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SUMMARY

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MORPHOLOGICAL ASPECTS OF THE THORACIC LIMB SKELETON IN BACTRIAN CAMEL

Cristian BELU, Anca ȘEICARU, Alexandru MANOLESCU, Sorina Andreea MIHAI, Petronela Mihaela ROȘU, Iulian DUMITRESCU, Mădălina Laura SÎRBU

University of Agronomic Sciences and Veterinary Medicine of Bucharest,
Faculty of Veterinary medicine, 105, Spl. Independentei, District 5, Bucharest, Romania

Corresponding author email: cristbelu@yahoo.com

Abstract

The paper describes in detail the particular aspects of the thoracic limb bones in two adult Bactrian camels. The working methods used were bone preparation and analysis of their macroscopic morphological features compared to large local domestic ungulates. The scapula of the camel has an intermediate aspect between those of equines and cattle. The humerus shows a reduction of the humeral neck and a less developed epicondylar crest. No delimitation between the articular surface of the proximal extremity of the radius and the semilunar surface of the ulna on the medial side was observed, which gives a maximum of consolidation of the zeugopodium, increasing the resistance to movement. The trapezius is absent from the second carpal bone row. The main metacarpal consisted of the fusion of metacarpals III and IV. The most important difference from the phalanges of equines and cattle is the lack of the sulcus that crosses in dorso-palmar direction the proximal joint surface.

Key words*: camel, forelimb, humerus, zeugopodium, autopodium.*

INTRODUCTION

Camelids, as compared to other domestic and farm ungulate, have been scientifically studied to a lesser extent. We used as study material the bones from two Bactrian camels, which belong to the Mammalia class, Cetartiodactyla order, Camelidae family, genus Camelus, *Camelus bactrianus* species (Bactrian camel or Asian/Mongolian camel). Recently, scientific research groups have begun to increasingly recognize and raise awareness of the importance of this species, as it is listed as critically endangered on the IUCN Red (International Union for Conservation of Nature). Like equines and cattle in our country, the Bactrian camel is socio-economically integrated into the lives of pastoral people in specific areas, represented by Central and East Asia's rocky deserts (Barat & Khomeiri, 2015; Gebreyohanes & Assen, 2017). It can better survive and easily move in drought conditions, representing a better means of transportation in the desert than other domestic animals. The camel shows different form of the distal extremity of the limbs as compared to cattle and horses, probably due to the anatomo-physiological particulars. These aspects should be known in order to offer the emergency medical care (Bani et al., 2008; Nourinezhad et al., 2014; Ocal, et al., 2004). Specialty literature is rather scarce as concerns studies regarding the osteology of Bactrian camelids. We have considered interesting and relevant the comparison between the thoracic limb bones in *Camelus bactrianus* and similar information existent in the literature for cattle and equines (Predoi et al., 2021).

MATERIALS AND METHODS

This study was carried out in the laboratory of the Anatomy discipline from the Faculty of Veterinary Medicine in Bucharest with the approval of competent authorities in the domain. The study material was represented by the bones obtained from an adult specimen (approximately 20 years old) from the Băneasa Zoo, Bucharest and a camel skeleton existing in the collection of the Comparative Anatomy laboratory of the Faculty of Veterinary Medicine Bucharest.

After skinning the corpse and removing the organs and muscle mass, the bone pieces were processed thermally in water with detergent for 4-6 hours. After completing this process, the

bones were cleaned under tap water and the remnants of soft tissues were manually removed. The bones thus obtained were placed in a 5% hydrogen peroxide (H_2O_2) was followed by washing in water and drying in a well-ventilated space. Following the analysis of the macroscopic morphological features, the pieces of interest were photographed.

RESULTS AND DISCUSSIONS

Forelimb zonoskeleton

In Bactrian camel, **the scapula** (Figure 1) is quite elongated in proximodistal direction. We found a ratio of 2:1 of the two proximo-distal and cranio-caudal axes, measured from the dorsal margin to the glenoid angle, respectively between the cervical and thoracic angles.

The lateral surface is crossed by an approximately rectilinear scapular spine, which increases progressively in height, starting from the dorsal edge, and reaching the maximum at the half of the bone.

Figure 1. Right scapula - lateral view:
1 – supraspinous fossa; 2 – infraspinous fossa; 3 – scapular spina; 4 – acromion; 5 – supraglenoid tuberosity; 6 – suprascapular cartilage; 7 – neck of the scapula; 8 – cervical angle; 9 – thoracic angle

The tuberosity of the scapular spine is elongated (reaching distally close to the origin of the acromion). Starting with its origin, the scapular spine is inclined over the infraspinatus fossa and, at the neck of the scapula, becomes perpendicular to the lateral surface of the bone. The ratio between the supraspinatus and the infraspinatus fossae is 1:2, identical to that of the horse. In the area of the supraspinatus fossa, near the detachment edge of the scapular spine, there is one of the two first-order nutrient foramina of this bone.

The medial surface has a superficial subscapular fossa. Its surface is crossed by several vertical lines for muscle insertion.

The dorsal margin of the scapula is strongly convex (contrary to cattle where it is approximately rectilinear). The suprascapular cartilage was largely ossified in the studied specimens. The cervical margin is extremely thin, almost sharp along its entire length. On its lateral side, in the rectilinear area, there is a narrow, rough band for muscle attachment.

The thoracic margin is very thick. On this edge, at the cranial limit of the neck, the second first-order vascular foramen was identified.

The cervical angle is very fine, while the thoracic angle is thick, tuberous, in the form of an elongated tubercle, oblique in the proximo-distal direction. The joint angle is supported by a long, thick neck. The glenoid cavity showed a cranio-caudal axis twice as long as the transverse one.

Compared to cattle, in which the acromion is widened, in camel it is cylindrical, thick, separated by a deep notch from the neck of the scapula.

The surface intended for insertion of the serrated muscles is extremely small, the lower limit reaching up to the middle of the cranial margin.

No glenoid notch similar to that of equines or cattle was observed, except, a wide notch present on lateral side of the thick and rough lip of the glenoid cavity.

Analyzing the general appearance of the scapula, we appreciate that it is intermediate, between that of equines and cattle.

Stylopodium

The **humerus** (Figure 2), although exhibiting a massive, thick body, due to the reduced ratio between its total length and width at the half of the bone, renders it a relatively slender appearance. As in equines, the humerus has three tubers: greater, lesser and intermediate, approximately equal. In camel, the tendinous groove located laterally to intermediate tubercle is much wider and shallower than the medial one. The insertion surface for the infraspinatus

muscle is circular (similar to cattle). The elongated, voluminous deltoid tuberosity is slightly drawn caudally.

As concerns the humerus, the essential differential element is the reduction of the neck that supports the head, which leads to an increase of the angle between the axis that passes through the center of the articular head and the axis of the shaft. Dissimilar to cattle and horses, the brachial groove is not well defined; the epicondyle crest was absent.

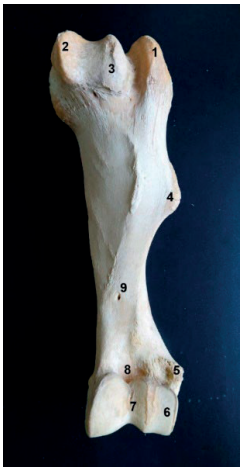

Figure 2. Left humerus - cranial view:
1 – greater cranial tubercle; 2 – lesser cranial tubercle; 3 – intermediate tubercle; 4 – deltoid crest; 5 – lateral epicondyle; 6 – humeral condyle; 7 – humeral trochlea; 8 – coronoid fossa; 9 – main vascular foramen

Zeugopodium

The bones of the zeugopodium are completely fused excepting the proximal and distal arches (interosseous spaces), which form a single piece, called in the literature radioulnar bone (Gupta et al., 2015), on which the boundaries of the two bones can be observed only by careful examination. Unlike cattle and horses, the Bactrian camel shows no diarthrodial joint surfaces. The oblique medial distal groove, characteristic for cattle and horses, could not be identified (Figure 3).

The proximal end of the **radius** was slightly widened transversely, forming two glenoid cavities, of approximately same dimensions, but with different appearance. The elevation that separates them is the highest eminence of this surface. The lateral surface is divided into two areas in continuity. Laterally, a cranio-caudally elongated cavity is observed. Its cranial half continues in the medial direction with a smaller articular area, which extends above the mentioned eminence with the medial cavity. The lateral articular surface, formed by the two subdivisions, deepens in the central part, the place where the lateral lip of the humeral trochlea enters.

Figure 3. Radius and ulna of the left limb - lateral view:
1 – olecranon; 2 – semilunar notch; 3 – body of the radius; 4 – proximal radio-ulnar arch; 5 – distal radioulnar arch; 6 – ulnar styloid process; 7 – body of the ulna

The medial articular surface continues caudally with the articular surface of the semilunar notch. In the middle of the dorsal surface of the entire joint surface is a rough area, the synovial fovea, relatively large. On the anterior margin of the articular surface protrudes a strong coracoid process, from which start two grooves, one medial and one lateral, the first being more inclined than the second. The connection between the olecranon and the proximal end of the radius is very strong. There are no diarthrodial joint surfaces present in domestic ungulates. We consider these elements a particularity that gives maximum strength to the zeugopodium, increasing the resistance in movement. Immediately below the anterior edge of the proximal surface, on the corresponding side of the proximal extremity of the shaft, there is a rough area, well defined, relatively large, corresponding to the biceps

tuberosity. The lateral tuberosity of the radius, delimited by a notch at an angle of 90˚ from the lateral articular surface of the proximal extremity, is very prominent and rough, being much more elevated than the bicipital one.
The distal end of the radius, slightly more voluminous than the proximal one, has a widened articular surface, with a concave-convex appearance in the cranio-caudal direction. More precisely, in the anterior part, we identified three true cavities, continued caudally with three condyles, their direction being perpendicular to the axis of the bone, as in equines. By careful examination of this extremity, we observed that the lateral condyle belongs ontogenetically to the ulna. There is a rough area, placed at the border between the medial and median articular formations of the distal extremity, which represents a synovial fossa.
On the anterior part of the distal extremity, there are two wide grooves, separated by a protrusion, for the common digital extensor and carpo-radial extensor muscles. The presence of three digital fossae was noticed, a wider medial one, a rough central one and a lateral one, slightly oblique. The body of the radius has the longitudinal axis slightly twisted: in the upper third, it is slightly curved, the convexity being directed medially, while in the middle and distal third, it is rectilinear. The medial margin of the radius shaft begins with a shallow, rounded part and then, after the first quarter, continues with a prominent ridge that runs caudo-distally in the second quarter, vertically from the middle of the bone to the distal quarter, where it fades, the edge remaining rounded and rough. The caudal surface of the radius forms a synostosis with the cranial surface of the ulna. Below the proximal extremity of the radius there is a canal about 5 cm long that replaces the proximal radio-ulnar arch. The distal radio-ulnar arch shows two narrow spaces, with a maximum length of 11-13 mm each, that separate the distal end of the ulna from that of the radius.
The **ulna** has a short olecranon as compared to the total length of the bone. The tuberosity of the olecranon is reduced and, when viewed caudally, is rounded in the proximal part and sharp in the distal part. It is slightly beveled on the rostral side, with a transverse notch similar to cattle, but much wider. The apex of the olecranon, slightly prominent, is rostrally directed and continued distally with articular surfaces. The medial surface is in continuity with the articular surface of the proximal extremity of the radius. The lateral one is interrupted by the synovial fossa of the humero-radial joint.

Basipodium
There are seven carpal bones, located in two rows, proximal and distal, as in equines, the trapezius being absent from the distal row (Figure 4).
The **accessory bone** *(Os carpi accessorium* syn. *Os pisiforme)* presents, as in equines, two articular surfaces, one for articulation with the ulnar condyle and another for articulation with the pyramidal. The articular surfaces of the accessory bone are concave and separated only by a short ridge. The caudal part of the bone progressively narrows to form a rounded tuber, slightly directed proximal, making the dorsal edge of the pisiform to be concave and shorter than the distal one, which is thick and convex. The groove of the metacarpal tendon of the carpoulnar extensor muscle was not identified.
The **ulnar bone** *(Os carpi ulnare* syn. *Os triquetrum)* does not resemble that of equines or cattle. It is an approximately parallelepiped bone, concave convex in the dorso-palmar direction. Dorsally, it articulates with the ulnar condyle and, partially, with the distal articular surface of the radius. The lateral dorsal and palmar surfaces are rough. Only the medial surface exhibits two articular surfaces, one proximal and one distal, located towards the dorsal margin, intended for articulation with the two congruent surfaces of the intermediate bone (lunate).

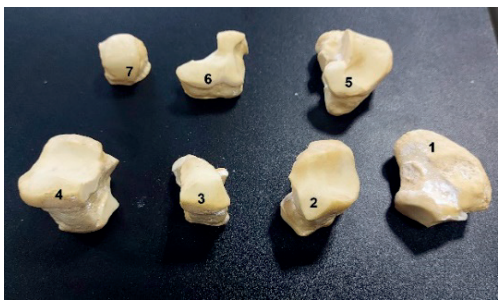

Figure 4. Carpal bones of the left limb - dorsoproximal view: 1 – accessory bone; 2 – ulnar bone; 3 – intermediate bone; 4 – radial bone; 5 – 4th carpal bone; 6 – 3rd carpal bone; 7 – second carpal bone

The **intermediate bone** *(Os carpi intermedium* syn. *Os lunatum)* is taller than wide, being smaller than the ulnar and radial bones. The distal articular surface of the intermediate bone consists of two elongated articular areas, separated by an elevation in the median plane of the bone. The dorsal, non-articular surface is high and slightly narrowed in the middle, as in cattle. The palmar surface, non-articular, shows a more developed tubercle, directed towards the median plane. This tuber has a joint surface for the scaphoid bone.

The **radial bone** *(Os carpi radiale* syn. *Os scaphoideum)* is the largest bone in the proximal row, with a parallelepiped appearance. It has the distal articular surface formed by two cavities, one superficial, anterior, and one deep, almost circular (intended for the convexity of the trapezoid). They are separated by an evident transverse eminence, perpendicular to the one described in the lunatum. Between the dorsal and medial surfaces, in the upper angle, a tubercle widened in transverse direction was observed.

The fourth carpal bone *(Os carpale quatrum IV* syn. *Os hamatum)* differs from that of bovines and horses, being flattened proximo-distally. Thus, the proximal and distal joint surfaces are very large. The proximal one is intended for articulation with the ulnar bone, as well as with the lunate bone. The articular surface of the fourth carpal bone with the ulnar bone is concave convex in dorso-palmar direction. The articular surface intended for the lunatum is subdivided into a dorsal area, oriented proximally and a palmar area that becomes almost vertical. On the palmar side is a tuber with a sharp distally directed prominence. The relatively regular dorsal non-articular surface (rectangular) is widened transversely and continues through a rounded angle with the lateral one. Due to the narrowing of the bone in palmar direction, we noticed a third surface of the outline, medio-palmar, with two articular surfaces, one dorsal and one palmar, separated by a wide and rough notch.

The **third carpal bone** *(Os carpale tertium III* syn. *Os capitatum)* is flattened and reduced, showing dorsally a triangular outline due to the presence of the palmar tubercle. It articulates with both the lunate and radial bones. The dorsal surface of the 3rd carpal is non articular, and the lateral one is congruent with the medio-palmar surface of the carpal bone. The medial surface has two central joints, each having a semicircular shape. These two surfaces are intended for the second carpal bone.

The **second carpal bone** *(Os carpale secundum II syn. Os trapezoideum)* is almost cubic shaped. The proximal articular surface is a true articular head, strongly convex in all directions.

Metapodium

The **main metacarpus** (Mc.) is formed by fusion of Mc. III and IV. The proximal end of the main metacarpus has a slightly widened articular surface represented by three areas: an approximately triangular, planiform, lateral one, intended for the carpal bone, separated by a prominence from the areas for the 3rd and 2nd carpal bones; between the last two areas there is a second, much smaller eminence. In the central part, caudal to the main prominence, is a deep synovial fossa.

Figure 5. Metacarpal bone:
1 – proximomedial tuberosity of the metacarpal bone; 2 – metacarpal III; 3 – metacarpal IV; 4 – dorsal longitudinal groove; 5 – distal articular surfaces

On the medio-proximal side, an elongated tuberosity occupies the proximal extremity of Mc. III and the medial extremity of Mc. IV.

The shaft is thick in the proximal and middle third and flattened in the distal third (Figure 5). The caudal surface of the shaft describes a wide convexity from bottom to top and is slightly

concave in transverse direction. The concavity is formed by two high and rough ridges, which limit this surface laterally and medially. On the dorsal surface of the shaft lies a longitudinal groove. The distal extremities of the two metacarpals that participate in the formation of the intermetacarpal groove are slightly divergent. An interesting feature, as compared to cattle, is the conformation of the joint surfaces of each participating bone. Thus, the condylar surfaces, separated by the eminences characteristic to ruminants, are placed caudally.

Acropodium

Phalanges of camels have a quite different appearance than those of bovines and horses. The most important difference from equines and cattle **phalanges** is the lack of the groove that crosses the proximal articular dorso-palmar surface, this being replaced by a shallow glenoid cavity that has contact only with the anterior, convex part of the distal articular surface of the metapodium bones (Figure 6).

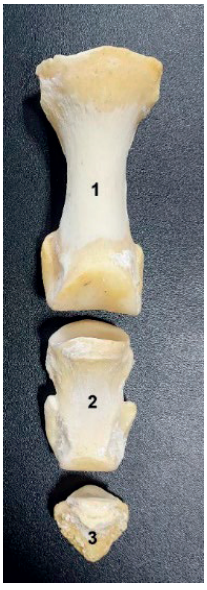

Figure 6. Phalanges of the anterior right limb:
1 – proximal; 2 – middle; 3 – distal

The proximal phalanx has a very long body, being quite symmetrical in respect to its longitudinal axis as compared to cattle. Its extremities are voluminous. The second phalanx is more flattened dorso-palmar than in cattle, and longer than in horse, and highly symmetrical in respect to its longitudinal axis. The distal phalanx is very short, with a pyramidal appearance. At the base of the distal phalanx, three angles slightly spaced from the joint surface, were noticed. One dorsal, corresponding to the pyramidal eminence and two lateral.

CONCLUSIONS

Compared to equines and cattle, the general appearance of camel scapula is closer to that of equines. The presence of the acromion makes a clear difference. The humerus as a whole can be easily confused with that of the horse. We consider that the reduction of the humeral neck and the reduced crest of the epicondyle in camels are specific elements of differentiation. The bones of the forearm, the radius and the ulna, are fused along the entire length of their shaft. The suture line is not visible. The radius has two glenoid cavities at the proximal extremity. The appearance of the distal extremity, relates much to the radius of horse. There are seven carpal bones, resembling those of equines. Exceptions are the ulnar bone (more like the lunatum bone) and the 4th carpal bone (with an intermediate shape between equines and the bovines).

Although we did not have the opportunity to perform functional studies, we can anticipate that in camel, the structure of the mid-carpal joint contributes to the mobility of the joint complex, much more than in bovines and equines. The extension of the convex surfaces on the palmar edge of the third and second carpal bones, supports this statement.

As a general conclusion, we appreciate that the morphology of the skeleton of the thoracic limb in the Bactrian camel presents common elements with both equines and cattle, but also specific elements that allow the differentiation of bones.

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STUDY REGARDING THE ADAPTATION STRESS IMPACT ON THE NUTRITIONAL BEHAVIOR IN DOMESTIC CATS

Iuliana CODREANU[1], Simona NICOLAE[1], Marian GHIȚĂ[1], Radu Marian ANGHEL[2]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest,
Faculty of Veterinary Medicine, 105 Spl Independentei, District 5, Bucharest, Romania
[2]CatShop Store & Services SRL, Bucharest, Romania

Corresponding author email: iulianacod@yahoo.com

Abstract

The adaptation stress activates the hypothalamic-pituitary-adrenal axis, which can lead to the modification of some behavioral manifestations. This paper highlights the adaptation stress effect on nutritional behavior, in direct correlation with plasma cortisol levels' changes as main physiological parameter involved in stress and adaptation mechanisms. The study was carried out on a group of 10 clinically healthy cats, housed in identical environmental conditions, each individual's behavior being studied performing ethograms on days 1, 5, 9 and 10 of accommodation, simultaneously with the evaluation of the serum cortisol levels. On the first day, significant changes in nutritional behavior were observed, resulting in the absence of watering in all individuals and the absence of feeding in 50%. Towards the end of the study (days 5 and 9) no significant variations of the nutritional behavior were observed anymore, data associated with the accommodation to the new living environment. The serological levels of cortisol variations were in accordance with the behavioral changes, registering significantly increased values ($p<0.05$) in 50% of the studied individuals, values that gradually decreased towards the end of the study period.

Key words*: nutritional behavior, adaptation stress, domestic cats.*

INTRODUCTION

Stress is an important phenomenon and its effects on domestic cats are being studied in detail today as it indicates a syndrome resulting from exposure of an individual to the influences of an unfavorable environment, hostile to its welfare.

The main object of study of this paper refers to the association of stress with the changes produced by it, both physiologically and behaviorally that have a direct impact on the nutritional behavior in domestic cats.

The stress factor can be emotional, but it has the effect of activating the hypothalamic-pituitary-adrenal axis, which determines a series of physiological consequences corelated with the increase secretion of certain hormones, especially cortisol (Amat, M. et al., 2016).

The central component of the stress response is the activation of the autonomic sympathetic nervous system, which causes the release of catecholamines from the medullary adrenal glands into the bloodstream (Beaver, B.V., 1976). Corticotropin-releasing factor (CRF) produced by the hypothalamus stimulates the pituitary gland to release many other hormones, such as ADH, oxytocin, prolactin, growth hormone and adrenocorticotropic hormone (ACTH). ACTH stimulates adrenal cortex function, resulting in the release into circulation of an increased amount of cortisol.

The complete absence of stressors is impossible, and a certain level of stress is even necessary for the cat to develop a malleability of neuroendocrine and behavioral responses. (Codreanu, I. et al., 2021; Simion, V. et al., 2019)

MATERIALS AND METHODS

The study was conducted on a sample of 10 cats aged 1-10 years, clinically healthy, vaccinated and dewormed according to age and without a medical history likely to generate an exaggerated response to the stress factors applied inevitably during this study.

The cats were accommodated at the Catshop Hotel, Bucharest, with the consent of the owners, a hotel dedicated to cats where 24/24 hour video recordings were made with the help

of surveillance cameras installed in each accommodation room.
The blood samples were collected in test tubes containing blood activator cloth used to extract serum for serum cortisol dosing, at set time intervals, respectively on days 1, 5 and 10.
The cats studied benefited from identical accommodation conditions, so there was no differentiating factor in terms of the organization or size of the rooms. The room temperature was maintained between 21°C - 23°C, and the humidity was around an average of 62%.
With the help of the recordings obtained through the surveillance cameras, individual daily ethograms were made, in which the exact recording of the nutritional behavior manifestations were followed.
The feeding of the cats was done by administering the types of food recommended by the owner. The amount of food offered was in line with the recommendations proposed by the manufacturer and ranged from 50 to 70 g of dry food per day divided into two meals, in the morning between 10:00 and 11:00 and in the evening between 19:00 and 20:00.
The watering was achieved by administering plain water Aqua Carpatica, at room temperature, in an amount of 70-100 ml, refreshed twice daily.

RESULTS AND DISCUSSIONS

Regarding the feeding behavior, the results obtained are presented in Figures 1-4 and synthetic Table 1.

