmol/L	7.875	3.2-7.5	8.25
TP	mmol/L	2.6575	1.33-2.90	2.54
CA	g/L	55.25	53-69	49.5
ALB	g/L	31.75	38-48	27.5
GLOB	g/L	23.75	15-28	21
ALT	U/L	44.75	20-61	59
ALKP	U/L	89.5	16-302	77.5
GGT	U/L	0	1.00-6.00	0
TBIL	µmol/L	< 2	2.00-12	2
AMYL	U/L	1232.5	326-2246	1259
LIPA	U/L	91.25	10-150	80.5

The groups with treated VGF2 implant were characterized by the presence of neoformation cells (2-3 rows), moderate fibrosis, proliferation of osteoblasts, homogeneous periosteum and new capillaries (Figure 2), and the groups with untreated implant showed necrosis phenomena, layers of cells suggesting bone reactivity to implant, fibrosis, dense collagen fibers, lymphocytes, fibroblasts, lymphocyte granulation tissue (Figure 3).

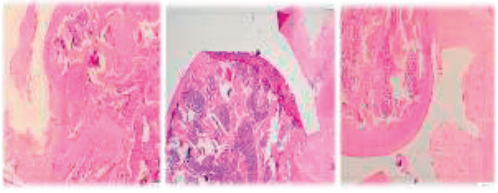

Figure 2. VGF2 implant: ob x 2 H.E., osteoblast proliferation and regeneration phenomena, moderate fibrosis

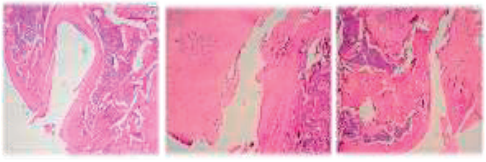

Figure 3. Untreated implant: ob x 2 H.E., necrosis and cells (lymphocytes, fibroblasts) that suggest bone reactivity to the implant

The survival rat was low, especially in the male groups in which the VGF2-treated implant was tested (20%); in sublot 1a, 4/5 rats died approximately 3 days after surgery, with no macroscopically observable clinical signs. Females with VGF2 implants had a survival rate of 71% as did male rats of group 2, in which untreated implants were fitted. Females in group 2 had a survival rate of 100%.

FGF2, VEGF and BMP4 are widely used for alveolar bone regeneration in the dental field, but it is not clear what growth factor should be recommended for alveolar regeneration. (Charoenlarp, 2017). Most studies have shown that BMP has a higher osteogenic capacity than FGF-2. (Nguyen et al., 2019) Although their application may result in associated complications on different cell types including not only osteoblasts, osteoclasts, but also bone marrow cells and should be studied in more detail to promote more efficient administration of FGF2, VEGF and BMP4. BMP and FGF2-induced bone formation is regulated by bone marrow cells (Nosho et al., 2020), BMP4, on the one hand, promotes bone regeneration and osseointegration around titanium implants, but on the other hand induces bone resorption when a BMP-coated implant it is inserted into a bone such as the mandibular one (Nosho et al., 2020). In long bone fractures, which are abundant sites in the bone marrow, FGF2 significantly accelerated fracture healing and callus formation (Nagayasu-Tanaka et al., 2017; Kawaguchi et al., 2001). BMP is known to stimulate the expression of genes associated with mineralization, but appears to have little or no effect on the expression of genes associated with cell proliferation. FGF-2 is known to be a potent inducer of vascular endothelial growth factor A (Hughes-Fulford, 2011). Furthermore, the effects of FGF-2 on BMP signaling appear to be dose-dependent; While a low dose of FGF-2 could increase BMP-associated bone formation, a high dose of