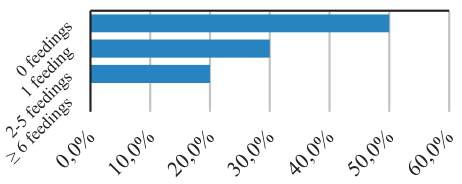

Figure 1. The feeding behavior on 1st day of the study

The feeding behavior - Day 1 results:
- The absence of feeding: 5 individuals (50%)
- One feeding: 3 individuals (30%)
- 2 - 5 feedings: 2 individuals (20%)
- ≥ 6 feedings: 0 individuals (0%)

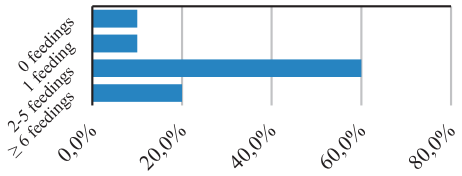

Figure 2. The feeding behavior on 2nd day of the study

The feeding behavior - Day 2 results:
- The absence of feeding: 1 individual (10%)
- One feeding: 1 individual (10%)
- 2 - 5 feedings: 6 individuals (60%)
- ≥ 6 feedings: 2 individuals (20%)

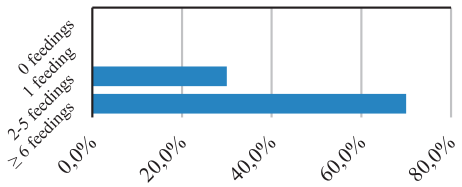

Figure 3. The feeding behavior on 5th day of the study

The feeding behavior - Day 5 results:
- The absence of feeding: 0 individuals (0%)
- One feeding: 0 individuals (0%)
- 2 - 5 feedings: 3 individuals (30%)
- ≥ 6 feedings: 7 individuals (70%)

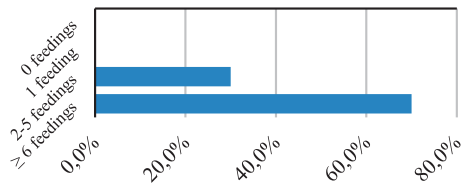

Figure 4. The feeding behavior on 9th day of the study

The feeding behavior - Day 9 results:
- The absence of feeding: 0 individuals (0%)
- One feeding: 0 individuals (0%)
- 2 - 5 feedings: 3 individuals (30%)
- ≥ 6 feedings: 7 individuals (70%)

Table 1. Synthetic table with the results regarding the feeding behavior of the studied group

No. of feedings / Day	0 feedings (%)	1 feeding (%)	2-5 feedings (%)	≥ 6 feedings (%)
Day 1	50 ***	30	20*	0
Day 2	10**	10	60	20
Day 5	0	0	30*	70***
Day 9	0	0	30*	70***

** p>0.01 – not significant differences*
*** p<0.05 - significant differences*
**** p<0.01 - distinctly significant differences*

Following the analysis of feeding ethograms in the studied group, it is observed that on the first day (day of accommodation), 50% of the animals did feed, representing statistically significant differences (*p <0.01*), compared to the other days on which the ethograms were performed. On the second day, the number of individuals that did not accept food decreased significantly to 10% (*p <0.05*), as on days 5 and 9 of the study, all individuals in the studied group fed, most of them more than 6 times.
Regarding the drinking behavior, the results obtained are presented in Figures 5-8 and synthetic Table 2.

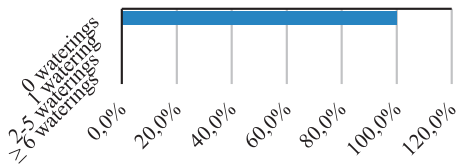

Figure 5. The drinking behavior on 1st day of the study

The drinking behavior - Day 1 results:
- The absence of watering: 10 individuals (100%)
- One watering: 0 individuals (0%)
- 2 - 5 waterings: 6 individuals (0%)
- ≥ 6 waterings: 2 individuals (0%)

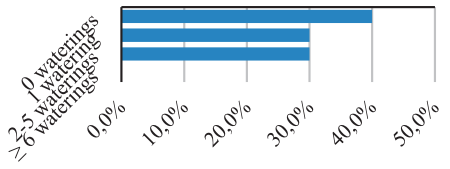

Figure 6. The drinking behavior on 2nd day of the study

The drinking behavior - Day 2 results:
- The absence of watering: 4 individuals (40%)
- One watering: 3 individuals (30%)
- 2 - 5 waterings: 3 individuals (30%)
- ≥ 6 waterings: 0 individuals (0%)

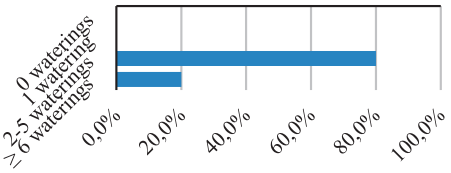

Figure 7. The drinking behavior on 5th day of the study

The drinking behavior - Day 5 results:
- The absence of watering: 0 individuals (0%)
- One watering: 0 individuals (0%)
- 2 - 5 waterings: 8 individuals (80%)
- ≥ 6 waterings: 2 individuals (20%)

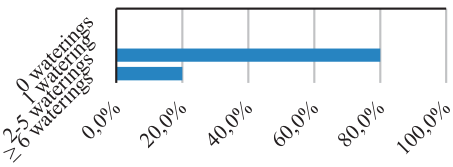

Figure 8. The drinking behavior on 9th day of the study

The drinking behavior - Day 9 results:
- The absence of watering: 0 individuals (0%)
- One watering: 0 individuals (0%)
- 2 - 5 waterings: 8 individuals (80%)
- ≥ 6 waterings: 2 individuals (20%)

Table 2. Synthetic table with the results regarding the drinking behavior of the studied group

No. of waterings / Day	0 waterings (%)	1 watering (%)	2-5 waterings (%)	≥ 6 waterings (%)
Day 1	100***	0	0	0
Day 2	40**	30	30	0
Day 5	0	0	80***	20*
Day 9	0	0	80***	20*

** p>0.01 – not significant differences*
*** p<0.05 - significant differences*
**** p<0.01 - distinctly significant differences*

Following the analysis of the ethograms in the studied group, it is observed that on the first day (day of accommodation), watering could not be observed in any of the subjects (0%) representing statistically significant differences (*p <0.01*), compared to the other days on which the ethograms were performed. On the second day, the number of individuals that did not accept water decreased significantly to 40% (*p<0.05*), as on days 5 and 9 of the study, all individuals in the studied group presented drinking behavior manifestations, most of them 2-5 times a day. As concerning the drinking behavior, water consumption is directly correlated with age, weight, activity level, but also with the type and amount of food that the cat consumes. (Codreanu, I., 2016; Stelow, E., 2020)

The serum cortisol levels were determined in 3 moments of the experiment: 1st day, 5th day and 10th day. The variations of the cortisol level in the studied group are presented in the synthetic graph bellow (Figure 9).

Cortisol dosing demonstrates significant changes in physiological values ranging from 1.5 to 5 µg/dL of blood serum. The results obtained can be summarized as follows: 5 individuals (50%) from the studied group showed serum cortisol values within physiological limits during the 3 dosing moments, while 5 individuals (50%) from the studied group showed significantly increased values of serum cortisol levels, predominantly on days 1 and 5.

These results demonstrate the validity of H. Selye's theory regarding the involvement of the adrenal cortex in stress response, through the hypersecretion of glucocorticoid hormones - mainly cortisol. (Tan, S. Y., & Yip, A., 2018)

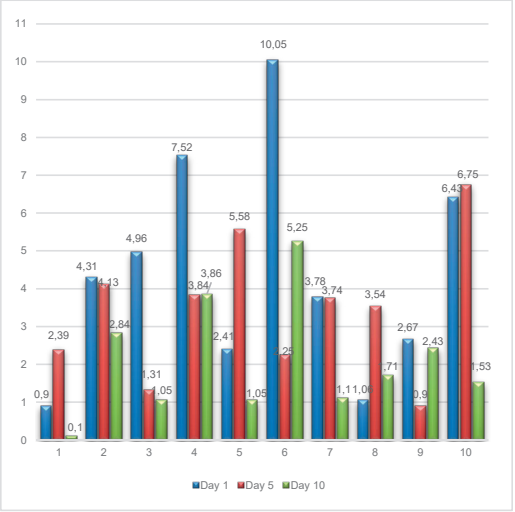

Figure 9. Dynamics of the cortisol variations in the studied group on day 1, 5 and 10 of the study (µg/dL)

CONCLUSIONS

During the experiment the stress caused by the environmental changes was manifested by the absence of feeding and drinking behaviors manifestations in the first days of the study and by increased values of serum cortisol, exceeding the physiological values in 50% of the individuals. Towards the end of the experiment, as the adaptation phenomenon occurred, the feeding and drinking behaviors were normal in all studied individuals, as well as the cortisol values dropped to normal physiological values, indicating a reduction of the stress effects on both behavioral and physiological parameters.

The experiment also highlights the importance of maintaining a suitable habitat, free of stressors, for domestic cats, this species being particularly responsive to stress.

By understanding the harmful effects of stress on cats, we can find more effective ways to prevent them. An essential component of preventive medicine is the interpretation of harmful stress and the clear expression of ways in which it can be reduced in intensity.

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MICROSCOPIC STUDIES OF THE CARDIOVASCULAR SYSTEM IN SHEEP

Valerica DĂNACU[1], Petronela Mihaela ROȘU[1], Carmen IONIȚĂ[1], Viorel DĂNACU[2], Bogdan GEORGESCU[1],

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, 105 Spl. Independentei, District 5, Bucharest, Romania
[2]ANSVSA, Bucharest, Romania

Corresponding author email: valericadanacu@yahoo.com

Abstract

This study was performed to determine the histostructural features of the heart wall and blood vessels in sheep. For this study, organs from normally developed, clinically healthy sheep of different ages were used. The samples were processed according to the usual histological techniques and stained by the methods Hematoxylin eosin, orcein and Mallory. Histological examination revealed that in the structure of the endocardium was observed the endothelium represented by a simple squamous epithelium, the endothelial cells have an elongated shape and arranged linearly. The subendothelial layer is represented by lax connective tissue and the subendocardial layer consists of well-represented lax connective tissue that continues with the interstitial connective tissue of the heart. The structure of the myocardium shows the contractile myocardium with a different thickness in the two ventricles and the excitoconductive tissue. The structure of the pericardium shows the visceral sheet represented by the serous pericardium and the parietal sheet represented by the fibrous pericardium. The circulatory system is made up of: macrovases made up of large caliber arterioles, elastic, muscular arteries, muscular veins and microvessels.

Key words*: endocardium, myocardium, epicardium, macrovessel, microvessel.*

INTRODUCTION

The function of the heart relies on the action of contractile cells, known as cardiomyocytes, specialized conducting cells that facilitate coordinating rhythmic contraction, extracellular matrix that provide mechanical support, as well as veins, arteries, and microvasculature to supply blood to the working muscle. Importantly, the heart vascular network, known as the coronary circulation, maintains perfusion of myocardial tissue with hemodynamics that are out-of-phase to the systemic circulation (Goodwill A.G., 2017).

Cardiac myocytes occupy approximately 75% of normal myocardial tissue volume, but they account for only 30-40% of cell numbers (Anderson, 2009). The majority of the remaining cells are non-myocytes, predominantly fibroblasts. Other cell types, such as endothelial or vascular smooth muscle cells, represent comparatively small populations (Lunkenheimer, 2006).

Histological examination, however, shows that the only muscular unit to be found within the myocardial walls is the cardiac myocyte itself. Our own investigations show that, rather than forming a continuous band, or being arranged as sheets, the myocytes are aggregated together as a three-dimensional mesh within a supporting matrix of fibrous tissue. Within the mesh of aggregated myocytes, it is then possible to recognize two populations, depending on the orientations of their long axes. The first population is aligned with the long axis of the aggregated myocytes tangential to the epicardial and endocardial borders, albeit with marked variation in the angulation relative to the ventricular equator. Correlation with measurements taken using force probes shows that these myocytes produce the major unloading of the blood during ventricular systole. The second population is aligned at angles of up to 40 degrees from the epicardium toward the endocardium (Anderson, 2009).

Concepts for ventricular function tend to assume that the majority of the myocardial cells are aligned with their long axes parallel to the epicardial ventricular surface. We aimed to validate the existence of aggregates of

myocardial cells orientated with their long axis intruding obliquely between the ventricular epicardial and endocardial surfaces and to quantitate their amount and angulation (Lunkenheimer, 2006).
Many animal studies have been performed to observe the formation sequences mature functional myocardocytes. In chicken embryos myocardocyte contractions may be observed only 36 hours after fertilization (Tokuyasu and Maher, 1987), and blood flow through the heart it begins 2 days after conception (Sissman, 1970). Myofibrils still uncompacted begin be visible in myocardocytes about 30 hours post-fertilization.
However, not much is known about the actual stage of sarcomere formation in human heart in vivo. At the age of 3-4 weeks, the human heart begins to contract, but does not he really knows to what extent sarcomeres are structurally developed (Mercola et al., 2011).
Although myocardial architecture has been investigated extensively, as yet no evidence exists for the anatomic segregation of discrete myocardial pathways (Smerup et al., 2009).
The visualization of the size, shape, and alignment of the myocytic arrays at any side of the ventricular wall is determined by the radius of the knives used, the range of helical angles subtended by the alignment of the myocytes throughout the thickness of the wall, and their angulation relative to the epicardial surface (Lunkenheimer, 2006).

MATERIALS AND METHODS

The research was conducted on permanent histological preparations of the heart and blood vessels of sheep clinically healthy. Histological specimens were prepared as follows: 10% formalin fixing, paraffin embedding and sectioning inclusion microtome. Large sections were stained on slides after staining following methods: hematoxylin eosin, orcein and Mallory (Bancroft, J.D. and A. Stevens, 1986).
By following successively and accordingly to the experimental plan, rigorous morphological studies were conducted using optical microscopy photomicrographs.
Histological preparations obtained were examined by light microscopy shooting device equipped with making photomicrographs.

RESULTS AND DISCUSSIONS

In the examined histological preparations it is observed as in sheep the endocardium of the left atrium is thicker and more opaque than that of the right atrium, and the collagen fibers are more numerous in the endocardium of the left atrium. As a histostructural aspect, the left ventricular endocardium appears thicker than the right ventricular endocardium.
The endothelium formed by a simple squamous epithelium arranged in a continuous layer on a thin basal membrane is observed. The subendothelial layer represented by a blade of loose connective tissue is observed. In its deep part, the collagen fibers appear more often, are arranged parallel to the surface of the endocardium and the elastic fibers form networks.
The level of the atria, the endocardium contains a continuous endothelium and a layer of loose connective tissue (Figure 1).

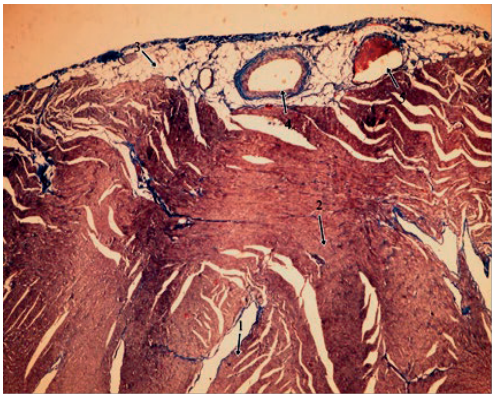

Figure 1. Heart, Mallory stain Overview, 4x objectiv:
1. Endocardium; 2. Myocardium; 3. Epicardium;
4. Artery; 5. Vein

The connective tissue is rich in elastic fibers, arterioles, veins, capillaries, nerve threads, adipocytes and elements of the excito-conductive system.
The right ventricular endocardium is thinner than the left ventricular endocardium.
In the structure of the myocardium, the myocardial fibers are organized in plexiform networks and are joined by means of intercalary discs or Eherth scalariform stria.
Their ends are often branched to form true anastomotic networks.

The areas of intercellular junctions appear in the form of transverse lines, with a winding trajectory, in a zig-zag pattern.
They are short and intersect cardiomyocytes at irregular intervals. The subendocardial space contains fenestrated elastic membranes and bundles of smooth non-muscular fibers, especially in the interventricular septum. At the level of the interventricular septum, blood vessels, nerves and elements of the excitoconductor system are observed.
In the wall of the right atrium, the sinoatrial node formed by a network of nodal cells is observed, in which the P cells are arranged centrally, and the T cells are arranged peripherally (Figure 2). Abundant loose connective tissue, blood vessels, nerve fibers, and nervous microglia are seen between the cells.
The cardiomyocytes have a cylindrical, elongated shape, the nucleus is located centrally (Figure 3).

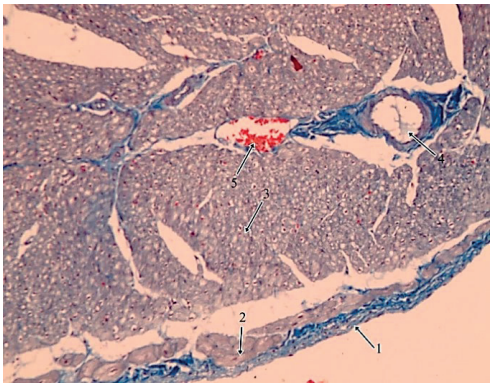

Figure 2. Heart, Mallory stain, 20x Objective:
1. Endocardium; 2. Nodal cells; 3. Myocardium;
4. Artery; 5. Vein

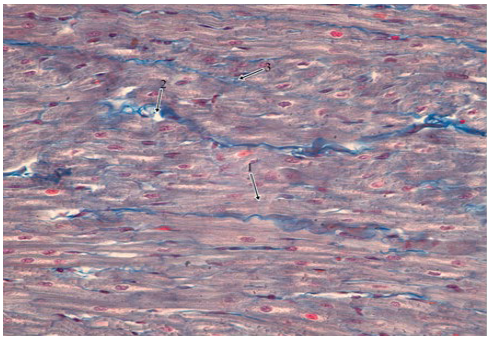

Figure 3. Heart, Mallory stain 40x Objective:
1. Cardiac muscle fibers; 2. Connective tissue;
3. Scalariform stria

Ventricular cardiomyocytes are larger than atrial cardiomyocytes. The working cardiomyocytes enters the structure of the atrial and ventricular myocardium. It is observed that they have morphological characteristics that clearly differentiate them from conducting cells.
The Purkinje anastomotic network is observed in the ventricular myocardium. These cells are smaller in size and are stacked with no orientation.

In the examined histological preparations it is observed that the contractile myocardium has a different thickness in the two ventricles (Figures 4, 5).
In the examined sections it is observed that the left ventricle has a much thicker wall than the right ventricle and the atria have a much thinner wall than the ventricular wall.

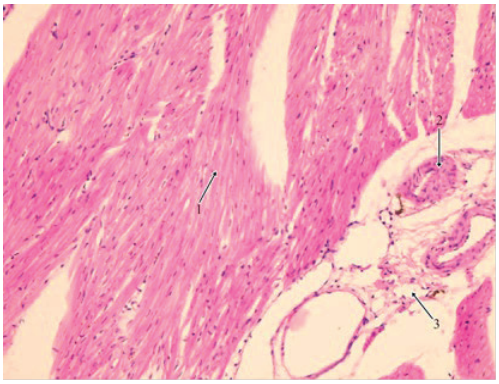

Figure 4. Heart, Hematoxylin and Eosin stain, 20x
Objective: 1. Cardiac muscle fibers; 2. Blood vessels;
3. Connective tissue

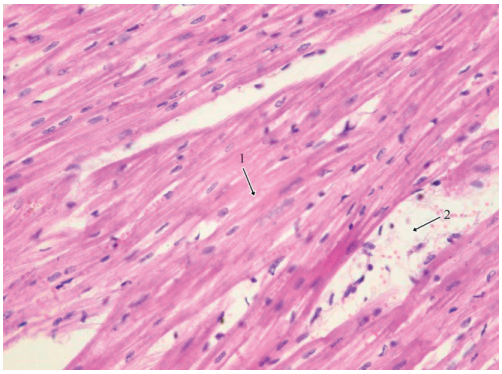

Figure 5. Heart, Hematoxylin and Eosin stain,
40x Objective: 1. Longitudinal section of muscle fibers;
2. Connective tissue

Mesothelium is represented by a simple, continuous squamous epithelium that covers the entire pericardium; The basal membrane of the mesothelium and the submesothelial connective tissue formed by a dense connective tissue, rich in elastic collagen fibers that form a continuous connective membrane, relatively thin in the superficial area, are observed.
In the deep part, the epicardial connective tissue has a looser structure and has numerous adipocytes (Figure 6).

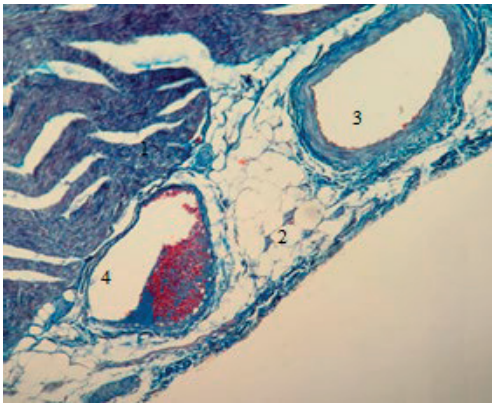

Figure 6. Heart, Mallory stain Overview, 20x Objective:
1. Myocardium; 2. Epicardium; 3. Artery; 4. Vein

It continues with the interstitial connective tissue of the myocardium.
Its structure shows the coronary arteries and their main branches, heart veins, lymphatic vessels and nerve fibers.
In the histological preparations examined from the arteries of elastic type it is observed that the endothelium is represented by a simple squamous epithelium, continuous, placed on a continuous basal membrane (Figure 7). Endothelial cells junction tightly with each other, but also with the basal membrane. They have numerous digitiform extensions.
The endothelium is a special type of epithelium with a semipermeable barrier function that separates the two compartments of the internal environment, blood plasma and interstitial fluid.
The endothelium has a high degree of specialization in mediating and actively monitoring the bidirectional exchange of small molecules, also having the role of restricting the transport of certain macromolecules.

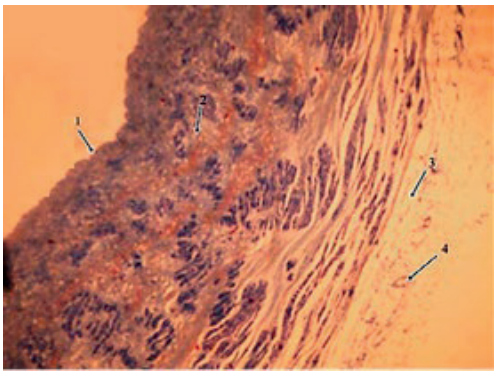

Figure 7. Aorta, Mallory stain Overview, 4x objective:
1. Intima; 2. Media; 3. Adventitia; 4. Vasa vasorum

The endarterium is located below the basal membrane and is made up of connective tissue rich in collagen, elastic and reticulin fibers. Among the fibers is connective tissue rich in glycoproteins and rare connective cells, especially fibroblasts.
The endarterium is separated from the tunica media by an internal elastic constraint. The internal elastic lamina is thin and difficult to distinguish from the first elastic blades of the tunica media.
Tunica media is the most developed structure. It consists of concentrically arranged fenestrated elastic slats, parallel, joined together by thin elastic fibers, few collagen fibers, smooth muscle fibers and sparse fibroblasts (Figure 8).

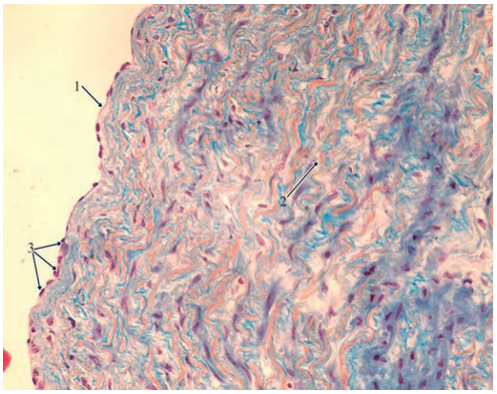

Figure 8. Aorta, Mallory stain, 40x Objective:
1. Intima; 2. Media; 3. Endothelial cell nuclei

The richness of lamellar elastic tissue and collagen fibers arranged on the lines of force

creates a very elastic structure and at the same time very resistant to blood pressure.
Tunica externa or adventitia is well represented, consisting of areolar connective tissue in which there are blood vessels (vasa vasorum) and nerve extensions (Figure 9).