FGF-2 suppressed it (Kawaguchi et al., 2001).
Bone is considered a rigid organ that supports and protects various vital organs in the body, including the bone marrow and the stem cell niche (Abdel Meguid, 2018). In this context, BMP is known to play a key role in the development of hematopoietic bone marrow (Kawai, 1994; Kusumoto, 1995). BMP may be suitable for application in the extramedullary medium, while FGF-2, in medullary bone regeneration.
Vascular endothelial growth factor-A (VEGF) is one of the most important growth factors for regulating vascular development and angiogenesis. Because bone is a highly vascularized organ and angiogenesis plays an important role in osteogenesis, VEGF also influences skeletal development and bone repair (Hu, 2016). During bone development, VEGF is a critical survival factor for epiphyseal chondrocytes so modulating VEGF levels in bones is a potential strategy for treating compromised bone repair and improving bone regeneration (Maes, 2004).

CONCLUSIONS

Despite the low survival rate, which did not show a direct correlation with the placement of the implants, the macroscopic clinical signs reached the proposed objectives, and neoformation bone tissue could be observed around the implant.
The results of the histological examination showed that titanium implants treated with growth factors and inducers of osteogenesis contribute to a faster bone regeneration, and the local effects are favorable.

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EVALUATION OF INDUCED METABOLIC SYNDROME OF OBESITY BY ADMINISTERING A PURIFIED DIET IN MICE

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Abstract

The worldwide prevalence of obesity has risen dramatically in the last decades. Obesity is associated with multiple medical conditions that appear in metabolic syndrome such as: type II diabetes, increased blood pressure, high triglyceride and cholesterol levels. The aim of this study was represented by the evaluation of diet-induced obesity, with the purpose of creating an experimental mouse model for testing food supplements and medication used in this syndrome. One group of C57BL/6 mice received an in-house purified hypercaloric diet, the second group received a standardized obesity diet and the third group received a control diet for a period of 60 days. The following aspects were assessed during the experiment: food intake, body weight, hematologic and biochemical parameters. On the final day, organ samples were collected (liver, kidneys and visceral adipose tissue) for necropsy and histopathologic examination. The obtained results showed that the administration of the in-house purified hypercaloric diet for a period of 60 days was optimal for installing obesity syndrome in mice. The use of unidirectional enriched diets presents an increased interest in current research for futher development of new therapeutic strategies in metabolic syndrome.

Key words: *metabolic syndrome, mice, obesity, purified diet.*

INTRODUCTION

Metabolic Syndrome (MetS) is characterized by the simultaneous occurrence of at least three of the following medical conditions: obesity, hyperglycemia, hypertension or dyslipidemia (Kaur, 2014).

Overweight and obesity are important clinical and public health concerns worldwide. The prevalence of obesity has increased dramatically during the last four decades and has reached epidemic proportions in both developed and developing countries (Hruby & Hu, 2015; Kelly et al., 2008). Globally, more than 1.9 billion adults aged 18 years and older are overweight, and of those, almost 700 million adults are obese (WHO, 2018).

Obesity is defined as an excessive or abnormal accumulation of adipose tissue in the body, associated with multiple medical conditions such as type 2 diabetes, hypertension,atherosclerosis, hyperlipidemia and arthritis (Alberti et al., 2006). Obesity is also associated with an important decrease in life expectancy and an increased risk of several cancer types (Engin, 2017). Obesity is considered a complex disease and has multifactorial etiology due to the interaction of both environmental and genetic factors and it is a result of the prolonged imbalance between caloric intake, basal metabolism and energetic consumption (Serra & Bautista, 2013; Lang et al., 2019).

In order to understand the pathophysiological basis of obesity and obesity-associated metabolic complications, it is imperative to develop animal models of MetS (Wong et al., 2016). Establishment of appropriate animal models mimicking MetS in humans is an

important concern for the biomedical research (Lutz & Woods, 2012).

Mice and rats are the most common animal models used in investigating obesity as they readily gain weight when provided with a high-fat diet (Speakman et al., 2008; Tristan et al., 2017). The aim of this study was represented by the evaluation of diet-induced obesity, with the purpose of creating an experimental mouse model for testing food supplements and medication used in this syndrome. Another purpose of the present study refers to the formulation, preparation and standardization of an in-house purified diet that might induce obesity in C57BL/6 mice, in comparison with an existing standardized obesity diet.