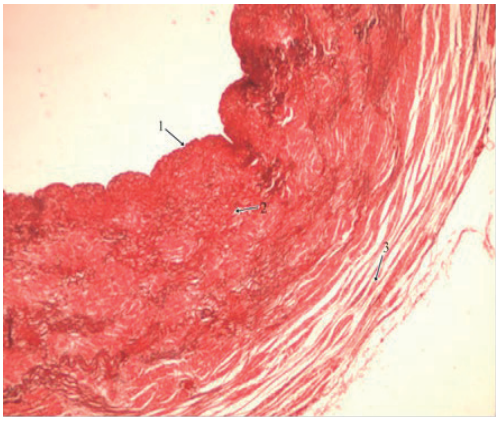

Figure 9. Aorta, Orcein stain Overview, 4x Objective: 1. Intima; 2. Media; 3. Adventitia

CONCLUSIONS

- The endothelium is a special type of epithelium with a semipermeable barrier function that separates the two compartments of the internal environment, blood plasma and interstitial fluid. The endothelium has a high degree of specialization in the mediation and active monitoring of bidirectional small molecule exchanges.
- Tunica intima is made up of a layer of endothelial cells supported by a basal membrane, a subendothelial layer made up of areolar connective tissue that may have a small number of smooth muscle fibers.
- Tunica media consists mainly of smooth muscle cells, arranged helically. Among them elastic and collagen fibers, proteoglycans and glycoproteins in are found varying amounts.
- Tunica externa or adventitia is composed mainly of collagen fibers and elastic fibers parallel to the long axis of the vessels. On the outside, the adventitia is lined by the connective tissue of the organ's blood vessels.
- The endocardium is similar to tunica intima of blood vessels and is bounded by a continuous endothelium consisting of a single layer of flattened cells. The left ventricular endocardium is thicker than the right ventricular endocardium.
- In the examined sections it is observed that the contractile myocardium has a different thickness in the two ventricles. The left ventricle has a much thicker wall than the right ventricle and the atria have a much thinner wall than the ventricular wall.
- The epicardium is a serous connective membrane covered by a single row of mesothelial cells.

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EVOLUTION OF THE BLOOD BIOCHEMICAL PROFILE OF THE HY-LINE VAR. BROWN HENS IN NATURAL MOULTING CONDITIONS

Cristiana GENES[1], Vasile GENES[2], Alexandru Cătălin GENES[2], Nicolae DOJANĂ[1]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest,
59 Marasti Blvd, District 1, Bucharest, Romania
[2]College of Romanian Veterinarians, Braila County Branch, 344 Galati Street, Braila, Romania

Corresponding author email: cristiana.genes@yahoo.com

Abstract

The purpose of this paper was to determine the evolution of the blood biochemical profile in 63-week-old Hy-Line var. Brown hens, in natural moulting conditions. A control group that showed no signs of moulting and a spontaneously moulted hen group were monitored for eight weeks. The analysed blood biochemical parameters were: glucose, total protein, uric acid, cholesterol, calcium and phosphate concentrations, and Ca/P ratio. All analysed parameters recorded different levels in the moulting period, compared to those in the period before this physiological process. The hens in the moulting group showed significant ($P<0.01$) variations in serum glucose compared to those in the control group where these variations did not differ significantly. Glucose raised by 19%, uric acid decreased by 2.9% and total serum proteins also decreased by 23.89% compared to the control group. Cholesterol of the moulting group reached its highest values raising with 100% and Ca/P ratio had a decreasing with 5.1% compared to the control group. Thus, it can be stated that the determination of the biochemical profile is useful in the evaluation of the physiological status of moulting hens.

Key words*: Hy-Line hen, biochemical profile, moulting.*

INTRODUCTION

Poultry meat is considered the healthiest because it has a lower fat content compared to other types of meat (Marangoni et al., 2015). Therefore, the worldwide increased demand for chicken was satisfied only by the impressive progress made by primary breeding companies in genetic selection (Buzala et al., 2016) to improve the growth rate, skeletal health, food conversion ratio (Nangsuay et al., 2017) and disease resistance (Parmentier et al., 2012), along with management improvements.

Another important sector of the poultry industry is egg production. Egg is one of the most affordable sources of animal protein, and it is therefore not surprising that the number of laying flocks is growing rapidy in developed countries such as India and China (Bain et al., 2016). The economic importance of raising poultry is given by a whole series of qualities that they have, especially their ability to produce various and valuable animal products, as well as their genetic ability to ensure large productions with low consumption, energy and feed. In some countries, at the end of the first production cycle, chickens are either sold as reform chickens or subjected to forced moulting. The choice to eliminated these reformed hens or to force their feeding by artificially inducing moulting depends on several economic factors. These factors include the availability and cost of replacement hens (North & Bell et al., 1990). Forced induction of moulting after 12 months of intensive production is frequently practiced by the commercial egg industry to increase the poultry productivity, thus inducing a second production cycle. Moulting of avian species can generally be defined as periodic loss and subsequent replacement of feathers. For most bird species, moulting involves resting. For the egg producer, there would be an unprofitable period of low egg production, which means the end of the active life of a flock. Although there are many data in the literature on this metabolic process, there are still questions about the triggers, metabolic processes that occur during moulting, hormonal mechanisms and how they influence the increase in the percentage of laying, including questions concerning the metabolic peculiarities of poultry, preceding or

initiating the moulting process. At the same time, no data are known on the particularities of carbohydrate, protein, lipid metabolism, as well as on the phenomena of degradation of reserves in the body, during moulting. Based on this data, the purpose of this paper was to determine the evolution of the blood biochemical profile in 63-week-old Hy-Line var. Brown hens, in natural moulting conditions.

MATERIALS AND METHODS

The experimental study was carried out in a commercial laying hen farm, located in Brăila county, specialized in raising laying hens, with a capacity of 361,600 cap., which uses as biological material the hybrid Hy-Line var. Brown, housed on the ground. The Hy-Line var. Brown is a highly specialized egg production tetra linear hybrid. This hen line has average body weight at 70 weeks, 2.25 kg, the color of the mineral peel is uniform, dark brown, the technological holding is from 18 to 80 weeks and the egg cycle is 62 weeks (Hy Line International, Galline ovaliole commerciali Hy-line Brown-Management Guide, 2021). There were made two hens' groups with 50 cap. each, a control group that did not show signs of moulting and an experimental group with spontaneously moulted hens that were collected and grouped in the same hall with the same microclimate conditions with the control group. The hens were identified at the beginning of the moulting period. They were collected, grouped, weighed and isolated, all in the same hall. The hens were monitored during the pre-moulting period as well as during the natural moulting period. The monitoring of the two groups was carried out and was considered completed when the hens from the experimental group completed the moulting and feathering process, and the laying percentage reached parameters similar to those of the hens in the control group. Blood samples were taken after the first collection of eggs and before the application of technological treatments. In order to perform the blood biochemical determinations, the venous blood samples were collected on Li-Heparin and centrifuged at 1500 rpm, thus retaining the plasma. Plasma samples were stored at -20°C until processing. The blood samples were collected weekly, both from the control and experimental groups during the moulting period, for eight weeks from the moment of appearance the moulting signs. Catalyst Dx Chemistry Analyzer (IDEXX) and Stat Spin VT Centrifuge (IDEXX) was used for blood biochemical determinations. The laboratory method used is based on the principle of dry biochemistry. The reagents used are integrated by dry biochemistry technology and specific for the tested biochemical parameters equivalent to slide test. Each slide test was made up of overlapping layers. Centrally, a circle was placed on the test slide, indicating where the test sample is pipetted, automatically placed by the analyser (https://www.idexx.com/files/catalyst-dx-operators-guide-en.pdf). The analysed blood biochemical parameters were: glucose, total protein, uric acid, cholesterol, calcium and phosphate concentrations, and Ca/P ratio was calculated.

RESULTS AND DISCUSSIONS

As shown in Table 1, the control group have been constant values for total serum proteins during the eight weeks of monitoring.

Table 1. The evolution of total serum proteins in the natural moulting hens during 8 weeks of monitoring vs. the control

	Age in weeks							
	Day 1	63	64	65	66	67	68	69	70
C	54.9 ±7.9	50.0 ±6.8	56.2 ±6.4	48.98 ±5.7	59.2 ±6.0	55.9 ±4.9	48.9 ±4.4	50.2 ±4.2	53.01 ±4.2
M	31.1± 6.1	28.1± 6.02	32.98± 6.01	30.1± 4.1	38.01± 5.89	47.05± 3.2	44.1± 4.2	44.9± 3.1	45.03± 3.2

C= control group; M=moulting group; Normal protein reference values: 35-40g/L

The values obtained in the case of the moulting group showed significant differences compared to the control group ($P > 0.5$), as follows: in the first week of monitoring, the value recorded was 28.1 ± 6.02, which continues to increase to 45.03 ± 3.2 in the last week, a fact explained in the literature by changing metabolism as a result of stress. The same results regarding the differences between the control and the experimental group were found in other studies, such as Arora et al. (2011) which reported on the Japanese quail a decrease in total plasma protein 3.50 g/dL vs. 5.56 g/dL during moulting compared to the pre-moulting period, returning to a value of 5.89 g/dL after moulting

(Arora et al., 2011). Puvadolpirod et al., 2000 reported an increase in total plasma protein levels in young hens after administration of glucocorticoid hormone preparations for stress induction (Puvadolpirod et al., 2000).

The increase in total serum protein has been reported by Puvadolpirod et al. (2000) following the administration of glucocorticoid hormone preparations for stress induction (Puvadolpirod et al., 2000).

The uric acid recorded in the present study, in the case of the control group, showed constant values throughout the monitoring period, respectively 4.3 ± 1.8 mg/dL in the first week and 4.8 ± 1.05 mg/dL in the eighth week. The group subjected to natural moulting, compared to the control, showed lower values, especially in the first week, 1.3 ± 0.15 (mg/dL), a period characterized by the phenomenon of profuse moulting, caused by stress, and metabolic changes (Table 2).

Table 2. The evolution of serum uric acid during in the natural moulting hens during 8 weeks of monitoring vs. the control

	Age in weeks								
	Day 1	63	64	65	66	67	68	69	70
C	4,2 ±1,6	4,3 ±1.8	4,8 ±1,1	4,6 ±1,2	4,7 ±1,3	5,04 ±1.5	4,4 ±1,5	4,6 ±0,96	4,8± 1,05
M	1,3 ±0,2	1,3 ±0,15	1,9 ±0,21	2,8 ±0,33	3,9 ±0,6	4,2 ±0,87	4,4 ±0,96	4,5 ±0,76	4,3 ±0,5

C= control group; M=moulting group; Normal acid uric reference values:1-7/l

Starting with the second week of monitoring, the concentration of uric acid increased steadily, which towards the end of the monitoring reached values close to those of the control group, namely 4.3 ± 0.5 mg/dL, which showed the end of the moulting period, regulating the metabolism to normal and physiological parameters as well as establishing the normal percentage of eggs.

At the same time, high plasma uric acid levels indicate the use of dietary protein for energy needs or conversion to other compounds (lipids) but may indicate a decrease in the body's protein during starvation. The results from this study are comparatively with those of Dunkley et al., 2007, who recorded that the plasma concentration of uric acid was minimal (2.7 mg/dL) after five days of starvation, again reaching values comparable to those of the control (at 5 mg/dL) after 12 days of starvation (Dunkley et al., 2007).

Regarding glucose, there were differences between glucose levels before and during the moulting period, with a higher level during the latter. In the case of the control group, a value of 197 ± 46 mg/dL was recorded in the first week of monitoring, and will increase steadily to 206 ± 25 mg/dL. The values of the group undergoing natural moulting were higher than those of the control group, respectively 153 ± 32 mg/dL in the first week, following that starting with the fourth week (212 ± 40 mg/dL) to increase and reach in the last week values of 214 ± 24 mg/ dL (Table 3).

Table 3. The evolution of blood glucose in the natural moulting hens during 8 weeks of monitoring vs. the control

	Age in weeks								
	Day 1	63	64	65	66	67	68	69	70
C	189 ±41	197 ±46	203 ±44	210 ±42	187 ±32	186 ±30	196 ±39	201 ±25	206 ±25
M	153 ±32	162 ±36	166 ±50	188 ±44	212 ±40	235 ±40	220 ±25	210 ±20	214 ±24

C= control group; M=moulting group; Normal glucose reference values C-145-198 mg/dL, M-130-270 mg/dL

Arora et al. (2011) reported that hens in the normal physiological period of egg production had higher blood glucose levels compared to hens in the moulting period, an increase of 31.93% and respectively 120.09% compared to post-moulting birds (Arora et al., 2011). McCormick et al. (1984) noted that liver glycogen has reached the minimum allowable levels due to zinc interfering with insulin secretion (McCormick et al., 1984). Hanafy et al., 2001 showed that there are no changes in blood biochemistry except for increased alkaline phosphate levels (Hanafy et al., 2001). The ovaries showed a reduction in the number of follicles, cessation of ovulation and hyperplasia of the germinal epithelium.

Regarding the calcium values on the first day of monitoring, in the case of the control group, was recorded 22.4 ± 6.1 mg/dL, a value that was constant during the first four weeks. This, in the following weeks and until the end of the monitoring, registered a decrease, but insignificant, compared to the first four weeks, reaching in the eighth week a value of 19.9 ± 3.1 mg/dL (Table 4).

The values of serum calcium, recorded in the case of the group subjected to natural moulting, were lower than those of the control group, respectively 9.1 ± 2.3 mg/dL on the first day of

monitoring, values that remained constant during the first four weeks, and which continued to increase gradually and steadily over the next four weeks, reaching 16.67 ± 3.2 mg/dL in week eight. Regarding the phosphorus monitoring, it showed variable values during the eight weeks of monitoring, in the case of the group subjected to natural moulting, being recorded in the first week values of 2.4 ± 0.7 mg/dL, so that in week five it reaches at values of 3.7 ± 1.2 mg/dL. The values obtained in our monitoring were similar with the literature results, which attributed the decrease in serum calcium to ovarian involution and uterine regression, due to the phenomenon of profuse moulting. Gildersleeve et al., 1983 reported low concentrations of this mineral, along with low concentrations of inorganic phosphorus, transaminases (GPT) and albumin (Gildersleeve et al., 1983). Brake and Thaxton (1979) showed that both total plasma calcium (Ca) and inorganic plasma phosphate (P) showed decreases in moulted hens compared to those that did not go through this process (Brake and Thaxton, 1979). They attributed this decrease in total plasma protein and total calcium to the loss of estrogen-dependent plasma complex phosphor-lipoprotein. One of the proteins in this complex is fosvitin 52, which binds a large proportion of total plasma calcium. Loss of fosvitine decreases total calcium concentrations to 5-6 mg/100 ml (Brake and Thaxton, 1979). Sexual rest led to the cessation of yolk synthesis, decreased total plasma proteins and total calcium levels. In addition, a decrease in estrogen levels is thought to cause a decrease in plasma inorganic phosphate.

Table 4. The evolution of serum calcium in the natural moulting hens during 8 weeks of monitoring vs. the control

	Age in weeks								
	Day 1	63	64	65	66	67	68	69	70
C	22,4 ±6,1	22,1 ±4,1	21,5 ±4,2	20,5 ±4,2	21,2 ±3,3	19,6 ±3,1	19,7 ±3,3	19,8 ±2,6	19.9 ±3,1
M	9,1 ±2,3	8,5 ±1,6	10,01 ±2,5	9,5± 2,2	11,98 ±2,1	14,7 ±3,02	15,98 ±3,5	16,05 ±3.6	16,67 ±3,2

C= control group; M=moulting group; Normal serum calcium reference values 520 mg/dL

At the same time, it is plausible that intestinal secretion of estrogen and parathyroid hormone (PTH) is responsible for elevated serum calcium levels in hens that are subjected to moulting, and its decrease is due to eggshell formation (Sykes, 1971). Regarding the Ca/P ratio, in the case of the control group, similar values were recorded during the eight weeks of monitoring, respectively 7.3 in the first week, with a constant but not decrease, up to 6.5 in the last week. The values of the group subjected to natural moulting were comparatively lower, compared to the control group, respectively 3.7 in the first week and 4.4 in the second, values which, however, started to increase with week five, reaching the end of the monitoring at 5.7, values close to the control group, in the same period. The recordings from the eighth week of monitoring the batch subjected to natural moulting tend to increase, to values similar to those of the control batch from the first week, which is attributed to the completion of the moulting process and the training of the wool in the case of Ca/P ratio (Table 5).

Table 5. The evolution of Ca/P ratio din the blood of the natural moulting hens during 8 weeks of monitoring vs. the control

	Age in weeks								
	Day 1	63	64	65	66	67	68	69	70
C	7,4	7,3	7,1	7,2	6,6	6,6	6,5	6,5	6,5
M	3,2	3,7	4,4	5,1	4,98	5,2	5,2	5,6	5,7

C = control group; M = moulting group; Normal Ca/P ratio reference values 3/5 L

There are differences between the cholesterol valuesbetween the control group and the one subjected to natural moulting.

In the case of the control group, the cholesterol values had constant variables during the eight weeks of monitoring but there were no differences between them, respectively 87 ± 20 mg/dL in the first week of monitoring, with constant values until week eight (96 ± 20 g/dL) (Table 6).

However, the same cannot be said about the values of the experimental group, which had significant and visible differences compared to the control group. The values recorded were higher compared to the other batch, respectively 205 ± 48 mg / dL, values that remained high and constant for three weeks, and which began to decrease, reaching 82 ± 16 mg / dL, in the last week monitoring. Gyenis et al. (2006) showed that plasma levels of total cholesterol and LDH cholesterol are constant and similar until the age of 17 weeks of laying hens, when

various developments are observed, LDH-cholesterol decreasing significantly (Gyenis et al., 2006). Kuenzel (2003) reported that during the night moult, a much more pronounced decrease in body weight was recorded (Kuenzel 2003). Halaby 1997 used for the induction of moulting, raw bitter mistletoe seed, which caused the cessation of egg production within 2 weeks of the first administration (Halab, 1997). It has been suggested that the complete withdrawal of food leads to the immediate cessation of egg production because the diet causes changes in the normal physiology with a preference for serum cholesterol, triglycerides and very low-density lipoproteins (VLDL), which are important for egg production (Baranczuk et al., 1995; Peebles et al., 2004). However, there are individual variations between birds and this theory is not generally valid.

Higher serum cholesterol levels have been found in hens near natural moulting. Large variations have been reported from chicken to hen in terms of serum levels (Griminger, 1976) which could make it difficult to determine the level of cholesterol in the blood.

Glucose raised by 19%, uric acid decreased by 2.9% and total serum proteins also decreased by 23.89% compared to the control group. Cholesterol of the moulting group reached its highest values raising with 100% and Ca/P ratio had a decreasing with 5.1% compared to the control group.

Table 6. The evolution of the serum cholesterol (mg/dL) in the natural moulting hens during 8 weeks of monitoring vs. the control

	Age in weeks								
	Day 1	63	64	65	66	67	68	69	70
C	86±22	87±20	97±15	75±15	98±18	102±20	82±16	84±17	96±20
M	106±38	205±48	195±37	182±39	127±25	128±21	97±17	84±16	82±16

C= control group; M = moulting group; Normal cholesterol reference values 3/5

CONCLUSIONS

With the exception of total serum proteins, all biochemical parameters recorded different levels in chickens in the moulting period, compared to those in the period before this physiological process.

Glucose raised by 19%, uric acid decreased by 2.9% and total serum proteins also decreased by 23.89% compared to the control group. Cholesterol of the moulting group reached its highest values raising with 100% and Ca/P ratio had a decreasing with 5.1% compared to the control group.

The determination of the biochemical profile is useful in the evaluation of l physiological status of moulting hens.

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AGGRESSION OF BROILER BREEDER MALES AGAINST FEMALES IN A POULTRY REPRODUCTION FARM: INJURIES AND POSSIBLE CONTRIBUTING FACTORS

Mihnea LUPU, Manuella MILITARU

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

Corresponding author email: mihnea.l96@gmail.com

Abstract

The present paper aims to present the aggressive behavior of broiler breeder males against females and its consequences. The research took place in a breeding poultry farm and the objectives were: to identify and describe the specific lesions found in females and to observe the behavior of the roosters in relation to the females in order to identify behavioral patterns. In the study, 15 corpses were examined and the specific lesions of aggression were described, analyzing the location, extent and acute or chronic nature of the wounds. Thus, it was found that the main areas where the lesions were found, are the head and torso and from the point of view of depth, superficial and deep lesions were identified. Another aspect observed was the clear differentiation of lesions caused by aggression from those caused by cannibalism. Following the behavior observation sessions, it was found that the courtship behaviour of the roosters was poor expressed or even missing, which led in forced mating, and resulted in the described lesions.

Key words*: the aggressiveness of roosters, broiler breeders, animal aggressiveness, poultry behavior.*

INTRODUCTION

Poultry farming, since the domestication of the first wild hens in the Asian jungle to the current level of intensive breeding, has been an important zootechnical branch throughout the contribution of very good quality animal products.

The aggressiveness of roosters towards chickens, in the populations of birds used for breeding, is a phenomenon encountered in poultry units with an intensive breeding system and has been described in the literature. This has been described in the case of roosters belonging to parental broiler lines (Millman et al., 2000).

The aim of the present study was to describe from an anatomopathological point of view the lesions found in the corpses of females, those lesions being produced by the aggressive behavior of roosters. In addition to the necropsy examination of the carcasses, the behavior of the roosters in the bird population was monitored and some observations were made in this matter.

The present study helps identify the aggressive phenomenon in bird populations and to also differentiate it from the cannibalism.

Another importance of the study is its interdisciplinary nature, as it includes both information appropriate to the pathology and observations on the aberrant behavior of roosters, related to etopathology.

MATERIALS AND METHODS

The present study was conducted on a breeding poultry farm for a period of 37 weeks. The studied population of birds is from the category of "slow-growing colored broilers". The breeding hall has a total area of 980 m². The entire hall was populated with 5.440 one-day-old females and 536 roosters of the same age.

At the age of 8 weeks, after some restructuring of the herd, 5.000 females and 450 males remained in the breeding hall.

The females and the males have been raised together since the age of one day old.

In the previous breeding cycle, a high rate of aggression of roosters towards females was observed, a phenomenon resulting in severe injuries, some of which lead to the death of many birds.

Based on these observations, the entire batch was monitored anatomopathologically, from the hall's population until week 37. The aim

was to identify, analyze and describe the lesions caused by the aggressive phenomenon and to identify possible causes of its onset. 15 corpses with visible signs of aggression were examined. During the examination, the location, depth, and age (acute / chronic) of the lesions were monitored.

The used necropsy technique was the classical one, comprising in general the external examination, the plucking, skinning and the internal examination of the corpses (Militaru et al., 2007; Ciobotaru, 2013).

The behavior was observed in 20-30 minute per session, after the birds got used to the presence of the observer. The aim was to identify the different behavioral patterns of males in relation to the females.

RESULTS AND DISCUSSIONS

Overview of lesions

There are two main locations of the lesions: on the head (Figures 1-4) and on the trunk (Figures 3 and 5). The lesions present on the trunk can be found either on the dorsal region of the trunk or on one or both lateral faces of the thighs. The distribution of lesions in the case of the 15 corpses examined in the study can be observed in Table 1.

Table 1. Number of cases depending on the location of the lesion

Location of lesions	Number of cases
Cranial / cervical	7 cases
Dorsal region of the trunk + side faces thighs	5 cases
Dorsal region of the trunk + lateral faces thighs + cranial / cervical	3 cases

Figure 1. 23-week-old hen with acute lesion in cranio-cervical region

Figure 2. 23-week-old hen with acute lesion in craniocervical region

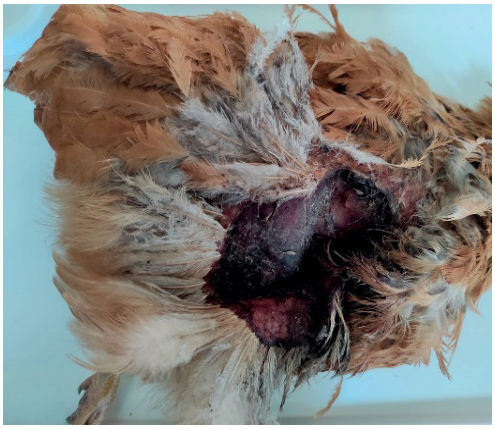

Figure 3. 29-week-old hen with an acute lesion in the lateral region of the thigh

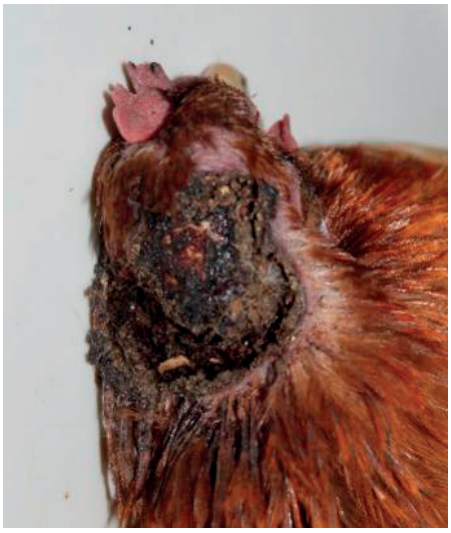

Figure 4. 27-week-old hen with subacute lesion in cranio-cervical region

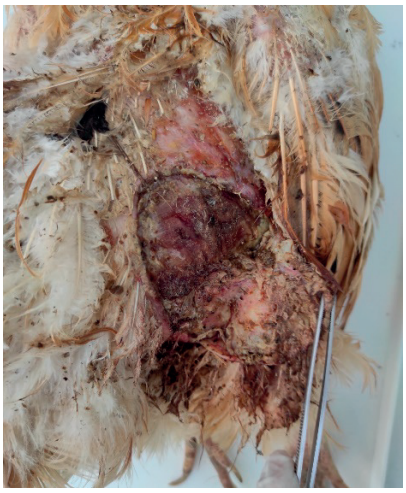

Figure 5. 35-week-old hen with an acute lesion in the lateral region of the thigh

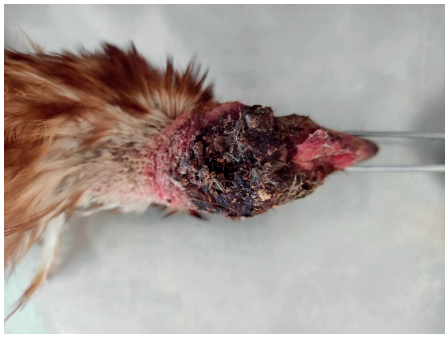

Figure 6. 37-week-old hen with acute lesion in the cranial region

The extent of the lesion and the depth of the traumatic agent's action

Looking from a depth perspective of the traumatic agent's action, the lesions include the skin, the subcutaneous connective tissue and the muscular surface.

The skin in the dorsal region of the trunk is torn apart by the claws of the roosters during the mounting process.

Once the skin is torn apart, during the attempts to perform the mount, the males tear apart the exposed muscles. Because of this fact, it is explained the presence of scratches of different degrees of depth, found on the surface of the corpse muscles (Figures 7-9).

Figure 7. 29-week-old hen with a wound on the side of her thigh. Perilesional fibrin deposition is observed

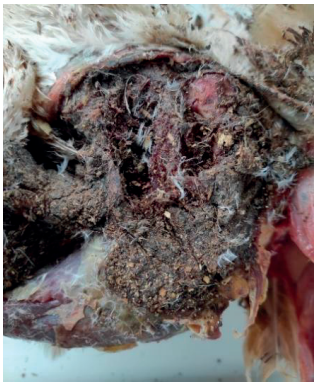

Figure 8. 30-week-old hen with deep tears in her thigh muscles

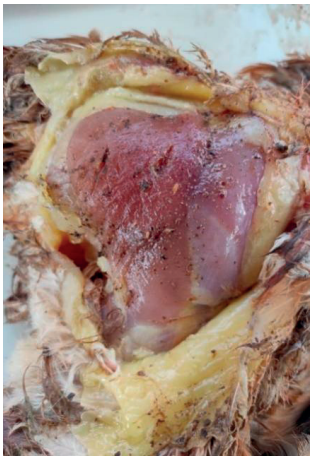

Figure 9. 36-week-old hen with superficial scratches on the muscles of the thigh

After the skin is torn apart, its edges retract. This process represents a vital local reaction due to the presence of muscle fibers in the skin architecture. This creates an entrance gate for bedding and feces, loaded with various microbial agents (Figure 10, Figure 11).
These residues migrate to the subcutaneous connective tissue, leading to the spreading of the inflammatory and infectious process, from the local level to the whole body. As a vital reaction of the body, the production of perilesional fibrin takes place, resulting in a delimitation of the lesion from the surrounding healthy tissues (Figure 12).

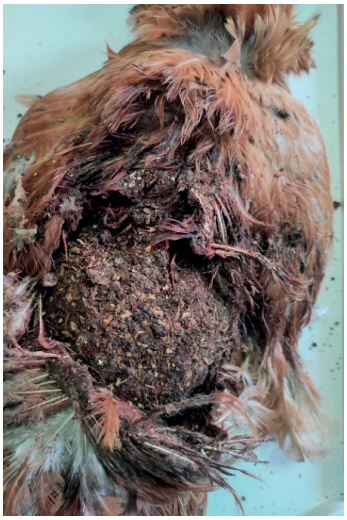

Figure 10. 26-week-old hen - the skin was torn and by opening the lesion remnants of litter penetrated to the subcutaneous tissue

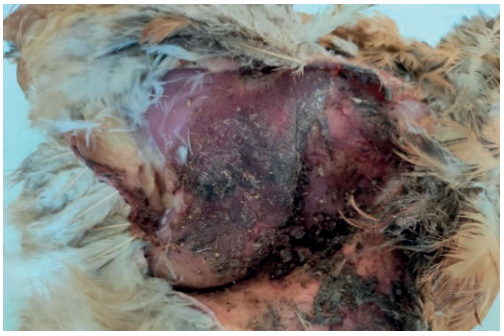

Figure 11. 29-week-old hen with a wound on the side of her right thigh. Litter and feces have entered the subcutaneous level

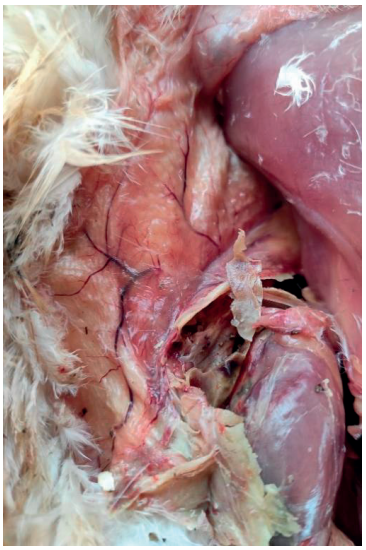

Figure 12. 30-week-old hen with a torso wound - perilesional fibrin deposition to the pectoral muscles is observed

The skin on the head or cervical area is torn apart by the beak of the roosters when they try to catch the hens to "step on" them.
Of the 15 cases examined in the study, 10 corpses showed extensive lesions and the remaining 5 corpses showed reduced lesions.
From the point of view of the age of the lesions, there are acute or chronic lesions. Of the 15 cases examined, 8 corpses had acute lesions, 5 corpses had chronic lesions and 2 corpses had both chronic and acute lesions.

The correlation between the age of the lession, extent, and the state of maintenance of the corpse

In the case of low-spread lesions, they become chronic and tend to heal. This type of lesion is usually found in the head area. These injuries, in most cases, do not result in the direct death of the birds. A predominance of chronic lesions were found in the head, in the case of cachectic corpses, with an inadequate state of maintenance. An explanation regarding this aspect is the following: the birds scared after the aggressive manifestation of the roosters, crowd towards the extremities of the breeding hall, avoiding in time to feed properly, this thing leading to their cachectic state.

In the case of acute, large-scale lesions, the death of birds occurs directly, either by hemorrhagic shock due to rupture of blood vessels, or by septic shock, due to the advancement of the infectious process in the body. In this situation, the state of maintenance of the corpses is good to very good (Table 2). The term "small" is characterized by low-spread wounds, which did not directly endanger the lives of birds.
The term "large" is characterized by very large wounds, which directly caused the death of birds either by hemorrhagic shock or septic shock.

Table 2. The correlation between the age and extent of the lesion and the state of maintenance

Cases	*Age of lesions*	*Extent of lesions*	*State of maintenance*
Case 1	Acute	Large	Very good
Case 2	Acute	Large	Very good
Case 3	Acute	Large	Very good
Case 4	Acute	Large	Very good
Case 5	Acute	Large	Very good
Case 6	Acute/chronic	Large	Good
Case 7	Chronic	Small	Inadequate
Case 8	Chronic	Small	Inadequate
Case 9	Chronic	Small	Inadequate
Case 10	Chronic	Small	Inadequate
Case 11	Acute	Large	Very good
Case 12	Acute	Large	Very good
Case 13	Chronic	Small	Inadequate
Case 14	Acute/Chronic	Large	Inadequate
Case 15	Acute	Large	Very good

The differences between aggression and cannibalism injuries

The literature defines cannibalism as an aberrant behavior, which is expressed by nibbling feathers and then the skin of other birds, in some cases reaching the almost complete consumption of an individual (Ioniță, 2014). In most cases, is determined by technological irregularities (Mitrănescu & Furnaris, 2012; Ioniță, 2014), but also some external parasitosis that causes birds to nibble their feathers, sometimes until the blood appears (Mitrea, 2011).
According to the definitions, cannibalism means the consumption of organs of an individual or parts of them by another individual of the same species. In the examined cases, the muscle masses are intact, with no signs of tissue deficiency. The only lesions were the tearing of the skin and the superficial scratches encountered in some cases on the muscular surface.
Comparatively, in Figure 14 is presented another case, where the specific signs of cannibalism can be observed. The intestinal mass of the bird was removed through the cloacal orifice, and part of it was consumed by the other chickens in the herd. Another case of cannibalism is shown in Figure 13. The lack of muscle mass in the preacetabular iliac region can be observed, which is consumed up to the bone level.

Figure 13. Case of cannibalism in a - the lack of muscles is observed, being highlighted the bone substrate

Figure 14. Case of cannibalism in the same population - the intestines extracted through the cloacal orifice are observed, which are partially consumed

Observing the behavior of birds

During the observation sessions, the following were found:
Failure of the specific courtship behavior by some roosters, resulting in forcing copulations.

Due to the lack of courtship, roosters no longer transmit their intention to mate chickens. For this reason, the hen is no longer adopting the squatting position, which then lead to males attacking them. They catch the skin of the cranio-cervical area with their beak, forcing them to climb on the backs of the females. In the hen's attempt to escape, specific lesions occur, or existing ones are aggravated; both in the head area and in the dorsal region of the trunk. The lack of courtship behavior of the roosters has been mentioned in the literature as one of the causes of aggressive manifestations of males (Millman et al., 2000). Fights between males have been observed, but in a normal level, without the hurting of the birds. Given the situation, one can discuss a sexual aggression and not an aggression based on dominance, because the identified and described lessions are produced during the mounting process and not from the competitive agression for resources.

The crowding of the chickens that show injuries in different evolutionary stages towards the extremities of the breeding hall in order not to come in contact with the aggressive males, which leads to the avoidance as time passes of food and water sources.

This manifestation could explain the cachexia of corpses with chronic injuries.

Inside the breeding hall there are chickens that show lesions in different stages (Figures 15 and 16).

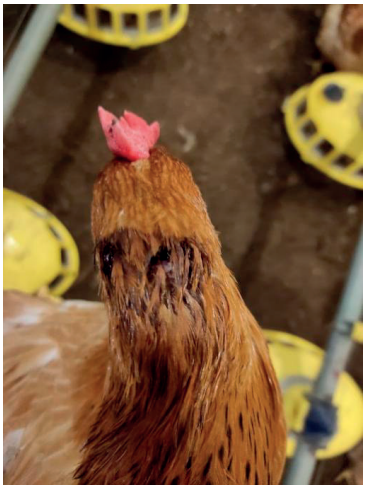

Figure 15. Live hen from the breeding hall with an acute cranial lesion

Figure 16. Live hen from the breeding hall with a chronic cranial lesion

The literature mentions as a cause of the lack of courtship behavior, the genetic over selection, focusing on the increased productivity of hybrids (Millman et al., 2000).

CONCLUSIONS

Following the aggressive behavior of roosters, the main locations of the lesions are: the head region and the dorsal region of the trunk. Some cases showed lesions both in the cranial region and on the dorsal area of the trunk.

A correlation was established between cachectic corpses and the presence of chronic, small lesions on them. Corpses with good or very good condition showed acute, large lesions.

The lesions produced as a result of the aggressive phenomenon are clearly differentiated from the lesions encountered in the case of cannibalism, in the same flock of hens.

In the present study, the cause of the aggressive behavior and the appearance of the described lesions is the lack or very weak expression of the specific courtship behavior of the roosters.

The lesions described are not specific to dominance fights, but indicate a phenomenon of sexual aggression.

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IMPACT OF THE ADDITION OF DIFFERENT FOREST BERRY FRUITS ON FUNCTIONAL, PHYSICOCHEMICAL AND SENSORY PROPERTIES OF YOGURT SHELF LIFE

Corina PREDESCU[1]*, Georgeta ȘTEFAN[1], Camelia PAPUC[2]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, 105 Spl. Independentei, District 5, Bucharest, Romania
[2]Academy of Romanian Scientists (AOSR), 54 Splaiul Independentei, 050094, Bucharest, Romania

*Corresponding author, email: durduncorina@yahoo.com

Abstract

The present study aimed to report the effect of black mulberry – BM (Morus nigra), black chokeberry – BC (Aronia melanocarpa), and blackberry – BB (Rubus fruticosus) fruits on some functional and physicochemical parameters and sensorial properties of yoghurt during refrigeration, compared to untreated yoghurt.
Yoghurt was prepared from cow milk, provided from a farm near Bucharest. The fruits used for the experiment were bought from a local market. Fruit puree was added at 0% and 5% (w/v). Yoghurt samples were collected after 1, 5, 10, and 15 days of refrigeration for analysis of several parameters [total phenol content (TPC), total anthocyanins content, antioxidant activity (AA), TBARS value, protein carbonyl, pH, titratable acidity, water holding activity, syneresis, and sensory evaluation]. The highest TPC was found in samples treated with BC, and the highest AA was in the samples treated with BC also. For all samples treated with berries puree, protein carbonyl, and TBARS values were lower than untreated samples. The sensory evaluation results revealed no statistical differences ($p>0.05$) between the acceptability of the three types of yoghurts. The addition of black mulberry (Morus nigra), black chokeberry (Aronia melanocarpa), and blackberry (Rubus fruticosus) puree fruits in yoghurt enhanced the lipid oxidative stability, decreased syneresis, and modified its sensorial properties in the acceptability limits.

Key words*: yoghurt, forest berry fruits, protein carbonyl, antioxidant activity, anthocyanins.*

INTRODUCTION

Yoghurt is nutritious and fortified with fruits and can provide an important concentration of biologically active compounds, such as phenolic compounds with antioxidant activity. Consuming fruits and yoghurt have been identified in all diets as indicators of healthy patterns. Fruits are relatively low in energy and are an excellent source of antioxidants, fibres, and polyphenols, promoting health (Fernandez & Marette, 2017; Predescu et al., 2016). On the other hand, yoghurt is a nutritious food because is a good source of dairy protein, calcium, magnesium, vitamin B12, essential fatty acids, and other essential molecules. Furthermore, it contains beneficial bacterial cultures, making it a potential source of probiotics. Yoghurt's sources of fermentation are *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* makes a unique fermented food matrix that provides added health benefits by enhancing nutrient absorption and digestion (Zepeda-Hernández et al., 2021). Lactic acid bacteria commonly used as starter cultures are known to produce antimicrobial substances such as bacteriocins and have great potential as food bio-preservatives (Bamgbose et al., 2021; Mokoena, 2017). Combining yoghurt and fruit could provide probiotics, dietary fibre (prebiotics), high-quality protein, important fatty acids, and a mixture of vitamins, phenolics and minerals that have the potential to exert synergistic effects on health. More recently, probiotics and prebiotics have been suggested to modulate the microbiota (Zepeda-Hernández et al., 2021; Tuohy et al., 2014). After more than 100 years, yoghurt is still the preferred dairy product with relatively high consumption, probably numerous health benefits. The acceptability by consumers of yoghurt as a functional dairy product remains very high and people of all ages have expressed their wishes to add it to their diet (Mokoena, 2017). This study investigated the supplementation of fermented dairy products

like yoghurt, with phenolic-rich products like some forest berry puree to optimize the benefits of probiotic products with prebiotic compound intake. Yoghurts are known to have unique characteristics that make them accepted by consumers. Therefore, it is important to clarify if the addition of berry puree may modify them positively or negatively. The addition of forest berry puree is expected to modify some specific aspects of yoghurt, for instance, total phenol content (TPC), total anthocyanin content, anti-oxidant activity (AA), titratable acidity, pH, water holding activity, syneresis, TBARS value, protein carbonyl and sensorial properties.

The present study aimed to produce yoghurt with a 5% addition of forest berry puree and to evaluate the functional, physicochemical characteristics, and sensorial characteristics, during storage for up to 15 days.

MATERIALS AND METHODS

Source of raw materials used for yoghurt preparation. Commercially packaged berries (no preservatives and no added sugars) from black mulberry – BM (*Morus nigra*), black chokeberry – BC (*Aronia melanocarpa*), and blackberry – BB (*Rubus fruticosus*); commercial pasteurized and homogenized cow's milk (Moara Domneasca Farm, Ilfov county, Romania); commercially pack starter culture for yoghurt production. This yoghurt starter consists of strains of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*.

Puree berry fruits preparation. Berries were poured into a blender, and additional water was added (w:v, 1:1) and blended until smooth. With the help of a spoon, the puree was gently pushed through a sieve. The puree was simmered for about 15 minutes.

Yoghurt preparation. The pasteurized milk was heated to 42°C and then inoculated with a yoghurt starter culture. It was mixed lightly and the obtained mixture was transferred to the beakers with lids. The yoghurt was left to ferment in a yoghurt fermenter until the pH reached the value of 4.6, without stirring, to let the formation of the curd, and the pureed fruit was added to a concentration of 5%, by manually mixing for five minutes. The yoghurt cups were then stored in the refrigerator until analysis was performed. Also, a control yoghurt (CY) was produced and was mixed also for five minutes without the addition of the fruit puree.

Preparation of hydroethanolic yoghurt extracts. Yoghurt samples (0.4 ml) were subjected to extraction with 9.6 ml ethanol (60%) for 2 h in a shaking water bath, at 50°C. Samples were then centrifuged at 5000 rpm for 15 min at 4°C and the supernatant was collected. The extracts were aliquoted and stored at −30°C until further analysis.

Total phenol content. Phenol content was measured using a modified Folin-Ciocalteau method (Deighton et al., 2000). 1000 µl of yoghurt extracts were mixed with 3 ml of water, and 250 µl of Folin-Ciocalteu reagent was added and incubated at room temperature for 1 min. Following the addition of 750 µl of 7.5% (w/v) sodium carbonate to the mixture, total polyphenols were determined after 1 h of incubation in the dark at room temperature. The absorbance of the reaction mixture was determined at 765 nm against a blank sample using a UV-VIS Jasco 670 spectrophotometer. Quantification was done concerning the standard curve of Gallic acid and results are expressed as mg/kg Gallic acid equivalents (GAE).

Total anthocyanin content. The total anthocyanin content was estimated by the pH differential absorbance method (Lee et al., 2005). Yoghurt extracts were diluted with pH 1.0 buffer (potassium chloride, 0.025 M) until the absorbance at 520 nm was around 0.5 when measured with the spectrophotometer, and the same dilution factor was used to prepare all samples for pH 4.5 buffer (sodium acetate, 0.4 M). If diluted samples were turbid, were therefore centrifuged before measuring absorbance at 520 and 700 nm (20-50 min after preparation). The diluted samples were read *versus* a blank filled with correspondent buffer. To calculate the total anthocyanin concentration, (expressed as mg/l cyanidin-3-glucoside equivalents), the following equation was used:

$$\text{Total anthcyanins}\left(\frac{\text{mg}}{\text{l}}\right) = \frac{A \times MW \times DF \times 10^3}{\varepsilon \times l}$$

Where, A = (A520 nm - A700 nm) pH 1.0 - (A520 nm - A700 nm) pH 4.5; MW (molecular weight) = 449.2 g/mol for cyanidin-3-glucoside

(cyd-3-glu); DF = dilution factor established in D; l = pathlength in cm; ε = 26,900 molar extinction coefficient for cyd-3-glu in l × mol^{-1} × cm^{-1} and 10^3 = factor for conversion from g to mg.

Antioxidant activity. Antioxidant activity was determined using the free radical DPPH• (2,2 diphenyl-1-picrylhydrazyl) method (Chen et al., 2003), with some modifications. Specifically, 1.0 ml diluted yoghurt ethanolic extract and 3.0 ml of DPPH• solution were added, vortexed, and allowed to stand at room temperature in darkness for 30 min. The absorbance of samples and blank (60% ethanol) was spectrophotometrically measured at 517 nm UV/VIS spectrophotometer Jasco 670 and quantified using Trolox as a standard.

Determination of thiobarbituric acid reactive substances (TBARS). One gram of each yoghurt was transferred in a 25 ml test tube and 5 ml of 50% glacial acetic acid in water were added. BHT (0.01%) was used to prevent further oxidation of the samples (Zeb & Ullah, 2016). The samples were shaken for 1 h and filtered. The filtrate was centrifuged. The extract of each sample (1 ml) was mixed with 1 ml 4.0 mM TBA reagent. The standard stock solution of MDA (1 mM) was prepared in glacial acetic acid. The calibration curve was constructed in the concentration range of 0.1 to 1.0 mM. The TBARS was calculated using the formula μmol/g of the sample:

$$\text{TBARS (μmol/g)} = \frac{Ac \times V}{W}$$

Where, *Ac* is the amount determined from the calibration curve and *W* is the weight of the sample taken while *V* is the volume in ml or dilution factor of the total extract prepared.

Determination of protein carbonyl. An aliquot of aqueous yoghourt solution (corresponding to ca. 2 mg protein) was incubated with 10 mM DNPH in 2 N HCl (0.5 ml final volume) for 30 min at room temperature (Citta et al., 2017). Yoghourt proteins were then precipitated with 10% TCA (final concentration) and recovered by centrifugation for 5 min at 1677 × g. Protein pellets were washed three times, with 1 ml of ethanol/ethyl acetate (50:50; v/v) to remove unreacted free DNPH reagent, and redissolved in 0.6 ml of 6 M urea. The carbonyl content was calculated by UV spectrophotometry (λ = 370 nm; ε = 22,000 M^{-1} cm^{-1}, Jasco 760 spectrophotometer (Levine et al., 1990). Protein concentrations were determined using the Bradford Protein Assay and results were expressed as nmol DNPH mg^{-1} protein.