The following aspects were assessed during the experiment: weight gain, food consumption, hematological and biochemical parameters and total body lipid level. By analyzing the results, we showed that the administration of the in-house purified hypercaloric diet for a period of 60 days was optimal for installing obesity syndrome in C57BL/6 mice.

MATERIALS AND METHODS

This study was approved by the Ethics Committee of Cantacuzino National Medical-Military Development Research Institute and approved by the competent authority. The animals were provided by Băneasa SFP (Specific Pathogen Free) Animal Facility area for rats and mice of Cantacuzino National Medical-Military Development Research Institute, Bucharest.

All aspects related to animal housing and care were undertaken in accordance with the national and international regulations concerning animal testing. The animals were kept under standard conditions, temperature 18-24°C, humidity 35-75% and cycle of lighting 12/12 h. The food and the water were administered *ad libitum* during the entire experiment period.

C57BL/6 mice, males and females, 9 weeks old, weighing 18-21 g were used in this study. Mice were randomly divided into three groups, based on the received diet. Each group was divided into 2 sub-groups with an equal number of males and females. Group 1 received in-house purified diet with 32.81% fat (ICO), group 2 received standardized obesity diet with 47% fat (Altromin 1080), for comparison purposes, and group 3 was considered the control group, which received a maintenance diet with 12% fat (Altromin 1081). The purified diets were administrated for a period of 60 days. Chemical composition and nutritional values of the diets are presented in Table 1.

Table 1. Chemical composition and nutritional values for each diet

Diet	ICO	1080 (Altromin)	1081 (Altromin)
Energy (Kcal/kg)	4821,17	4553	3501
Protein (%)	18.47	18	23
Fat (%)	32.81	47	12
Moisture (%)	3.83	5.1	7.8
Fiber (%)	4.08	4.7	4.6

Animals were daily inspected and food consumption was recorded for each group once a week. Weight measurements were performed for each mouse every 14 days during the entire feeding period.

Blood collection from the retro-orbital sinus was performed on days 0, 30 and 60, under general anaesthesia, using a cocktail of acepromazine (5 mg/kg) and ketamine (100 mg/kg). For hematological tests, blood was sampled in EDTA pre-conditioned tubes and IDEXX ProCyte Dx 5 Diff analyzer was used. For biochemistry, blood was sampled in lithium–heparin pre-conditioned tubes and tests were performed on VetTest 8008 Chemistry Analyzer.

On the final day, animals were euthanized using anaesthetic overdose. Liver, kidneys and visceral adipose tissue were collected for necropsy, weighing and histopathological examination. Total body lipid content was determined from the animal carcass by using petroleum ether extraction.

Statistical analysis

All data are shown as mean values for each group. Statistical comparisons were performed using the Microsoft Excel T-test for independent groups and one-way analysis of variance for comparison of means of parameters within the same group. P-values < 0.05 were considered statistically significant.

RESULTS AND DISCUSSIONS

Body Weight

Compared to the maintenance diet (1081) and the standardized obesity diet (1080), mice fed with in-house purified diet (ICO) recorded the greatest weight gain, 48.9% in males and 41.2% in females. The results of the weight measurements during the study are graphically represented in Figure 1.

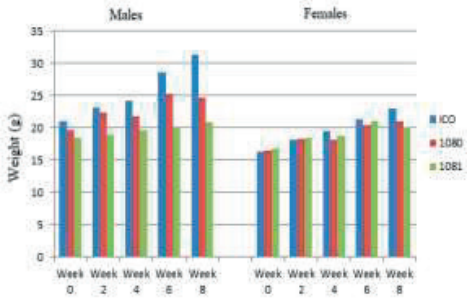

Figure 1. Weight measurement in males and females along 60 days study

Food Consumption

Food intake was determined weekly for each diet and an average consumption/animal/day was calculated for the entire feeding period. Group 1 recorded the highest food consumption, with an average of 2.68g/mouse/day in males and 3.91g/mouse/day in females. The results of the food intake during the entire study are graphically represented in Figure 2.

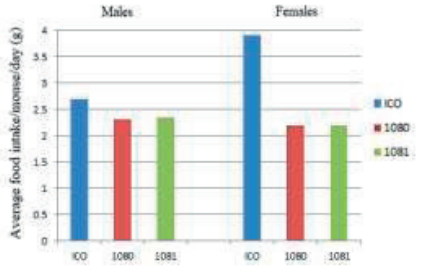

Figure 2. Average food consumption/mouse/day (g) for each diet

Biochemical Results

We focused on the most relevant biochemical parameters in obesity syndrome: cholesterol (CHOL), glucose (GLU), alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

The mean GLU value for ICO diet was significantly increased compared to 1080 and 1081 in both genders, reaching a maximum value on day 30. GLU level slightly decreased by the end of the study for ICO diet but remained higher compared to the other two groups (Figures 3 and 4).

The mean CHOL values were significantly increased for ICO group compared to control group, reaching the maximum value in day 30 in males and maintaining the high value during the entire period of the experiment. In females, CHOL level increased continuously until the end of the study (Figures 5 and 6).

The aminotransferases values were variable and did not entirely reflect the changes induced by the obesity diet compared to control diet. In males, mean ALT value on day 60 was higher in ICO fed group compared to the other 2 groups. In females, ALT value was higher for ICO group compared to 1080 group but lower compared to control group (Figure 7). Mean AST value was similar for ICO fed group and control group on day 60 (Figure 8).