Titratable acidity and pH. Titratable acidity is the total amount of all the available hydrogen ions in a solution and was expressed as g lactic acid /100 g of yoghurt (Dimitrellou et al., 2020). 10 ml of yoghurt were transferred into a beaker and using the same pipette, 90 ml of distilled water were added. Lactic acid was titrated with 0.1 N NaOH using a digital titrator (TitroLine easy). The pH was measured with an electronic pH meter (Mettler Toledo pH Meters), and before each determination, the pH meter was first calibrated by 2 buffers 4 and 7.

Water Holding Capacity. A sample of about 10 g of yoghurt (Y) was centrifuged for 20 min at 5000 rpm and at 20°C (Sidira et al., 2017). The whey expelled (WE) was removed and weighed. The water holding capacity (WHC) was calculated using the next equation:

$$WHC(\%) = \frac{Y - WE}{Y} \times 100$$

Where, WE are whey g expelled and Y is the initial yoghurt sample in g.

Syneresis. Syneresis was determined using 50 g of unstirred yoghurt spread evenly on a filter paper in a funnel at 4°C. After 5 h of drainage, the volume of collected whey was measured, multiplied by 2, and expressed as syneresis (%) (Sidira et al., 2017).

Sensory evaluation of yoghurt samples. The sensory analysis of yoghurt samples was conducted by 10 panellists selected from graduated students in the Control and expertise of food products, using a 7-point hedonic scale: 1 – 'Strongly disliked'; 2 – 'Moderately disliked'; 3 – 'Slightly disliked'; 4 – 'Indifferent'; 5 – 'Slightly liked'; 6 – 'Moderately liked', and 7 – 'Strongly liked'. Yoghurt samples were coded with numbers and randomly tested for appearance, colour, consistency, taste and smell. Mineral water and cracker biscuits were available as neutralizers between samples to avoid carryover effects. The test was performed in 3 replications, on the seventh day of yoghurt refrigeration time (Varedesara et al., 2021).

Statistical analysis. The statistical analysis was done using the Statistical Analysis System

Program, version 20.0 (SPSS Inc., Chicago, IL, USA) at a significance level of $p < 0.05$. The measurements were performed in triplicate for each sample and results were expressed as mean value ± standard deviation.

RESULTS AND DISCUSSIONS

Total phenol content. Black mulberry (*Morus nigra*), black chokeberry (*Aronia melanocarpa*), and blackberry (*Rubus fruticosus*) fruits are good sources of phenolic compounds, especially anthocyanins. Therefore, the total anthocyanin and total phenol content of yoghurt samples containing fruit purees were analysed during their shelf-life period. As presented in Table 1, total phenol contents in yoghurt containing BM, BC and BB were both higher than the control yoghurt (CY). This effect was significant ($p < 0.05$) for the first seven days of storage. The difference in total phenol content of fortified yoghurt with fruits purees (BC > BM > BB).

Table 1. Total phenol content, total anthocyanin and antioxidant activity of tested yoghurts

Yogurt sample	Control yoghurt	Black mulberry	Black chokeberry	Blackberry
Fruit puree	0%	5%	5%	5%
Time (days)	Total phenol content (mg/kg)			
1	15.24±1.24	39.14±4.15	41.56±5.40	26.17±2.31
5	15.21±3.01	40.25±3.07	43.20±3.14	29.02±1.92
10	17.21±1.20	42.21±1.32	47.81±1.25	31.74±2.11
15	18.90±1.98	39.10±3.21	43.31±1.67	29.31±1.67
Time (days)	Total anthocyanin (mg C3GE/l)			
1	0.14±0.09	3.58±0.65	4.54±0.42	3.24±0.56
5	0.11±0.08	3.25±0.22	4.31±0.52	3.28±0.36
10	0.14±0.06	3.15±0.20	4.88±0.54	3.15±0.54
15	0.12±1.98	3.10±0.21	4.25±0.65	3.04±0.31
Time (days)	Antioxidant activity (μmol TE 100 g^{-1})			
1	19.14±1.54	37.25±4.22	42.31±3.52	27.28±2.36
5	14.51±3.21	25.21±1.65	37.54±2.12	24.24±1.56
10	9.24±1.26	18.45±1.20	34.88±1.54	18.85±1.54
15	5.90±1.98	17.10±1.21	28.25±2.65	17.04±1.11

Results are presented as means ± SD

An increase in total phenols was observed for all yoghurts during the first 10 days of storage. A similar evolution in the phenolic content during refrigerated storage has been presented by (Raikos et al., 2019) and was attributed to the formation of compounds that react with the Folin-Ciocalteu reagent. For instance, proteolysis of milk proteins may release amino acids with phenolic side chains, such as phenylalanine and tyrosine, which could contribute to the increase in total phenol content. In addition, the phenolic compounds added to yoghurt may suffer glycoside hydrolysis or C-ring cleavage and release of simple phenolics such as phenolic acids (Raikos et al., 2019). After the first ten days of storage, the total phenol content in the yoghurt sample had a small reduction, which was similar to other research on yoghurts containing different fruit extracts. Polyphenols interact with milk proteins forming insoluble complexes, and this is reflected by reducing the total free polyphenol content in the yoghurt sample (Raikos et al., 2019). However, significant levels of polyphenols were detected even after fifteen days of storage especially in BC, followed by BM and BB. All concentrations of total phenolic content were higher than CY even after 15 days of refrigeration.

Total anthocyanin content. Anthocyanins are important to the food industry because of their attractive and stable pink-red colour in acid foods like yoghourts and their antioxidant power. The total anthocyanin content of different types of yoghurts was analysed. As shown in Table 1, the addition of BM, BB and BC purees increased the total anthocyanin content in yoghurt compared to the control yoghurt.

Antioxidant activity. Generally, the DPPH free radical activity test is used to measure the antioxidant ability of a sample. DPPH radical solution has maximum absorption at 517 nm, but in the presence of an antioxidant, it reduces the absorption measurement by DPPH free radical scavenging. Therefore, the antioxidant property of a compound is expressed as DPPH• ability to scavenge free radicals (Varedesara et al., 2021). The results of the present study showed (Table 1) that all yoghurts had a higher potential for DPPH free radical scavenging. The bioactive fruit purees exert their antioxidant activity through various mechanisms such as inhibition of lipid

peroxidation, inhibition of protein oxidation, and free radicals scavenging.

Determination of thiobarbituric acid reactive substances (TBARS). To quantify the spontaneous lipide peroxidation of yoghurt occurring during the shelf life, a test based on TBARS production was used. First of all, adding berries puree to yoghurts show lower levels of TBARS, especially in the presence of BC (Table 2). In addition, it was observed a slight increase in lipoperoxidation for all treated yoghurts, and especially for control yoghurt. However, the levels of lipid peroxidation were relatively low reaching a maximum of about 21.21±2.11 µmol/g (Table 2). In the presence of berries, the TBARS level was even lower in the presence of CB, BM and BB. In addition, during the shelf life, a constant increase in TBARS formation until the 15th day was observed. This behaviour was found both in the presence and in the absence of berries. However, in the absence of berries puree, a lower TBARS production was observed for control yoghurt alone. Similarly, the presence of berries determines a decrease in lipid peroxidation of yoghurt compared to yoghurt without berries.

Determination of protein carbonyl content. During the fermentation process of yoghurt, proteins are partially hydrolysed in compounds susceptible to oxidation (Citta et al., 2017).

Table 2. TBARS value and protein carbonyl content of tested yoghurts

Yogurt sample	Control yoghurt	Black mulberry	Black chokeberry	Blackberry
Fruit puree	0%	5%	5%	5%
Time (days)	TBARS value (µmol/g)			
1	14.11±0.87	14.78±1.62	14.03±0.32	15.02±1.33
5	16.21±1.54	16.05±1.87	15.02±1.21	15.31±1.64
10	19.45±2.11	17.87±1.51	17.91±1.61	17.41±1.95
15	21.21±2.11	20.25±2.14	19.41±1.21	20.51±2.25
Time (days)	Protein carbonyl content (nmol DNPH/mg protein)			
1	3.38 ± 0.32	3.28 ± 0.37	3.48 ± 0.51	3.21 ± 0.32
5	5.37 ± 0.97	4.3 ± 0.39	3.47 ± 0.48	5.44 ± 0.42
10	6.39 ± 1.30	5.38 ± 0.40	5.49 ± 0.35	6.45 ± 0.31
15	9.38 ± 1.36	7.34 ± 0.38	6.46 ± 0.51	8.43 ± 0.39

Results are presented as means ± SD

The yoghurt samples (control, and puree berries treatments) were also tested for protein carbonyl formation during the shelf life, on the same days on which lipid peroxidation was measured. The resulting amount of protein carbonyl (Table 2) was very similar in the presence of puree berries, indicating that berries were able to prevent protein oxidation.

A higher protein carbonyl value was shown for control yoghurt. This result is quite the same as that obtained for the TBARS value (Table 2) indicating that the antioxidants of yoghurt are capable of protecting against lipid and protein oxidation. During the shelf life of 15 days, the proteolytic activity of bacteria in yoghurt proceeds (Germani et al., 2014) and determines fragmentation and oxidation, particularly in control yoghurt. Protein oxidation is higher for BB compared with BC and BM at the end of the treatment (Table 2).

Titratable acidity and pH. pH is the negative logarithmic measure of the ionic strength of hydrogen in aqueous solutions and reflects the degree of acidity or alkalinity of aqueous solutions.

Table 3. pH and titratable acidity of tested yoghurts

Yogurt sample	Control yoghurt	Black mulberry	Black chokeberry	Blackberry
Fruit puree	0%	5%	5%	5%
Time (days)	pH			
1	4.65 ± 0.38	4.58 ± 0.47	4.66 ± 0.44	4.59 ± 0.53
5	4.53 ± 0.16	4.41 ± 0.33	4.38 ± 0.33	4.45 ± 0.34
10	4.37 ± 0.29	4.41 ± 0.41	4.39 ± 0.52	4.37 ± 0.42
15	4.19± 0.34	4.47 ± 0.54	4.32 ± 0.45	4.25 ± 0.63
Time (days)	Total acidity (%)			
1	0.91±0.11	1.04±0.18	0.90±0.08	1.10±0.01
5	1.05±0.11	1.11±0.11	1.01±0.08	1.14±0.05
10	1.02±0.17	1.14±0.16	1.11±00.11	1.14±0.04
15	1.01±0.11	1.12±0.14	1.16±0.09	1.15±0.05

Results are presented as means ± SD

In particular, for yoghurt production standards, pH and titratable acidity are the most important tests for increasing yoghurt shelf life and better acceptance by the consumer. Acidity is the consequence of lactic acid production, by the fermentation of yoghurt carbohydrates. pH and titratable acidity are considered the most important tests and also acceptable indicators for yoghurt storage (Varedesara et al., 2021). The results of changes in pH and acidity (Table 3) during the maintenance process showed that pH values decreased over time in all treatments and acidity increased. During the production

and storage of yoghurt, the starter bacteria cause the production of lactic acid and consequently, increase the titratable acidity and decrease the pH. The decreasing pH and increasing acidity during storage have been reported in most related studies (Varedesara et al., 2021; Dimitrellou et all., 2020). The results related to the pH and acidity of yoghurt values showed that the addition of fruit purees didn't have a remarkable effect on the pH and acidity, in all storage periods. On the last day of treatment, the highest pH and lowest acidity values were observed in the treatment containing BM.

Water Holding Capacity. Measuring the value of water holding capacity is one of the most important physical tests to measure the quality of yoghurt. WHC represent the gel capacity to prevent the separation of the aqueous phase from the continuous phase of the casein network. As it is shown in table 4, WHC decrease during the 15 days of storage for all tested yoghurts.

Table 4. Syneresis and water holding capacity of tested yoghurts

Yogurt sample	Control yoghurt	Black mulberry	Black chokeberry	Blackberry
Fruit puree	0%	5%	5%	5%
Time (days)	Syneresis (%)			
1	31.10±3.45	34.41±3.12	33.31±3.38	35.21±3.24
5	32.48±3.14	36.51±3.11	35.99±3.98	36.82±3.03
10	35.14±3.13	37.21±3.21	36.36±3.89	38.57±3.02
15	37.94±3.68	37.94±3.68	37.81±3.51	39.11±3.31
Time (days)	WHC (%)			
1	71.10±5.45	69.41±5.12	70.81±4.51	63.57±5.02
5	67.48±5.14	65.51±5.11	69.31±4.38	61.21±5.24
10	66.14±5.13	62.21±5.21	65.99±5.98	59.82±5.03
15	60.94±4.68	61.94±4.68	62.36±6.89	58.11±5.31

Results are presented as means ± SD

Syneresis. Syneresis represent the separation of whey and is one of the most common defects in fermented milk products like yoghurts (Amatayakul et al., 2006). This defect may have a negative impact on its acceptability by the consumers due to the undesirable appearance, and limit the shelf life of the product. Overall, the consumers correlate syneresis with the potential yoghurt microbial infection (Dimitrellou et al., 2019; 2020). Moreover, previous studies have correlated the addition of fruit with reduced viscosity and increased syneresis of yoghurts. In order to reduce syneresis, the addition of milk solids or stabilizers can be added to yoghourts. In the present study, the syneresis increased during the yoghurt refrigeration for 15 days, and BB treatment showed the highest syneresis at the end of the treatment (Table 4).

Sensory evaluation of yoghurt samples. The most important factors in accepting or rejecting products and taking satisfaction from their consumption are sensory characteristics (Shori et al., 2018). In the present study, sensory analysis of yoghurt samples was determined based on colour, aroma, softness, taste, and overall liking.

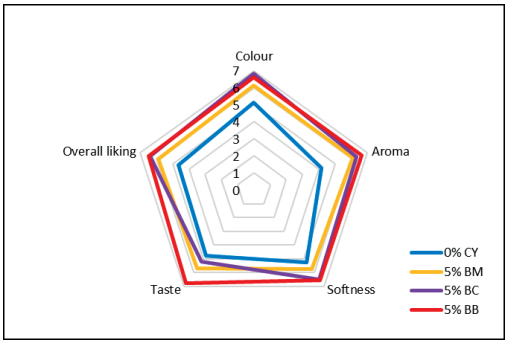

Figure 1. Sensory evaluation of yoghurt samples (CY = control yogurt; BM = yogurt with black mulberry; BC = yogurt with black chokeberry; BB = yogurt with blackberry)

According to the statistical analysis results, the sensory attributes were increased for yoghurt samples added with fruit puree (Figure 1). Overall, the three puree treatments were appreciated by evaluators. The colour and the softness of the yoghurts sample treated with BC and BB were appreciated more than the BM treatment. The aroma results showed that all three added puree fruits were preferred to CY and BB was appreciated as the most fragrant yoghurt of all samples. The taste evaluation showed probably a personal preference for the fruits added to the yoghurt, BB>BM>BC>CY. For all evaluated attributes, a higher sensory score was observed for BB yoghurt.

CONCLUSIONS

Significant levels of phenols were detected even after fifteen days of storage especially in

BC, followed by BM and BB. The bioactive fruit purees exert their antioxidant activity through various mechanisms such as inhibition of lipid peroxidation, inhibition of protein oxidation, free radicals scavenging. The addition of BM, BB and BC purees increased the total anthocyanin content in yoghurt. The syneresis increased during the yoghurt refrigeration for 15 days, and BB treatment showed the highest syneresis at the end of the treatment. WHC decrease during the 15 days of storage for all tested yoghurts. The decreasing pH and increasing titratable acidity during storage were observed for all yoghurts. In the presence of CB, BM and BB berries, the TBARS level was lower than control yoghurt (CY). Protein oxidation is higher for BB compared with BC and BM at the end of the treatment. A higher sensory score was observed for BB yoghurts, for all evaluated attributes.

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MORPHOLOGICAL FEATURES OF THE SKULL IN THE EURASIAN BROWN BEAR (*Ursus arctos arctos* - Linnaeus, 1758): CASE STUDY

Petronela Mihaela ROŞU, Bogdan GEORGESCU, Cristian Romeo BELU, Letiţia PURDOIU, Sorina Andreea MIHAI, Eduard GUREŞOAE, Valerica DĂNACU

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

Corresponding author email: rosupetronelamihaela@yahoo.com

Abstract

This study aims to analyze and describe the morphological characteristics of the skull in the Eurasian brown bear (Ursus arctos arctos) in Romania. The morphological particularities of the skull are essential elements for the recognition of the species. The data from the literature on the morphology of the skull in this species are insufficient and focused mostly on the morphometric particularities of the skull. For the present study, we examined a skull from an adult bear, that is part of the collection of the Anatomy department. The bear is a protected animal and is hunted only under conditions established by law. The observational analysis of the morphological features of the skull led to the following conclusions: the skull is compact, elongated and relatively narrow on the dorsal part, the zygomatic processes of the frontal bone are very short and lacking in supraorbital foramen, the external sagittal crest is reduced, the lacrimal bone has a single lacrimal foramen, the articular surface for the mandible is represented by a transversal elongated articular cavity, being delimited in an caudal direction by the retroarticular process, the accessory palatine foramina are very small.

Key words*: bear, skull, zygomatic process, external sagittal crest.*

INTRODUCTION

The Eurasian brown bear *(Ursus arctos arctos)* (Linnaeus, 1758) belongs to the Order *Fissipeda,* an order that includes carnivorous mammals, belonging to the *Ursidae Family,* Genus *Ursus*. It is a species that lives all over Europe, and in Romania it can be found in the mountainous area (about 6000 specimens), where their number is constantly growing, due to restrictive measures regarding the hunting of this species. (Cotta & Bodea, 1969; Şelaru & Goicea, 2005)

There is a considerable lack of literature regarding the morphological features of the skull in the brown bear, as opposed to morphometric studies, which are far more common. (Yousefi, 2016; Mihaylov et al., 2013). Moreover, data regarding the morphological aspects of skulls in other various species of carnivores has been found. (Atalar et al., 2009; Getty, 1975; Jackson, 2011; König & Liebich, 2004); Movahhedi et al., 2014).

This study was conducted on a skull of a specimen of Eurasian brown bear (*Ursus arctos arctos*), and its aim was to present key particularities based on which the Eurasian brown bear can be differentiated from other species of carnivores.

Taking into consideration that the number of hunted or captive exemplars in Romania is very low, the morphological particularities of the skull in this species have been scarcely studied.

MATERIALS AND METHODS

The study material was represented by a Eurasian brown bear skull (*Ursus arctos arctos*), belonging to the collection of the Anatomy department. The most interesting aspects were described and photographed. The description, identification and approval were made in accordance with the 2017 *Nomina Anatomica Veterinaria* (NAV).

RESULTS AND DISCUSSIONS

In the Eurasian brown bear (*Ursus arctos arctos*), the dorsal surface of the skull is characterized by an elongated and relatively narrow appearance. The nuchal crests are relatively high, reunited in the dorsal median plane, forming a reduced occipital protuberance.

The parietals are convex on the whole surface, the external sagittal crest is short and slightly prominent in the caudal third of the parietal suture, dividing into two very short temporal lines. The zygomatic processes of the frontal bone are very small in size, having a ventro-caudal orientation and lacking of the supraorbital foramen at the base (Figure 1).

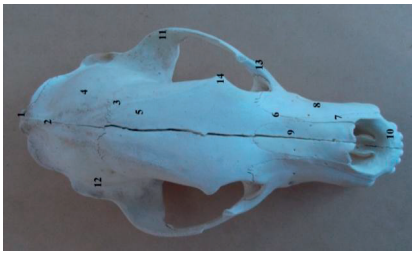

Figure 1. Dorsal face of the skull in Eurasian brown bear (*Ursus arctos arctos*) - 1. External occipital protuberance; 2. External sagittal crest; 3. Temporal line; 4. Parietal bone; 5. Frontal bone; 6. The nasal processes of the frontal bone; 7. The nasal processes of the incisive bone; 8. Maxilla; 9. Nasal bone; 10. The incisive bone; 11. The zygomatic process of the temporal; 12. The temporal bone; 13. The zygomatic bone; 14. The zygomatic process of the frontal

The frontal bones are narrow and elongated, covering almost half of the dorsal face of the skull, ending in the rostral extremity by two narrow and sharp extensions, representing the nasal processes of the frontal bone.

The nasals do not articulate on the sides with the maxilla, they articulate only with the nasal processes of the frontal bone and of the incisive bone, whereas in the rostral extremity, it ends slightly bifid, delimiting a small notch (Figure 2).

The lateral surface of the skull of the Eurasian brown bear (*Ursus arctos arctos*) has an incomplete orbit. The zygomatic process of the frontal bone is very short; so it does not join the zygomatic arch.

The temporal fossa is elongated and slightly convex. The orbito-temporal crest is strongly highlighted. The fossa of the lacrimal sac is narrow. The lacrimal duct is delimited by the lacrimal bone and the maxillary bone. At the level of the lacrimal bone, a single lacrimal foramen can be observed (Figure 3).

Figure 2. Eurasian brown bear skull (*Ursus arctos arctos*) - dorsal face - 1. Nasal bone; 2. The incisive bone; 3. Maxilla bone; 4. Vomer bone; 5. Nasal processes of the frontal bone; 6. Infraorbital foramen; 7. The zygomatic bone; 8. The zygomatic process of the frontal bone

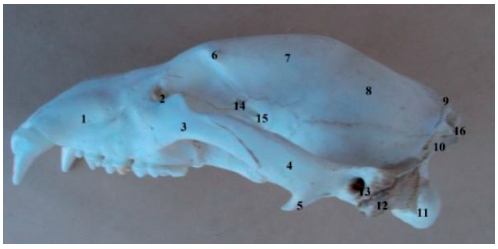

Figure 3. The lateral face of the skull in Eurasian brown bear (*Ursus arctos arctos*) - 1. Maxilla; 2. Lacrimal foramen; 3. Zygomatic bone; 4. The zygomatic process of the temporal; 5. Retroglenoidal process; 6. The zygomatic process of the frontal bone; 7. Temporal line; 8. Parietal bone; 9. External sagittal crest; 10. Nuchal crest; 11. Occipital condyle; 12. The mastoid process of the petrous temporal bone; 13. External auditory canal; 14. Ethmoidal foramen; 15. Optic foramen; 16. External occipital protuberance

The maxillary foramen is very wide and elongated in appearance, being located under the fossa of the sac of the lacrimal gland, and communicates with the infraorbital foramen through a short infraorbital duct. Between the maxillary and the lacrimal foramen, we can observe another foramen, possibly of vascular nature (Figure 4).

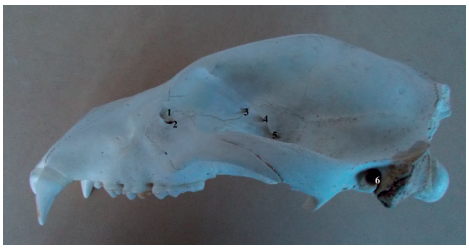

Figure 4. The lateral surface of the skull in Eurasian brown bear (*Ursus arctos arctos*) - 1. Lacrimal foramen; 2. Vascular foramen; 3. Ethmoidal foramen; 4. Optic foramen; 5. Orbital fissure; 6. External auditory canal

In the caudal extremity of the maxilla, behind the tubercle and under the maxillary foramen, there are two other foramina, the sphenopalatine foramen and the caudal palatine foramen, separated by a small bony blade arranged transversely, thus giving the area a layered appearance.

On the lateral face of the maxilla, an infraorbital foramen can be seen, extended in the rostral direction by a small fossa. The zygomatic process of the maxilla is arranged ventromedial in the rostral part of the zygomatic arch, being articulated with the lacrimal bone under the maxillary foramen. The zygomatic bone has two processes: one of them being located in the reduced rostrum and another long one, situated in a latero-caudal position.