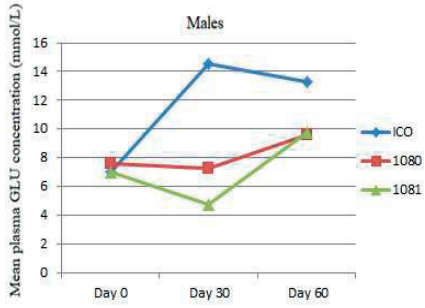

Figure 3. Glucose (GLU) measurements in males during the study

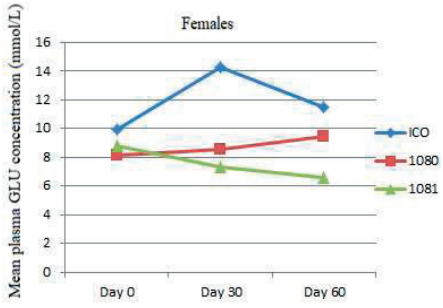

Figure 4. Glucose (GLU) measurements in females during the study

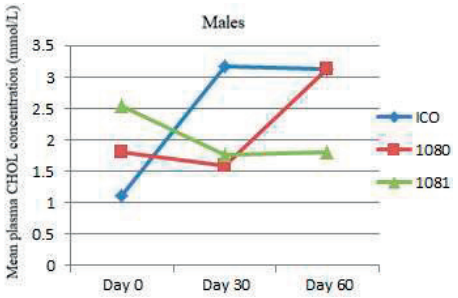

Figure 5. Ccholesterol (CHOL) measurements in males during the study

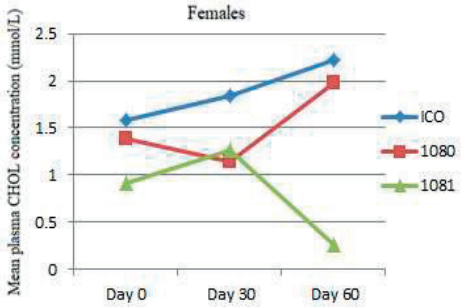

Figure 6. Glucose (GLU) measurements in females during the study

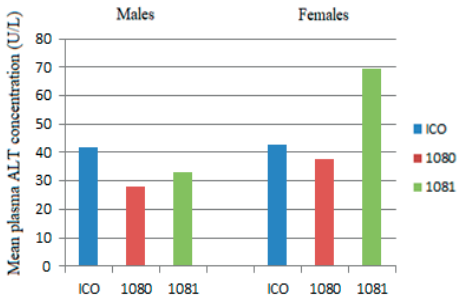

Figure 7. Alanine aminotransferase (ALT) measurements in males/females on day 60

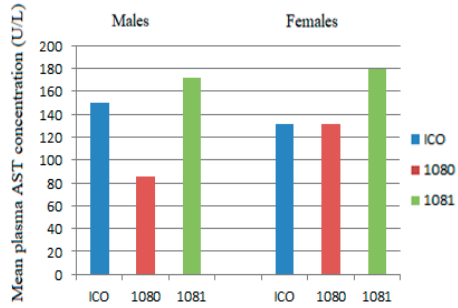

Figure 8. Alanine aminotransferase (AST) measurements in males/females on day 60

Hematological Results

The reference interval for hematology was based on the average values obtained in a previous experiment on C57BL/6 mice fed with a high fat diet, in animals provided by the same animal facility and the use of the same analysis laboratory (Popoiu et al., 2020).

Our study revealed a higher total leukocytes count (WBC) in ICO fed group for males compared to control group. For the same high-fat diet, lymphocyte count (LYM) and platelet count (PLT) were increased in males, compared to control group. In females, these values were similar for all groups. RBC count showed slight variations in the case of animals fed with diets for inducing obesity compared to control group mice.

Other hematologic parameters registred low degree variations and there were not considered relevant for the present study.

The mean hematological parameters on the final day are presented in Table 2.

Table 2. Hematological parameters on day 60 for both genders

Diet	ICO		1080 (Altromin)		1081 (Altromin)	
Parameter	♂	♀	♂	♀	♂	♀
RBC 10^12/L	8.83	8.82	8.84	8.31	8.89	8.6
WBC 10^9/L	3.59	1.57	2.26	1.17	2.06	1.10
LYM 10^9/L	1.77	0.87	1.67	0.49	1.04	0.81
PLT K/µL	735	570	468	509	426	538

Total Body Lipid

Petroleum ether extraction (Soxhlet technique) is a commonly used method of isolating lipids and determining the total body lipid content.

ICO fed group recorded the highest average body lipid/mouse (g) compared to 1080 group and control group. In males, body lipid percentage was significantly increased for ICO diet compared to the other two groups (Table 3).

Table 3. Average body lipid content (g) and total body lipid percentage (%) for both genders

Diet	Body lipid content (g)		Body lipid percentage (%)	
	♂	♀	♂	♀
ICO	2.95	0.98	10.23	4.89
1080	1.22	0.79	5.43	3.98
1081	0.78	0.40	3.66	2.3

Necropsy

On the final day, liver and kidneys were collected for necropsy, weighing and histopathological examination.

Organ examination revealed enlarged liver and dystrophic liver appearance in animals fed with obesity diet compared to control group. No pathological changes were observed in kidneys examination.

Animals in ICO group had increased organs and carcass weight compared to groups 2 and 3 in both genders. Values obtained by weighing the organs and the carcass for each group are presented in Table 4 and graphically represented in Figure 9.

Histopathological results will be reported in a future paper, as data will be available.

Table 4. Average weight merasurments in organs and animal carcass on final day

Diet	Liver weight (g)		Kidneys weight (g)		Carcass weight (g)	
	♂	♀	♂	♀	♂	♀
ICO	1.47	1.23	0.45	0.34	28.69	20.46
1080	1.12	1.14	0.41	0.32	22.8	19.18
1081	1.08	0.95	0.38	0.29	21.36	17.53

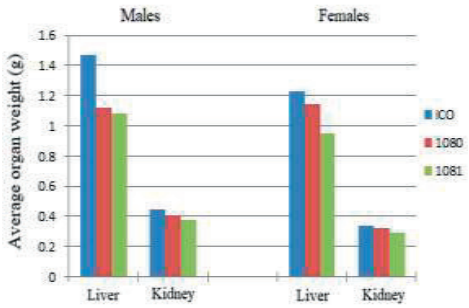

Figure 9. Average weight merasurments in organs and animal carcass on final day

In light of the rapid growth of the obesity rate and concerns over the health effects of obesity, diet-induced obesity animal models are precious resources for the biomedical research . This models allow us to create a controlled environment in order to study and understand the mechanisms involved in obesity development and as well as its effects.

For obesity animal models, unidirectional enriched diets with high fat content, play an important role.

Diverse high energy diets have been used to induce obesity and related metabolic disorders in rodent models, though the dietary mediation has not been absolutely standardized. (Sasidharan et al., 2013). These diets consist of a simple exchange of carbohydrate-derived calories with fat-derived calories and are being compared to a standard chow diet as control.(Lang et al., 2019; Sampey et al., 2011). Herein, we describe a diet-induced obesity model in C57BL/6 mice based on feeding with an in-house purified diet and a standardized obesity diet in comparison to a maintainance diet.

Our results showed significant changes in the body weight, caloric intake, glucose and cholesterol metabolism, inflammation indicators and adipose tissue, for in-house high-fat diet compared to control diet.

In another study, the body weight was increased after 2 weeks of high-fat diet feeding and the hyperglycemia reached the maximum level around the 4th week (Della Vedova et al., 2016). Fraulob et al. (2010) showed that mice fed with high-fat (60%) diet exhibited greatly increased body mass, fat pads and total plasma cholesterol.

Following the administration of the diet for obesity, the body weight of the mice was increased in both genders starting with day 30 of the study, and the glucose values were increased in males and had similar values as the control in females (Popoiu et al., 2020).

Toita et al. (2018) showed in their study that serum ALT levels were similar between high-fat diet and normal diet fed mice. However, a normal serum ALT value may not guarantee absence of hepatic inflammation. In our study, serum levels of hepatic enzymes were higher at the same time with the increase of the body weight only in males.

Total body mass and liver mass following 14 weeks of high fat diet were also significant increased, while kidney mass was not positively correlated to adipose tissue in C57BL/6 mice (Wooten et al., 2016).

Several studies relate inflammation to obesity, an indicator of inflammation being the increase of the number of leukocytes (WBC), as our results also showed. The WBC count was positively correlated with percentage of total body lipid.and fasting plasma leptin

concentration. Evidence suggests that leptin and the leptin receptor are part of a pathway which stimulates hematopoiesis (Wilson et al., 1997).

Panagiotakos et al. (2005) found higher rate of inflammatory markers, including a 17% higher WBC count in participants with a central obesity as compared with those whose body fat was distributed normally.

Jamshidi & Seif (2017) showed a relationship between central and general adiposity and WBC count as an inflammation factor, and higher count of platelets count in obese subjects.

The origin of inflammation during obesity and the underlying mechanisms that explain its occurrence are not yet fully understood, but pro-inflammatory cytokines play a central role, the adipose tissue being the main source of inflammatory cytokines. (Rodríguez et al., 2013).

Lipid infusion and a high-fat diet activate hypothalamic inflammatory signaling pathways, resulting in increased food intake and nutrient storage (Thaler & Schwartz, 2010).

In order to confirm the onset of inflammation at tissue level, there is a need for additional histopathological tests,which will be reported in a future paper, as data will be available.

CONCLUSIONS

The comparative analysis of the animal body weight, blood parameters and body lipid content showed better results in ICO purified diet then standardized obesity diet (1080) in inducind mice obesity. Data obtained in this study has shown that the changes in measured indicators during the experiment can be related to the metabolic syndrome of obesity in mice. We will further correlate this results to the data obtained in histopathologic examination.

ICO purified diet is a reliable diet in inducing mice obesity and can be used for standardizing animal models of metabolic syndrome as new preventive strategies and constant research is needed.

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