The zygomatic process of the temporal situated in a latero-rostral position, presents ventrally an elongated latero-medial, deep glenoid cavity, behind which there is a prominent retroglenoidal process (Figure 5).

The tympanic bulla is reduced and the muscle process is short. The mastoid process of the temporal petrous part is well developed and is located between the temporal and occipital squamous parts.

The external auditory canal is considerably highlighted. Behind and at the base of the retroarticular process is located the retroarticular foramen. The stylomastoid foramen is located behind the external auditory canal.

At the level of the orbital hiatus, the ethmoidal foramen, the optic foramen, the orbital fissure, the foramen rotundum and the rostral alar foramen open together, being separated by a bony blade arranged transversely (Figure 6). At the level of the pterygoid process of the sphenoid, can be seen the arrangement of the alar canal, through which the anterior alar foramen communicates with the posterior alar foramen**.** Behind the caudal opening of the alar canal, the *foramen ovale* can be observed.

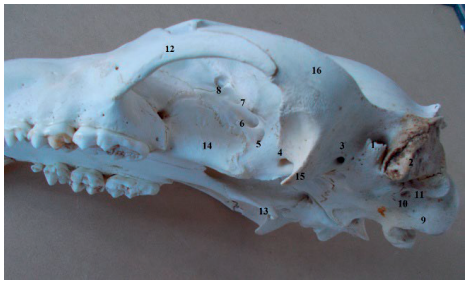

Figure 5. Lateral-ventral face of the skull in Eurasian brown bear (*Ursus arctos arctos*) - 1. External auditory canal; 2. The mastoid process of the temporal piramide; 3. Retro-auricular foramen; 4. Posterior alar foramen; 5. Anterior foramen; 6. Optic foramen; 7. Orbital fissure; 8. Ethmoidal foramen; 9. Occipital condyle; 10. Hypoglossal nerve foramen; 11. Paracondylar processes; 12. Zygomatic bone; 13. Pterygoid hamulus 14. Palatine bone; 15. The retroglenoidal process; 16. The zygomatic process of the temporal bone

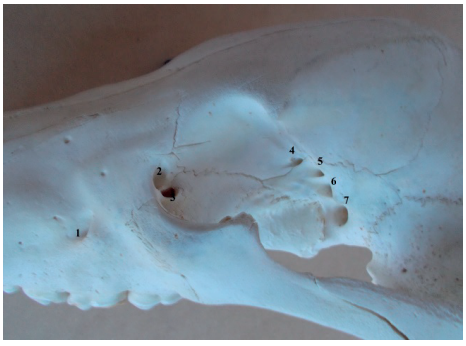

Figure 6. The lateral face of the skull in the Eurasian brown bear (*Ursus arctos arctos*) - 1. The infraorbital foramen; 2. The lacrimal foramen; 3. Vascular foramen; 4. Ethmoidal foramen; 5. Optic foramen; 6. Orbital fissure; 7 Foramen rotundum

The diastema or interalveolar margin is occupied almost entirely by the canine alveolus.

At the level of the maxilla, the premolar and molar alveolar reliefs can be noticed, especially at the level of the carnassial tooth.

The ventral surface of the skull of the brown bear (*Ursus arctos*) presents at the caudal extremity, the convex condyles of the occipital, delimiting a wide and oval-looking foramen magnum. The paracondylar processes are obvious, with the free extremity drawn ventrocaudal, delimiting with the condyles of the occipital a narrow and deep ventral condylar

fossa. The foramen of the hypoglossal nerve is located at the level of the condylar fossa.
The basioccipital is wide, and on the sides, there are two small, ventrally-oriented processes, at the base of which a small fossa is observed.
The tympanic bulla is reduced, flattened dorso-ventral, and the muscular process is barely outlined.
The carotid foramen is located in the rostro-ventral plane, behind the tympanic bulla, and the jugular foramen is situated in a ventro-cadual direction, separated by a small bony blade.
Between the tympanic bulla, the paracondylar processes and the mastoid process, is situated an elongated and relatively deep fossa in ventro-latero-caudal position, in which are located the stylomastoid foramen and the articular surface for the tympanohyoid. The foramen lacerum is not present.
In the oral extremity of the tympanic bulla, at the border between it and the basisphenoid wing, the musculo-tubal duct is located laterally, and medially there are two superimposed foramina, separated by a thin bone blade, the one on the upper floor being represented by the foramen spinosum, and the one on the lower floor of a foramina, probably vascular (Figure 7).

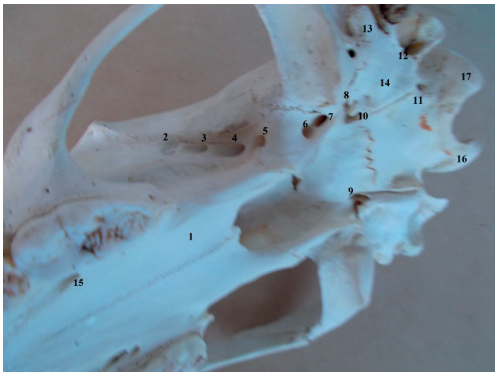

Figure 7. The ventral face of the skull in Eurasian brown bear (*Ursus arctos arctos*) - 1. Horizontal processes of the palatine; 2. The ethmoidal foramen; 3. Optic foramen; 4. Orbital fissure; 5. Foramen rotundum; 6. Caudal alar foramen; 7. Foramen ovale; 8. Musculo-tubal canal; 9. Spinous foramen; 10. Vascular foramen; 11. Jugular foramen; 12. Stilo-mastoid foramen; 13. External additive canal; 14. Tympanic bulla; 15. Major palatine foramen; 16. Paracondylar process; 17. Occipital condyle

The bodies of the basisphenoid and the presphenoid are broad, and the pterygoid bone has a highly underlined and ventro-caudal oriented hamulus. The pterygopalatine crests are long and oriented laterally into their caudal extremity. They delimit a wide guttural opening.
The articular surface for the mandible is arranged on the ventral side of the zygomatic process of the temporal bone, being represented only by a transversely elongated glenoid cavity, behind which is arranged a well-grown retroglenoidal process and rostral curvature. At the level of the caudal surface of the retroglenoidal process, a retroauricular foramen is observed in a dorso-caudal position.
The palatine arch, made by the palatine processes of the incisive bone, the palatine processes of the maxilla and the horizontal processes of the palatine, narrows in the caudal extremity.
The horizontal processes of the palatine are much elongated in the rostral extremity, ending sharply, having a relatively triangular appearance.

Figure 8. The ventral face of the skull in Eurasian brown bear (*Ursus arctos arctos*) - 1. Palatine fissure; 2. The interincisive canal; 3. Major palatine foramen; 4. The palatine process of the incisive bone; 5. Horizontal processes of the palatine; 6. Basisphenoid; 7. Bazioccipital; 8. Glenoid cavity; 9. The retroglenoidal process; 10. Caudal alar foramen; 11. Foramen ovale; 12. Jugular foramen; 13. Foramen of the hypoglossal nerve; 14. The paracondylar process; 15. The mastoid process of the petrous part of the temporal bone; 16. Occipital condyle; 17. Foramen magnum

At the border between the rostral extremity of the palatine bone and the caudal extremity of the palatine process of the maxilla, near the dental alveoli, the major palatine foramina are arranged on the lateral parts. They extend rostrally with a small palatine grooves, reaching the level of the palatine fissures. Behind the major palatine foramina, we can observe a small accessory palatine foramen. The palatine fissures are wide in the rostral extremity, ending sharply in the caudal part and being arranged near the canine alveoli (Figure 8).
In the caudal third of the interincisive fissure, there is a small interincisive canal.
The nuchal face of the Eurasian brown bear (*Ursus arctos arctos*) has a relatively quadrilateral appearance.
In the dorsal plane, at the level of the nuchal face, a reduced external occipital protuberance is observed, from which the well nuanced nuchal crests descend on the lateral parts. The external occipital crest is well developed in the upper part, being reduced in size near the for a-men magnum. On either side of the external occipital crest, below the nuchal ridges, there is a thickened tubercle for muscle insertion (Figure 9).
The paracondylar processes are short and do not exceed the occipital condyles in the ventral direction.

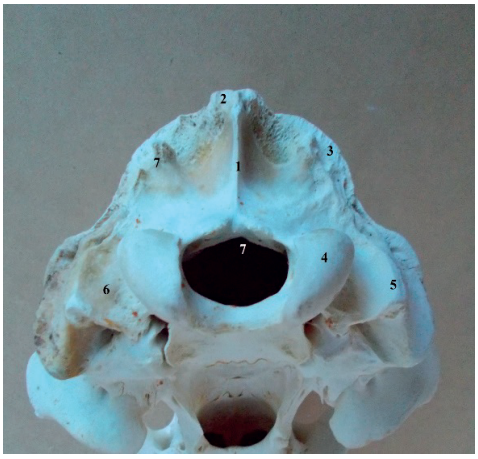

Figure 9. Ventral face of the skull in Eurasian brown bear (*Ursus arctos arctos*) - 1. External occipital crest; 2. External occipital protuberance; 3. Nucal crest; 4. Occipital condyle; 5. Paracondylian process; 6. The condylar fossa; 7. Foramen magnum; 7'. Tubercle for muscular insertion

The mandible of the Eurasian brown bear (*Ursus arctos arctos*) is formed by a paired bone. The mandibles are articulated in the rostral part by synchondrosis.
The body of the mandible is short. The diastema is evident, presenting the canine alveolus (Figure 10).
The horizontal branch of the mandible has a relatively convex ventral edge, ending in a noticeable angular process, curved dorsally and flattened dorso-ventral, and slightly excavated on the dorsal part
On the lateral surface of the horizontal branch of the mandible, there is a mental foramen, accompanied by 2-3 accessory mental foramina (Figure 11).
At the level of the curved branch of the mandible, on the lateral side, a deep and relatively triangular-looking masseteric fossa can be seen. On the medial surface, the pterygoid fossa is superficial, and the mandibular foramen is placed near the caudal margin.

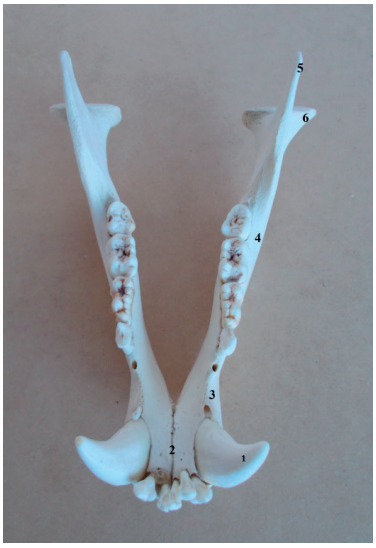

Figure 10. Eurasian brown bear mandible (*Ursus arctos arctos*) - dorsal view (original photo) - 1. Canine; 2. The body of the mandible; 3. Interdental space (diastema); 4. The horizontal branch of the mandible; 5. Coronoid process; 6. Condylar process

The coronoid process is strongly developed, with a rounded free end and slightly caudally oriented. The condylar process is short and has a convex surface. The corono-condylar notch is elongated and not very deep.

Figure 11. Eurasian brown bear mandible (*Ursus arctos arctos*) - side view (original photo) - 1. Mental foramen; 2. Accessory mental foramen; 3. Masseter fossa; 4. Coronoid process; 5. Condylar process; 6. Angular process; 7. Interdental space (diastema)

CONCLUSIONS

The skull of the brown bear has an elongated and relatively narrow dorsal face.

The external sagittal crest is short, not very prominent, and the zygomatic processes of the frontal bone are very reduced.

The nasal bones do not articulate with maxilla.

The orbit is incomplete, there is an orifice between the maxillary and the lacrimal foramen, we assume it is of vascular nature.

The foramen rotundum and the rostral alar foramen are separated by an osseous blade.

The external auditory canal, circular in section, is strongly highlighted.

The tympanic bulla is reduced and flattened dorso-ventral, the muscular process is barely represented, and there is no *foramen lacerum*.

Between the tympanic bulla and the wing of the basisphenoid, there are two medial overlayed foramina, a spinous foramen being dorsal, and a ventral vascular foramen.

The coronoid process of the mandible is well-developed, with the free extremity rounded and slightly drawn caudally, and the condylar process is short, with a convex surface.

The angular process of the mandible is curved dorsally, flattened dorso-ventral and lightly excavated on the dorsal face area.

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EFFICIENCY AND THERAPEUTIC CONDUCT OF SURGICAL PROCEDURES FOR MATURE CATARACTS IN A DOG, CASE REPORT

Elena BONEA, Cornel IGNA

Banat's University of Agricultural Sciences and Veterinary Medicine "King Michael I of Romania" from Timisoara, Faculty of Veterinary Medicine, 119 Calea Aradului, Timisoara, Romania

Corresponding author email: drboneaelena@yahoo.ro

Abstract

Cataracts are not typically brought to the attention of a veterinarian until owners report their dogs bumping into different kind of things, because when a cataract is fully developed, mature, it leaves the eye functionally blind. The patient of this study is a male old dog, 15 years old, completely blind, with mature bilateral cataract. The quality of his life had a very low score, the dog injured himself with the objects around, his behaviour changed and became retired, sleeping most of the time and became aggressive with owner and dogs around. Because the only effective and recommended treatment is surgery, for economical owner's reason, for the right eye of the dog we chose the manual extracapsular cataract extraction surgical procedure. Short follow-up surgery, dog became visual animal, but irreversible complications occurred, so, after 3 months' time, for the left eye of the dog we proceed with the phacoemulsification method of cataract extraction with the implantation of an intraocular artificial lens. Both eyes surgical methods had good post operatory results, but long-term for the second method results were superior.

Key words*: dog cataract, cataract surgery, phacoemulsification, intraocular lens*

INTRODUCTION

A cataract is a permanent change in the thin, clear, highly organized protein fibers that make up the lens of the eye of the dogs.

This change transforms the clear protein into a milky, white, opaque one. Once the proteins change throughout the entire lens, it is like a frosted pane of glass, and as the lens thickens it becomes more difficult for the pet to see around. When the opacity covers about 60% of the total lens area, visual impairment often becomes apparent.

If the opacity progresses to 100% of the lens, in which case the cataract is fully developed (mature), the dog will be functionally blind in the affected eye (Grogan, 2020). Degree of maturation of cataract are: incipient - earliest lens changes, focal opacity of the lens and radiations or "spoke" shaped opacities; immature - the cataract affects the entire lens, but does not completely abolish the tapetal reflection; mature - lens totally opaque, fundic reflex absent, vision lost with the lens usually normal size; hyper mature - resorption causing decrease in total lens volume and wrinkling of anterior capsule and may have areas of fibrosis and dystrophic calcification (Kecová et al., 2004).

Usually, cataracts are not typically brought to the attention of a veterinarian until owners report their dogs bumping into different kind of things or furniture.

Diagnosis of cataracts is set up on the ophthalmic examination findings and they are seen as an opacification of the lens. A full physical examination, clinical history and, when warranted, a comprehensive hematologic and blood chemistry, should be pursued for patients presenting with cataracts.

Clinically the mature and hyper mature cataracts can be easily seen like a white disk behind the iris. The incipient and immature cataracts may be observed easily with retro illumination of the eye after pharmacologic pupil dilation (i.e., tropicamide or atropine administration topically). Small opacifications of the lens may be observed as dark or light areas within the lens.). Retro illumination will also allow the observer to evaluate the tapetal reflection, thereby allowing differentiation between immature and mature cataracts, if the entire lens is affected. Ophthalmic evaluation with retro illumination and direct focal illumination will help differentiate cataracts from nuclear sclerosis, a normal aging change of the lens caused by increased density of nuclear lens

fibers. Identification of the location of the cataract within the lens and further characterization of the cataracts typically require advanced training and slit-lamp bio microscopy performed by a veterinary ophthalmologist.

According to Hlinomazová and Vlková, 2003, after clinical examination of the patient, the preoperative examination should include also a complete ophthalmologic exam. Also, intraocular pressures (IOP) for both eyes need to be checked to see for underlying glaucoma or uveitis.

There are many studies for dog lenticular disorders or with lens changes, for all degrees of cataract and cataract treatment options and their follow-up (Davidson et al., 1991; Nassise et al., 1990, 1991; Gaidon et al., 1991; Lim et al., 2011; Fischer and Meyer-Lindenberg, 2014; Ionascu, 2013). When the dog's quality of life is being significantly affected by the loss of vision resulting from cataracts, the only efficient and recommended treatment is surgery with the lens extraction (Lim et al., 2011). There are two methods for lens extraction in dogs: extra-capsular surgical technique when the lens is extracted through a corneal or scleral incision line (Patil et al., 2014) and intra-capsular lens phacoemulsification, or removal of lens material through small incision with fragmentation, emulsification and aspiration (Davidson et al., 1991; Gaiddon et al., 1991, 2000; Gilger, 1997; Nasisse et al., 1990, 1991).

In patients for which cataract surgery is not an option (e.g., severe cardiac disease precluding anesthesia, clients have declined surgery, etc.), supportive topical therapy should be instituted long-term to help prevent complication such as lens-induced uveitis and glaucoma secondary to cataracts (Park et al., 2009).

CASE PRESENTATION

This case report describes the treatment of a bilateral cataract in a dog by two different surgical method, one for each eye and the use of an intraocular lens in one eye.

The patient of this case study has the name Unic, it is a male dog, 15 years old, cross-breed, medium size, 18 kg, overweight, non-neutered, completely blind. His owner reported that his dog was injuring himself with the objects around, his behavior changed and became retired or sleeping and sometimes even got aggressive with him or with the other dogs from the house.

At clinical examination we noticed that the dog was blind, and thorough slit lamp examination, we noticed white disks behind both iris' eyes because of the lens opacification. After pharmacologic pupil dilation (i.e., tropicamide topically) mature cataracts were observed easily with retro illumination of the eye. We set up the diagnostic of mature bilateral cataract for our patient. Tonometry show that both Unic's eyes had normal intraocular pressure (IOP) with value of 13 mm Hg on the right eye and 15 mm Hg on the left eye, this investigation shown that no underlying glaucoma occur as a cataract complication.

Thorough slit lamp examination we checked the possible visual interference in cornea (opacity, edema, pigmentation) or anterior lens capsule and the state of suspensory apparatus of the lens (signs of lens subluxation). No fundoscopy exams were done in order to check retinal detachment or progressive retinal atrophy and no electroretinography was done in order to check the retinal function, at the dog's owner request.

Blood work is a very important diagnostic tool that provides a significant amount of information about a pet's health. We tested blood glucose level; dog value was 102 mg/dl (normal dog values 80-120 mg/dl), which could have revealed diabetes, which is a systemic disease that can accompany cataract (Falca et al., 2011).

We did also a biochemical blood profile in order to assess the function of internal organs. Biochemical analyses of dog's blood had the next results: alkaline phosphatase (ALP) U/I 33 (<68), amylase U/I 1005 (<1289), bilirubin mg/dl 0.1 (0.5), glutamine transferase GGT U/I 15 (<20), alaninaminotransferase ALT U/I 50 (<89), cholesterol mg/dl 220 (110-300), creatinine mg/dl 0.8 (<1.8), urea mg/dl 30 (<54), all the parameters we tested were in range of dog normal values.

We also did a Complete Blood Count of the dog, including red and white cell count and the measure of hemoglobin. Values we count were as follows, all of the dog were normal values (we represented in parentheses the normal dog's values): white blood cell count (WBC) 10.6 x 10^3/mcl (4-12 x 10^3/mcl), red blood cell count

(RBC) was 6.2 x 10-6/mcl (5.7-10.5 x 10^6/mcl), hemoglobin (HGB)- 14 g/dl (9-16 g/dl), hematocrit (HCT) 48% (38-52%), corpuscular volume (MCV) 55.9 fl (40-60 fl), corpuscular hemoglobin (MCH) 18.2 pg (15-20 pg) corpuscular hemoglobin concentration (MCHC) 33.5 g/dl (32-36 g/dl) , platelets (PLT) 210/mcl 160-420/mcl), segmental neutrophils 52% (51-72%), Lymphocytes 30% (8-35%), Monocytes 6% (1-9%), (Eos) 4% (0-9%), Basophils 2% (0-2%), all the parameters were in range.

We discuss the cataract surgical treatment options with the owner. Together, we decided to start with one of the eyes, the right one, with the surgical extra-capsular lens extraction method.

Surgery was performed under general anesthesia, which consisted of premedication with diazepam (intravenous, dose 0.5-1 mg/kg, Diazepam Terapia SA, Romania) and ketamine (8 mg/kg, Ketamidor, Richter Pharma AG, Austria), and narcotic induction with Propofol 1% (3-6 mg/kg - self-dosing, Fresenius SE&C, Germany), after which the subject was intubated and connected to the closed-loop, assisted-breathing inhalation anesthesia apparatus, the anesthesia being maintained throughout and maintenance with isoflurane (at concentrations ranging from 5% to 0.5%, Anesteran, Rompharm Company, Romania) vaporized in oxygen using intermittent positive pressure ventilation.

From 2 h before surgery, atropine, tropicamide with phenylephrine, dexamethasone, flurbiprofen sodium and gentamycin eyedrops were administered every 30 min.

For a good visualization of the anatomical components of the eye we used a magnifying glass with lamp. The surgical instruments we used were the ophthalmological ones: curved Castroviejo corneal scissors, corneal forceps, conjunctival forceps, Castroviejo straight corneal forceps, Mackool Barraquer 0.5 mm spatula, dog eye speculum, single-use eye cannula.

The eyeball was fixed with an eye speculum that allows easy access to operative area with less injury (Figure 1). A stab incision was made through the sclera above the cornea using a surgical blade that was extended with corneal scissor (Figure 2). After the entry in anterior chamber, anterior chamber collapsed which was partially filled with saline water to prevent injury to corneal epithelium. Then, capsule was tear with double bended 23 G needle, which was passed horizontally and then twisted to 90º to perform capsulotomy. We used a colorant blue to marked the anterior capsule.

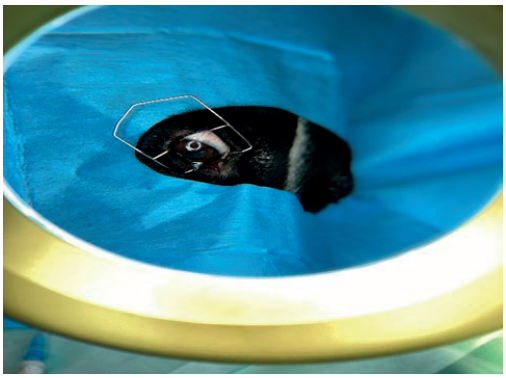

Figure 1. The aspect of the eyeball with the eye speculum through the magnifying glass

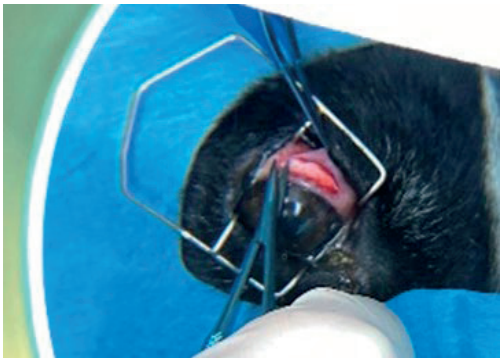

Figure 2. The incision on the sclera

Then, slowly proceed to capsulorhexis, when the capsule was tear at weaker equatorial region of lens. After removal of anterior capsule, the cortical and nuclear material of lens luxated in the anterior chamber (Figure 3) from where we removed them, without disturbing posterior lens capsule, with the use of a small lens loupe (Figure 4). We perform a gentle irrigation of anterior chamber using saline water, and after, we closed the sclera and the conjunctiva suturing with 8-0 mononylon in three separate stitches, after filing the anterior chamber with saline water, with a good aspect of the eye, with no hemorrhagically residues. Dog received an intraconjuctival injection with gentamycin and dexamethasone, and his owner was recommended to follow a schedule of dosage

and prescribed with dexamethasone and enrofloxacin for initial one-week general treatment after surgery and topical eye drops with gentamycin and dexamethasone.

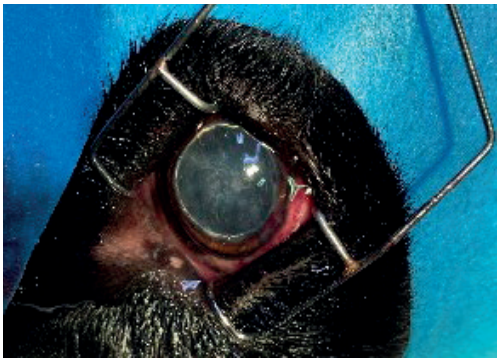

Figure 3. The luxation of the lens after the capsulorhexis

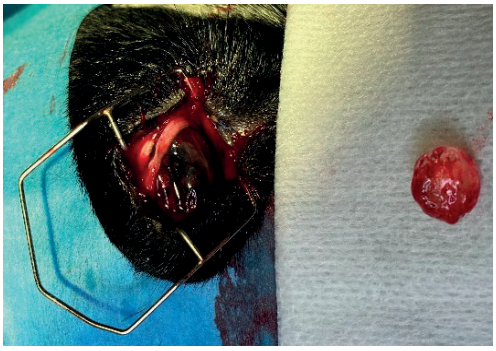

Figure 4. The aspect of the eye after removing the lens and the aspect of the removed lens

The dog was examined at days 7, 14, 21, one and three months after the surgery was performed. At day 7, the dog eye was examined, and uveitis occurred post-surgery, so he was prescribed general treatment with dexamethasone until day 30. At 14-day examination, a cloudiness in the upper half of the eye corneas was noticed. Corneal opacification progressively advanced and when we re-examined the dog at day 30 after the surgery (Figures 5, 6), also posterior capsular opacification (PCO) developed and the dog had partial loss of vision with his right eye. No other post-operatory complication was observed.

Reexamination dog's eyes after 3 months, when owner came back to the Clinique with the same problem, that the dog cannot see, we noticed that the post-operatory corneal opacification made his right-eye blind again.

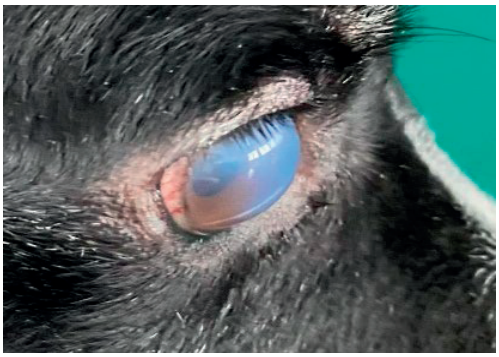

Figure 5. Aspect of the cornea at day 30 after surgery

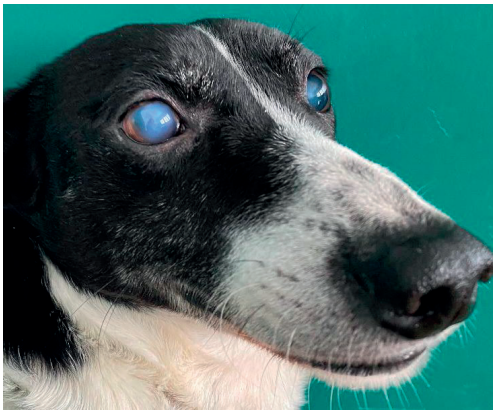

Figure 6. Aspect of the corneal opacity of right eye at day 30 post-surgery and mature cataract of left eye

Discussing surgical treatment options with the owner again, and taking in consideration the many studies that consider phacoemulsification surgical method with intraocular lens implantation, as a highly successful procedure to restore vision in dogs with cataracts, with few post-operatory complications (Nassise et al., 1991; Wilkie and Colitz, 2013), we proceed with this method for dog's left eye surgical treatment. This procedure uses an ultrasonic device to break up and remove the mature lens from the dog's eye, and is the same procedure that it used in cataract surgery on people.

For this surgical method we used a surgical microscope, with magnification in 5 steps, with two 10x tilting binoculars with wide field, 50W LED lighting system, with articulated mobile arm and with the possibility of horizontal and vertical movement. Pre-operatory preparations were the same as first method.

During phacoemulsification technique, we started with a small incision at the edge of the

clear cornea to enter in the anterior eye chamber. We injected trypan blue using a canula in the anterior chamber for better visualization of the lens capsule for performing the capsulorhexis (Figure 7), and then with a viscoelastic substance, we filled the entire anterior chamber of the eye.

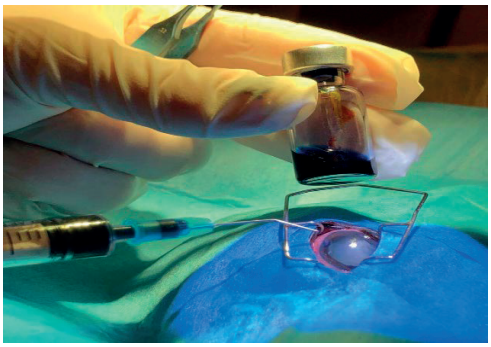

Figure 7. Injection of trypan-blue in the anterior eye chamber

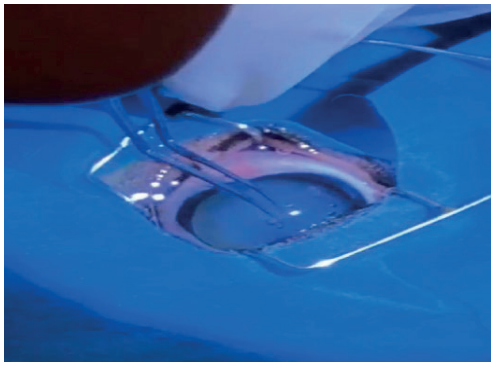

Figure 8. Capsulorhexis procedure

We performed the circular capsulorhexis continuously in the equatorial area (Figure 8), with the aid of a Utrata forceps and a capsulorhexis cystotome, then followed by hydrodisection, rotation and phaco emulsification of the lens core with the phaco device sonde. The fracture technique used was phaco-chop and the phacoemulsification parameters were set in 60% of ultrasonic power, 40 cc/min, 12 pulses/sec.

The aspiration of the cortical waste was performed with the irrigation/aspiration pen of the device (Figure 9). Then, we used a thin haptic intraocular lens (IOL) made of silicon elastomer and acrylate/methacrylate polymers, foldable, for implanting through the small corneal incision (2.5-3.5 mm).

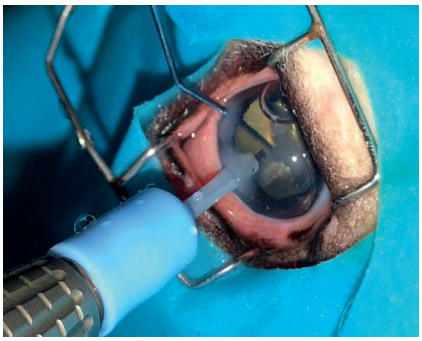

Figure 9. Phaco-chop fragmentation of the lens, with irrigation and aspiration of cortical waste

The IOL came sterile with single-use preloaded injector for easy handling and insertion. We previously folded the IOL using an IOL-holding forceps, then we inserted it into the IOL cartridge and after, through the incision, into the capsular bag from where we removed the mature lens.

Finally, it was performed the aspiration of the viscoelastic substance and the injection of an air bubble on the anterior chamber, and hydration of the surgical borders. A two stitches suture using 8-0 mononylon was made on the main incision.

Dog received an intraconjuctival injection with gentamycin and dexamethasone, and his owner was recommended to follow a schedule of dexamethasone and enrofloxacin for initial one-week general treatment after surgery, and topical eye drops with gentamycin and dexamethasone. The animal's appetite was back to normal immediately post-surgery and all vital parameters remained unchanged.

The dog was examined at days 7, 14, 21 and one month after the surgery was performed.

At day 7, a small corneal inflammation was noticed at the incision area. Topical eye drops like previously, were recommended for two more weeks, two times per day.

No other post-surgical complication was noticed until day 30.

Two months after surgery the dog presented for a recheck examination. The animal was reported to be active, with normal food and water intake and perceived to be visual and comfortable.

DISCUSSIONS

Cataract surgery in companion animals is considered an elective procedure that aims to improve the animals' quality of life.

Because of the considerable progress in cataract surgery in recent decades, lens opacities can now be operated on with very good short-term success (90-95%), taking into account that proper patient selection is essential to maximize the probability of a successful surgical outcome (Fischer and Meyer-Lindenberg, 2014).

According to Patil et al., 2014, manual extra capsular cataract extraction was standard and the most popular method of lens removal in dog for many years in which, the anterior lens capsule, the lens cortex and nuclear material were extracted. Although the approach of extra capsular cataract extraction is primitive, it has been still recommended in cataract cases of dog due to size, density of cataractous lens and thick capsule (Patil et al., 2014), also for being an economical advantageous method that doesn't need a special equipment. For all these reasons, and mostly because of financial reasons, together with the dog owner's we chose and proceed with this method for dog right eye.

First days after the surgery, the dog became very active, comfortable and has regained his vision. Most dogs will have enough vision to tackle obstacle, boost confidence and avoid life-threatening accidents and improve overall their quality of life, even they remain with aphakia at eye, like in this right dog eye. After 14 days post-surgery, the dog started to develop a corneal opacification and posterior capsular fibrosis that led to definitive opacification of cornea at the next re-examinations, that made him again blind. If postoperative complication can be prevented (PCO in our case right eye), then the extracapsular cataract manual extraction can be an alternative to owners which can't afford lens cost and would like to resolve vision loss in their pet.

Development of corneal opacity might be due to excessive handling, as this case selected had mature cataract, this is in agreement with Dziezyc, 1990 and Joy et al., 2011.

According to many studies, the phacoemulsification surgical method and intraocular lens implantation is a highly successful procedure to restore vision in dogs with cataracts (Nassise et al., 1991; Wilkie and Colitz, 2013). Nowadays, even in veterinary medicine, the method of choice in most cases is phacoemulsification, or removal of lens material through small incision with fragmentation, emulsification and aspiration (Davidson et al., 1991; Gaiddon et al., 1991, 2000; Gilger, 1997; Nasisse et al., 1990, 1991). The only disadvantages are the costs of equipment and materials of this procedure.

According to Sigle and Nasisse, 2006, surgical success rates have increased over time with refinements of surgical technique, but a surgical success is not guaranteed. Surgery is considered to have failed when dogs develop painful and/or blinding complications such as endophthalmitis, retinal detachment, or glaucoma. Our results with this method for dog's left eye were successful and we didn't have any complications like the studies describes.

In addition to the advantages of improved vision in pseudo phakic eyes, there are several studies suggesting that posterior capsular opacification (PCO), a common complication after cataract surgery in humans and animals, may be mitigated by the IOL. This is supported by several studies that showed that the lens design, material and chemical properties could decrease PCO (Nishi et al., 2004; Buehl and Findl, 2008). According to these, we used a soft acrylic haptic IOL for the dog left eye. Two months post-surgery, the results were good and no PCO was observed in our dog case.

In the recent past there were studies of Davidson et al., 2013, valuating the IOL size and dioptric power in pet dogs, concluded that most dogs will see much better when an artificial lens (IOL - 41D) is implanted inside the lens capsule.

According to Konopińska et al., 2021, phacoemulsification method is considered to significantly reduce endothelial cell damage, which is preventing the posterior capsular opacification, corneal opacification, but despite all the improvements in the techniques used in cataract surgery and lens design, statistically, PCO was reported to occur after surgery in some cases.

Advantages of phaco-chop include also reduction of zonular and capsular stress because forces are directed toward an opposing instrument and the phaco tip is kept in a central 'safe zone' in the middle of the pupil (Kecova

and Neaas, 2004). But, because of the mature cataract, the time of this technique was longer, because of the difficulty of ultrasound to chop the cataract of its hardness and complication according to literature may appear (Davidson et al., 1991; Nasisse et al., 1990, 1991).

In conclusion, our case report reveals good long-term results after the phacoemulsification surgical method with intraocular lens implantation.

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ANTI-ANTHRAX VACCINATION IMPACTS ON IMMUNITY IN EXTENSIVELY RAISED DAIRY COWS

Paul-Adrian BOR[1], Gheorghița DUCA[2], Carmen Dana ȘANDRU [1, 2], Diana OLAH[1], Marina SPÎNU[1, 2], Emoke PALL[1, 2], Constantin CERBU[1], Florina MARIAN[1], Adrian POTÂRNICHE[1], Aurel VASIU[1]

[1]University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, 3-5 Calea Manastur, Cluj-Napoca, Romania
[2]Institute of Research and Development for Montanology Cristian, Sibiu, Romania

Corresponding author email: aurel_vasiu@yahoo.com

Abstract

Anti-anthrax vaccination is stressful for animals, with a potential negative impact on some immune effectors. This research aimed to investigate the extent of those and estimate the effectiveness of vaccination. Twenty-three extensively raised dairy cows and 11 calves were sampled before and two weeks after the anthrax vaccination (R 1190 Stamatin strain). Blood samples were collected, subjected to blood counts and the N/L ratio was calculated as a stress index. The total Ig (24%o zinc sulphate precipitation) and circulating immune complexes (CIC) (4.2% polyethylene glycol precipitation) were quantified from the serum samples.
The N/L ratio was of 0.79 ± 0 .59 before and 0.58 ± 0.45 after the vaccination in adult animals, while in calves it increased significantly (0.61 ± 0.25 and 1.11 ± 0.68, respectively). The total Ig concentrations supported a lesser immunization of the calves than in dairy cows (6.95 ± 2.09 versus 12.10 ± 6.68 Vernes degrees, respectively) supporting the more stressful effect of the primary vaccination than of the booster one. Nevertheless, the antibody clearance was enhanced in the younger animals (5.4 ± 0.25 versus 1.0 ± 0.1 ODU, respectively). Repeated stimulation is the substrate for an enhanced adaptive response to vaccination in cattle.

Key words*: dairy cows, anthrax, vaccination, immunity, extensive raising.*

INTRODUCTION

Within the category of farmed, cattle have a major socio-economic, health, biological and ecological importance in the economy of numerous states, having a positive economic impact on the rural environment and representing a substantial source of income for many communities around the world (Thornton, 2010). Still, farmers involved in cattle breeding, in spite of providing support to the local markets and jobs for other members of the community (veterinarians, technologists/ mechanics and distributors of animal products), have to face numerous difficulties, such as those imposed by expanding environment protection and animal health and welfare legislation. The carbon fingerprint limitations are also likely to influence the cattle farming (Herrero et al., 2013; Rotz et al., 2013).
The societal development will trigger further progress in breeding technologies, improved nutrition strategies and perfected preventive measures applied to farmed animal diseases to intensify the production potential of bovine. Human health concern based on a nutritional opinion diverted from red meat or cow milk consumption could still heavily moderate the increase in numbers and size of bovine farms. Cattle-farming is one of the main branches of animal production, which provides people and the processing industry with particularly valuable products and by-products such as milk and meat as food products, and hides, bones, hooves, horns and manure as by-products (Pawlak and Kołodziejczak, 2020), especially in developing countries.
The historically-supported close proximity of people and animals, bovine included, leading to domestication, has also enhanced the progression of a new host-pathogen relationship and jump over species for various microbes (Schiffman et al., 2002). Thus, numerous diseases of bovine could show a zoonotic character.
Anthrax is considered to be a non-contagious zoonotic disease, albeit the soil represents the main source for *Bacillus anthracis*.

Nevertheless, there are risk categories of farmers, caretakers, veterinarians, slaughterhouse workers or personnel involved in handling carcases or by-products, which are exposed to anthrax infection from animal sources (Rume et al., 2020).

Achieving high-performance production is conditioned by the health of the animals, which are exposed to a multitude of technological factors on the farm, sometimes inducive of undesirable effects. One of the most widespread operations to support health is immune prophylaxis, performed through vaccination operations. Depending on the epidemiological situation, the vaccination protocols applied in cattle farms are diversified, some of them being included in the national strategic program.

Although vaccinations have long been considered a revolutionary means of preventing disease (Turnbull, 1991; Misra, 1996), more and more recent research reveals adverse effects attributed to these vaccinations, especially repeated ones, caused either by the included antigenic structures (hypersensitivity, allergy, anaphylactic shock) or adjuvants used (local inflammatory reactions, anaphylactic shock) (Fasanella et al., 2001).

All these types of reactions are based on an exaggerated reactivity on the part of the immune system.

This research aimed at investigating the stress levels, expressed by neutrophile/lymphocyte ratios and humoral immune responses (total Ig and circulating immune complexes' levels) and estimating the effectiveness of vaccination.

MATERIALS AND METHODS

Biological material. The experiment was performed on dairy cows (n = 23) and young bovine, both males and females (n = 11) over two month of age, reared extensively. The animals were vaccinated according to the Strategic Program, with a live vaccine containing the attenuated R 1190 Stamatin strain, acapsulogenic, oedematogenic and immunogenic.

Venous blood samples were collected on EDTA, for blood smears and on a clotting agent, for serum collection, before and one month after the vaccination.

N/L ratio (Chung et al., 2015). The use of the leukogram, more precisely the calculation of the N/L ratio as a stress indicator (Davies et al., 2008, Hickman, 2017) was used by multiple researchers in medicine and ecology to monitor stress levels in different species. The method is more precise than investigation the level of corticosteroid levels, which make the baseline measurements difficult under stressful field conditions.

For that, blood samples collected on EDTA where smeared at a 30 degree angle and then stained with a Panoptic method after drying (Kit for Fast Staining in Haematology (Fast Panoptic) for clinical diagnosis).

The staining involved the following steps:

a) Dipping the slide in a holder with the fixative for fast staining (Panoptic No. 1) 5 x 1 second each time followed by the drainage of the excess liquid over filter paper;

b) Submerging the slide in another holder with the Eosin (Panoptic No. 2) for fast staining, 5 x 1 second each time and draining;

c) Dipping the slide in a holder with Blue for fast staining (Panoptic No. 3), 5 x 1 second each time and draining;

d) Rinsing the smear with Buffer solution, pH = 7.2;

e) Finally, draining the slide and examining it under the microscope, magnification x 100.

The leukocyte populations were expressed as a % and the N/L ratio was calculated.

Circulating immune complex measurements (Khokhlova et al., 2004)

Circulating immune complexes (CIC) measurement allows the evaluation of the molecular clearance capacity of the physiological mechanisms of the host.

For this, sera were separated from the clotted blood by centrifugation at 2500 rpm for 10·min and kept at –20°C until tested. The CIC precipitating agent was represented by a 4.2% polyethylene glycol (PEG) solution in borate buffer, while buffer-treated samples served as controls for borate-induced precipitation. The reaction was performed in a 96-well-plate to enhance spectophotometrical readings. 196.7·µl of borate buffer and PEG solution, respectively, were mixed with 3.3·µl of each serum, in parallel wells, in duplicate. The samples were allowed to precipitate at room temperature (20-21°C) for 60·min, then read

spectrophotometrically (optical densities, OD) at a wavelength of 450·nm in the test plate (d = 0.5·cm) (multichannel spectrophotometer SUMAL PE2, Karl Zeiss, Jena, Germany). The calculation of CIC levels was performed according to the formula:

$$\mathbf{CIC\ (units) = (OD_{PEG} - OD_{borate}) \times 10^3}$$

Immunoglobulin measurements (Khokhlova et al., 2004). Opsonins or total immune-globulins, represent humoral effectors within the 'first line of defence' against aggressors. The colloidal stability of gamma globulins is lower than that of serum albumins at a pH·7.4, therefore low concentrations of metal salts precipitate the immunoglobulin (24‰). For this, a volume of 193.4·ml of a 0.024 mg/100 ml zinc sulphate solution in barbital buffer was mixed with 6.6·ml of serum and allowed to precipitate for 30·min at room temperature (20-21°C). Optical density (ODU) then was read spectrophotometrically (l = 475·nm, d = 0.5·cm) and used to calculate the concentration in Vernes degrees, by multiplying the optical density with 100.

Statistical processing of the data. Averages of CIC and total Ig for both samplings along with the standard deviations were calculated. The Excel program was used to calculate the significance of the differences between the two samplings by means of the t- Student test.

RESULTS AND DISCUSSIONS

Vaccination generally represents a beneficial method of positively influencing the immune system that has facilitated the eradication of numerous communicable diseases, due to the specificity of the response. The practical application of immune prophylaxis relying on antigenic components isolated from microorganisms is an approach based on understanding the pathogenetic mechanisms, on the analysis of the host's protective response to pathogens, and on the use of immune system regulation by stimulating responses on behalf of T and B lymphocytes.

Most vaccines are injected, a route that involves disadvantages, a practical and also an immunological one. Inoculations are painful and expensive requiring specific materials and a trained operator, and therefore mass vaccination procedure is laborious. Immunologically, this pathway does not always mimic the usual track of pathogens into the body and may not stimulate the immune system properly, for example, if the infection site is one of the mucosae. In addition, inoculation, through the pain caused and the negative reflex induced, exerts an immune suppression, thus, due to increase in corticosteroid levels, the expected result is not achieved.

The present study attempted to establish a relationship between systemic immune protection in extensively reared cows on a family farm by looking at how humoral and cellular immune effectors are affected by the routine anti-anthrax vaccination.

Thus, the non-specific humoral immune response (total immunoglobulins, CIC) as well as the status of leukocytes involved in the first line of defense was investigated in these animals, aiming at establishing the influence of a compulsory immune prophylactic operation with recognized stress inductive effects.

The results obtained showed that the N/L indicator allows estimate the degree of stress induced by the bovine vaccination against cattle (Figure 1).

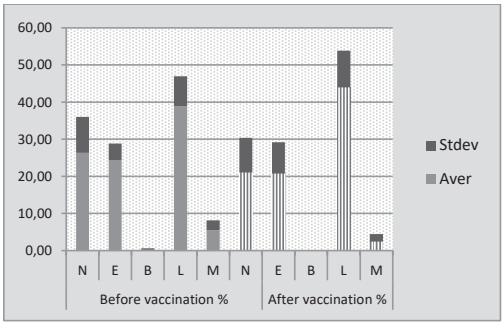

Figure 1. Changes of leukocyte populations subsequent to vaccination (x ± s) in adult animals, subject to repeated vaccination

It has been shown that there is an interrelationship between the N/L ratio (Figure 2) in young cattle in which the stressful effect of vaccination is obvious, with the adaptive cellular immune profile diminished in this category of animals.

Opsonin levels increased in adult animals significantly ($p<0.05$), probably due to the repeated vaccination procedure (Figure 4).

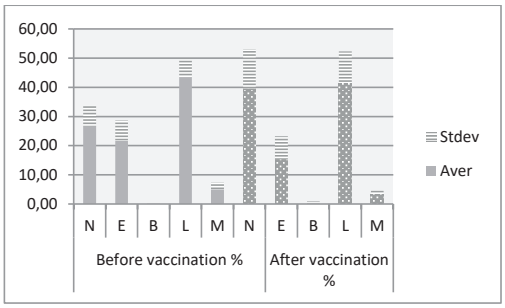

Figure 2. Changes of leukocyte populations subsequent to vaccination (x ± s) in animals subject to primary vaccination

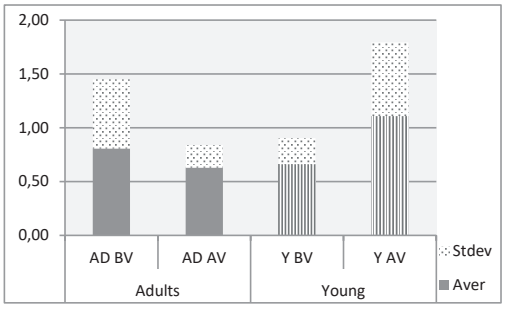

Figure 3. Variation of the N/L ratios (stress indicator) after the vaccination (x ± s)
Legend: AD-adults, Y-young, BV-before vaccination, AV-after vaccination

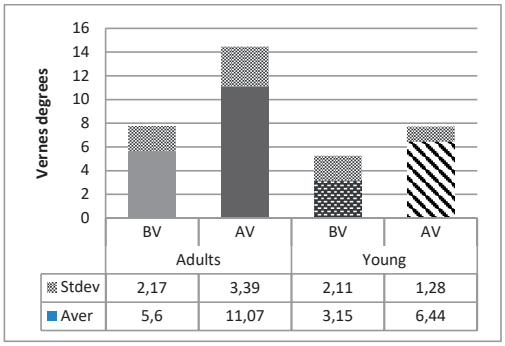

Figure 4. Changes in total Ig levels in the experimental groups after the vaccination (x ± s)
Legend: AD-adults, Y-young, BV- before vaccination, AV-after vaccination

The increase in the Ig levels in young animals, although less than in the adult ones, clearly underlines the possible stimulating effect of the vaccination on the immune system and the general immediate protection of these animals. The lesser values were supported by the higher stress levels indicated by the increased N/L ratios in young animals after vaccination.

Similarly, the detected CIC concentrations showed that there is no relevant increase in this parameter in adult animals, while in young animals, the increase of CIC levels was significant ($p<0.01$), corresponding to the increased possibility of deposition of those in target organs (Figure 5).

In the case of the young group, unlike in adults, the clearance capacity of CIC was statistically significantly decreased post-vaccination ($p<0.05$), probably due to the increased stress levels suggested by the N/L ratio values.

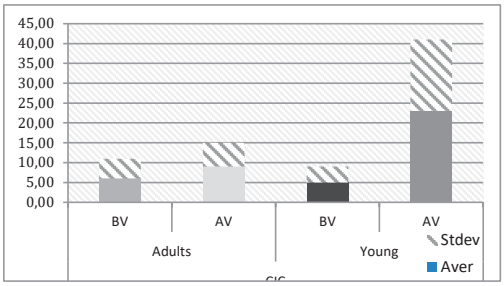

Figure 5. Changes in CIC levels in the experimental groups after the vaccination (x ± s)
Legend: AD-adults, Y-young, BV-before vaccination, AV-after vaccination

CONCLUSIONS

The significantly increased N/L ratios in calves indicated a more severe stress in the latter. In spite of this, there was an increase of the antibody levels but a decrease of their clearance (increased CIC levels) which could lead to pathogenic effects of the deposited complexes. Repeated anti-anthrax vaccination could therefore be the substrate for an enhanced adaptive response to vaccination in adult, but not in young cattle.

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*** Kit for Fast Staining in Haematology (Fast Panoptic) for clinical diagnosis

*** OIE Terrestrial Manual 2018, Section 3.1, Multiple species, Chapter 3.1.1. Anthrax, p. 311-320

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MANAGEMENT AND COMPLICATIONS OF A STRANGLES OUTBREAK

Anca BULGARU[1], Elena NEGRU[1], Horia DINU[1], Dragoş LUPU[1], Mihai DANEŞ[2], Doina DANEŞ[1]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, 105 Splaiul Independentei, District 5, Bucharest, Romania
[2]Faculty of Veterinary Medicine Spiru Haret University, 256 Basarabia Avenue, District 2, Bucharest, Romania

Corresponding author email: anca.floarea@ymail.com

Abstract

The present study is a report of a strangles outbreak in a horse breeding farm. The clinical signs began to appear in the first week of July, and included coughing, purulent nasal discharge, lymph node abscesses and fever. Bacteriological tests confirmed Streptococcus equi ssp. equi infection. The horses were treated with Penicillin 12 mg/kg, from the onset of the clinical signs: 38 of the 42 affected horses showed improved clinical status after 5-10 days of therapy. The other 4 foals were diagnosed Rhodococcus equi induced pneumonia, and they were placed under rifampicin (7.5 mg/kg) and claritromicin (7.5 mg/kg) treatment. Of the 38 horses treated successfully with penicillin, 6 horses aged 3 to 6 months, were later dignosed with Streptococcus equi ssp. zooepidemicus induced pneumonia, and were also treated with rifampicin and clarithromicin. By the end of September, none of the horses present on the farm showed any more signs of respiratory infections. Daily monitoring of the herd, active bacteriological surveillance and early onset of antibiotic therapy were key factors in avoiding severe complications of strangles and limiting mortality.

Key words*: Equine strangles, horses, respiratory infection, Streptococcus equi, Streptococcus equi ssp. Zooepidemicus.*

INTRODUCTION

Strangles is one of the most frequently diagnosed and important infectious diseases of horses worldwide. The disease is highly contagious, affects mainly young horses, but mature, naive horses may also be susceptible (Piche, 1984). The etiological agent of strangles is *Streptococcus equi* subspecies *equi* (*S. equi*), a β-hemolytic Lancefield group C bacterium, first identified in 1888 (Schutz, 1888). After an incubation period of 3 to 14 days, the acute form of the disease is characterized by fever, abscessed lymph nodes of the head and neck, dyspnea and anorexia. Following the onset of fever, in the first 24 hours, the affected animals develop bilateral serous or mucoid nasal discharge, that later becomes mucopurulent (Neamat-Allah and Damaty, 2016). Initially, submandibular and retropharyngeal lymph nodes are enlarged and firm, and 7 to 10 days later they begin to fluctuate and may rupture and drain into the guttural pouches and via the nasopharynx, or directly through the skin, into the environment. In susceptible populations, the infection can reach a morbidity of up to 100%, but a low mortality rate (10%), if the appropriate treatment is implemented. Most of the affected horses fully recover within an average period of 3 weeks (Roberts, 2014). Following infection, if horses recover without antibiotic treatment, 75% of them develop a protective immune response that lasts up to 5 years. There is evidence that treatment with penicillin during the acute stage of strangles can impair the persistence of antibodies against *S. equi*, decreasing the duration of the serological response (Pringle et al., 2020). However, during an emerging outbreak, the early start of antibiotic therapy increases the chances of complete recovery and can prevent complications (Boyle et al., 2018). In approximately 20% of *S. equi* infections, severe complications can occur, such as metastatic infections (“bastard strangles”) (Berlin et al., 2013), suppurative necrotic bronchopneumonia, guttural pouch empyema and chondroids, purpura hemorrhagica, muscle infarction and rhabdomyolysis (Constable et

al., 2017). Also, there is evidence that on extremely rare occasion, *S. equi* infections can become zoonotic, causing infections of the central nervous system in humans (Kerstens et al., 2021).

MATERIALS AND METHODS

The outbreak of strangles emerged in a horse breeding farm, housing at the time 142 horses: 8 yearlings, nine 2 year old horses, 31 foals aged 1 to 6 months, 4 stallions, 70 mares used for reproduction and 20 mares used as surrogates in the reproduction process. The dynamics of the size and structure of the population were due to the newborn foals during that period (31 foals were born between January and July). Occasionally, some of the horses traveled abroad for competitions. None of the horses had left the farm in the months before the outbreak; however, 4 new, clinically healthy mares were introduced to the population in June, approximately 4 weeks before the outbreak began in the first week of July. The first horses to exhibit symptoms of strangles were 4 of the yearlings and 5 foals aged 3-6 months: the horses presented with inflamed submadibular lymph nodes, cough, purulent nasal discharge and slightly elevated rectal temperatures, ranging between 38.8°C and 39.5°C. Nasal swab samples were collected for bacteriological investigation and antibiotic susceptibility tests. The affected animals were placed under treatment with penicillin, 12 mg/ kg bodyweight, administered intramuscularly, for 5-7 days. The infection spread rapidly throughout the stable, and within 3 days, 11 other foals, aged 1 to 4 months, showed similar symptoms and were placed under therapy. Due to the large number of recent births and the characteristics of the farm (limited capacity of the stables, limited staff), an adequate quarantine area, in order to limit the spread of the infection was not set. By the end of July, all of the foals, yearlings, one of the 2 year-old horses and 2 adult mares had shown symptoms of strangles, with various degrees of severity, and were treated accordingly. The affected horses were examined daily, rectal temperatures, abnormal respiratory noises and the evolution of the clinical signs being recorded. Non steroidal anti-inflammatory medication (flunixin 1.1 mg/kg bodyweight) was administered intravenously in cases where temperature exceeded 39°C. Horses with persistent cough and abnormal respiratory sounds (crackles and wheezing) received, in addition to antibiotic, bronchodilator and mucolytic medication (Venti Plus) as an orally administered product containing clenbuterol hydrochloride (0.8 g/kg bodyweight) and dembrexin hydrochloride (0.3 mg/kg bodyweight). There were 42 cases of *S. equi* infections confirmed on the farm in July. Clinical signs observed throughout the breakout included:

- submandibular and retropharyngeal lymph node inflammation - lymph nodes enlaged, firm, and painful to palpation; this was the first symptom noticed in all the affected animals, regardless of age. After 4-5 days, the lymph nodes became fluctuant, ruptured and drained, mainly via the nasopharinx;
- bilteral mucopurulent nasal discharge - present in all the affected animals;
- coughing, initially dry, later became productive - very frequent in all young animals; the infected mares presented only dry cough in the first 2-3 days after the clinical onset;
- fever - the majority of the horses registered slightly elevated body temperature (38.8-39.5°C) at onset of the disease, that returned to normal after 1 – 3 days of antibiotic therapy;
- dyspnea and abnormal respiratory sounds (wheezing and crackling) - present in some of the more severe cases of strangles in 1 to 6 month old foals. Approximately 50% of the affected foals presented these symptoms, along with persistent productive cough, requiring up to 10 days of antibiotic therapy;
- guttural pouch empyema - one of the more severely affected foals, a 3 month old filly, developed guttural pouch empyema.

Of the 42 affected horses, 4 foals aged 2-3 months did not respond to the penicillin treatment, presenting with elevated temperature (39.3-39.6°C), lethargy, cough and dyspnea, 7 days into the antibiotic therapy. Based on the history of the farm, *Rhodococcus equi* pneumonia was suspected. Pulmonary ultrasounds and bacterial examinations of nasal

swab samples were performed in order to confirm the diagnosis. The foals and their mothers were separated from the herd and were treated with rifampicin 7.5 mg/kg and clarithromicin 7.5 mg/kg, orally, twice daily for 8 weeks, orally administered probiotics, mucolytic medication, flunixin 1.1 mg/kg, when necessary to decrease high temperature, and vitamin C, 1 g/animal/day, for 5 days, intravenously. The foals were closely monitored daily, and pulmonary ultrasounds were performed weekly in order to assess the evolution of the pulmonary lesions. One of the 4 foals was unresponsive to the treatment and died 15 days after the *R. equi* infection was confirmed. Necropsy and bacteriological examinations were performed in order to establish the cause of death.

Of the 38 horses affected by *S. equi* infection and treated successfully with penicillin, 6 horses aged 3-6 months reported recurrence of respiratory distress, coughing and purulent nasal discharge 2-3 weeks after the remission of the initial symptoms. The clinical signs indicated towards a relapse of strangles, and nasal swab samples were collected for bacteriological examination. One 4 month old filly presented more severe symptoms such as peracute respiratory distress, complete loss of appetite, high fever (40.1°C) and died the day after the symptoms were first observed. Necropsy was performed and samples of lung tissue were collected for bacteriological examination. Nasal swab and lung tissue cultures identified *Streptococcus equi* ssp. *zooepidemicus*, and the 5 remaining foals were placed under treatment with rifampicin 7.5 mg/kg and clarithromicin 7.5 mg/kg, per os, twice daily, according with results of the antibiotic susceptibility test. They were also given probiotics and mucolytic drugs during the treatment, and vitamin C 1 g/animal/day, for the first 5 days, intravenously.

For the bacteriological examinations, Columbia agar with 5% sheep blood was used for seeding and isolation of the bacterial strains. The identification of the isolates was performed based on cultural characteristics, morphological aspects examined microscopically using the Gram stain method and biochemical characteristics, determined using API strips (Biomerieux): API 20 Strep for *S. equi* and *S. equi* ssp. *zoopeidemicus*, and API Coryne for *R. equi*. The interpretation of the API test results was performed according to the producer's instructions, using the provided software. The *R. equi* isolate was also seeded on selective culture media: MacConkey agar and Lowenstein-Jensen agar. For the antibiotic susceptibility tests, performed by disc diffusion method, Liofilchem antimicrobial discs were used, and the results were interpreted according to Liofilchem and EUCAST standards.

RESULTS AND DISCUSSIONS

Bacteriological examinations. Cultural and morphological characteristics of *Streptococcus equi* ssp. *equi* and *Streptococcus equi* ssp. *zooepidemicu*s were identical: the colonies were small, smooth, translucent, shiny, β-hemolytic on Columbia blood agar after 20 hours incubation at 37°C; microscopic examination revealed Gram positive, chain forming cocci.

R. equi colonies were medium-sized, whitish, mucoid, confluent and presented weak alpha-hemolysis on Columbia blood agar, after 48 hours incubation at 37°C. On MacConkey agar, the colonies were transparent, shiny and lactose positive. On Lowenstein – Jensen agar, the culture developed slowly, becoming evident after 72 hours incubation at 37°C. The morphological examination of the cultures revealed Gram- positive polymorphic bacteria, with a tendency to form chains.

Biochemical characteristics. The catalase and oxidase tests were negative for the two *Streptococcus* stains. The *R. equi* isolate was catalase positive and oxidase negative. The biochemical characteristics of the three isolates, determined using API (Biomerieux) tests, are detailed in Tables 1, 2 and 3.

Table 1. Biochemical characteristics of the *Streptococcus equi* ssp. *equi* isolate

VP	HIP	ESC	PYRA	AGAL	BGUR	BGAL	PAL	LAP	ADH	RIB	ARA	MAN	SOR	LAC	TRE	INU	RAF	AMD	GLYG
-	-	-	-	-	+	-	+	+	+	-	-	-	-	-	-	-	-	+	+

Legend: VP – Acetoin production (Voges Proskauer), HIP – Hydrolysis of hipuric acid, ESC – esculin, PYRA – PYRrolidonyl Arylamidase, AGAL – α-GALactosidase, BGUR – β-GIUcuRonidase, BGAL – β-GALactosidase, PAL – Alkaline Phosphatase, LAP – Leucine AminoPeptidase, ADH – Arginine DiHydrolase, RIB – Ribose, ARA – Arabinose, MAN – Mannitol, SOR – Sorbitol, LAC – Lactose, TRE – Trehalose, INU – Inulin, RAF – Raffinose, AMD – Amidon, GLYG – Glycogen, "+" - positive result, "-" – negative result.

Table 2. Biochemical characteristics of the *Streptococcus equi* ssp. *zooepidemicus* isolate

VP	HIP	ESC	PYRA	AGAL	BGUR	BGAL	PAL	LAP	ADH	RIB	ARA	MAN	SOR	LAC	TRE	INU	RAF	AMD	GLYG
+	-	-	-	-	+	-	+	+	+	+	-	-	+	+	-	-	-	+	+

Legend: VP – Acetoin production (Voges Proskauer), HIP – Hydrolysis of hipuric acid, ESC – esculin, PYRA – PYRrolidonyl Arylamidase, AGAL – α-GALactosidase, BGUR – β-GIUcuRonidase, BGAL – β-GALactosidase, PAL – Alkaline Phosphatase, LAP – Leucine AminoPeptidase, ADH – Arginine DiHydrolase, RIB – Ribose, ARA – Arabinose, MAN – Mannitol, SOR – Sorbitol, LAC – Lactose, TRE – Trehalose, INU – Inulin, RAF – Raffinose, AMD – Amidon, GLYG – Glycogen, "+" - positive result, "-" – negative result.

Table 3. Biochemical characteristics of the *Rhodococcus equi* isolate

NIT	PYZ	PYRA	PAL	βGUR	βGAL	αGLU	βNAG	ESC	URE	GEL	GLU	RIB	XYL	MAN	MAL	LAC	SAC	GLYG
+	+	-	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-

Legend: NIT - Potassium nitrate, PYZ - Pyrazinamidase, PyrA - Pyrolidonylarylamidase, PAL - Alkaline phosphatase, βGUR - β-glucuronidase, βGAL - β-galactosidase, αGLU - α-glucosidase, βNAG - N-Acetyl-β-glucosaminidase, ESC - Esculin, URE - Urease, GEL - Gelatin, GLU - Glucose, RIB - Ribose, XYL - Xylose, MAN - Mannitol, MAL - Maltose, LAC - Lactose, SAC -Sacchrarose, GLYG - Glycogen, "+" - positive result, "-" – negative result.

Antibiotic susceptibility test. The *S. equi* ssp. *equi* strain was susceptible to multiple antimicrobials, including penicillin, resistant to gentamicin, and intermediately susceptible to enrofloxacin (Table 4), in accordance other researcher's findings (Erol et al., 2012). The *S. zooepidemicus* isolate was resistant to all tested antimicrobials, with the exception of rifampicin (Table 4).

Similar to other studies (Giguere, 2017; Lupu et al., 2021), it was determined that the R. equi isolate was susceptible to rifampicin and clarithromicin (Table 4).

Table 4. Antibiotic susceptibility test results for the bacterial isolates

Streptococcus equi* ssp. *equi	**Susceptible:** ampicillin, amoxicillin + clavulanic acid, penicillin, trimethoprim + sulfomethoxazole, cloramphenicole, cefazoline, marbofloxacin, rifampicin. **Intermediately susceptible:** enrofloxacin. **Resistant:** amikacin, tetracycline, gentamicin, doxyciclin, kanamicin.
Streptococcus equi* ssp. *zooepidemicus	**Susceptible:** rifampicin. **Intermediately susceptible:** cloramphenicole, doxyciclin, erythromycin, enrofloxacin, trimethoprim + sulfametoxazole, cefixime **Resistant:** cephalotin, gentamicin, streptomycin, penicillin, norfloxacin
Rhodococcus equi	**Susceptible:** gentamicin, doxyciclin, rifampicin, norfloxacin, clarithromicin. **Intermediately susceptible:** spectinomicin, streptomycin **Resistant:** cefixime, vancomycin, oxacillin, trimethoprim + sulfomethoxazole, cephalotin, clindamycin.

Pulmonary ultrasounds performed on the foals affected by *R. equi* infections, at the onset of the disease, showed the presence of abscesses of various sizes and inflammation of the pleura. Towards the end of the treatment, the abscesses were no longer visible, the surface of the lung appearing slightly irregular, possibly due to the presence of scar tissue.

Post-mortem examinations. In the case of *R. equi* induced pneumonia, necropsy findings included pleurisy and suppurative bronchopneumonia, with the presence of multiple abscesses measuring between 2 mm and 1.5 cm in the caudal lobes and foci of pulmonary atelectasis.

R. equi was isolated from purulent material collected from the abscesses in the lung tissue.

The pneumonic lesions of the foal that died of peracute respiratory distress were consistent with serohaemorrhagic pneumonia with focal pulmonary necrosis. *S. equi* ssp. *zooepidemicus*

was isolated in pure culture from samples collected from the lung tissue.

Management and treatment. The entire herd was monitored constantly during the outbreak, by the members of the medical team and the groomsman. The affected animals were examined daily, recording the rectal temperature, the presence or absence of nasal discharge, the characteristics of the lymph nodes and respiratory sounds.

The two adult mares, the yearlings and the 2 year-old that showed symptoms of strangles recovered after 5 days of treatment with penicillin, without any complications. Of the 31 foals aged 1 to 6 months, 50% had mild symptoms, such as inflamed lymph nodes and nasal discharge, and required 5-7 days of treatment for a complete recovery. 16 foals had more severe manifestations, such as persistent productive couch, fever, copious mucopurulent nasal discharge, pathological respiratory sounds and abscess formation in the submandibular lymph nodes. Penicillin administration was prolonged for up to 10 days, until the abscesses drained and the nasal discharge subsided. The bronchodilator and mucolytic medication aided in clearing the airways of secretions and relieving the cough.

Of the 4 foals with *R. equi* infection, one died 2 weeks after the onset of the symptoms, due to extensive lung damage. The other 3 foals responded well to the treatment: after the first week of therapy, the temperature and appetite returned to normal and they were no longer lethargic. After 4 weeks of treatment, the foals were no longer coughing, and imaging showed a decrease in the size of the pulmonary abscesses. The treatment was continued for another 4 weeks, until the abscesses were no longer visible.

The 5 foals treated for *S. zooepidemicus* pneumonia fully recovered after 5 weeks of treatment.

CONCLUSIONS

The present study highlights the different clinical patterns of strangles and the efficacy of early onset of antibiotic treatment in preventing the development of severe complications and death.

Daily monitoring of the herd allowed the medical team to prescribe personalized treatment for each affected horse, depending on their clinical condition.

Active bacteriological surveillance was a key factor in discrimination between strangles and the other respiratory infections that showed similar clinical pattern.

The antibiotic susceptibility tests allowed for a targeted and safe therapeutic approach. Infection with *S. zooepidemicus* demonstrates the imperative of carrying out a complete bacteriological investigation, including the antibiophenotype of each isolated strain: the strain identified in this case was susceptible to rifampicin alone.

In the case of *R. equi* infected foals, pulmonary ultrasounds were a useful tool in assessing the remission of lesions, and in adapting the duration of the treatment.

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THE INFLUENCE OF THE ANTI- *Mycoplasma agalactiae* VACCINE STRAIN ON THE HUMORAL IMMUNITY IN SHEEP

Gheorghița DUCA[2], Carmen Dana ȘANDRU [1, 2], Diana OLAH[1], Mariana RUSU[2], Constantin CERBU[1], Marina SPÎNU[1, 2], Florinel BRUDAȘCĂ[1], Emoke PALL[1, 2], Adrian POTÂRNICHE[1], Aurel VASIU[1]

[1]University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, Faculty of Veterinary Medicine, 3-5 Calea Mănăștur, 400372, Cluj-Napoca, Romania
[2]Institute of Research and Development for Montanology Cristian, Sibiu, Romania

Corresponding author email: emoke.pall@usamvcluj.ro

Abstract

Vaccination against contagious agalactia of sheep, an OIE declarable, highly economically impacting disease, is the most widespread method to reduce the disease prevalence. This research monitored the influence of M. agalactiae S/94 and AG6, two commercial vaccines for sheep, on systemic humoral immunity in two flocks of ewes (n1=95, n2=220, respectively). Blood was sampled from both flocks before and one month after the booster vaccination and subjected to total Ig (24%o zinc sulphate precipitation test) and circulating immune complexes' (4.2% polyethylene glycol precipitation test) evaluation. There were significant differences (p<0.001-0.004) in total Ig levels for both strains (7.68±2.63 and 23.68±5.7 for S/94 and 11.1±3.58 and 26.11±3.4 Vernes degrees for AG6, before and after the vaccination, respectively), but no differences in CIC concentrations (p=0.08 and p=0.59, for S/94 and AG6) between the samplings or the strains (p=0.342) were found. A strong positive correlation (r=0.889, p<0.05) was established between total Ig and CIC levels for the AG6 but not the S/94 strain, therefore other influential factors (individual, adjuvant, frequency of vaccination) should be investigated.

Key words*: vaccination, M. agalactiae, S/94/AG6 strain, humoral immunity.*

INTRODUCTION

Mycoplasmic mastitis (contagious agalactia) in sheep and goats represent one of the most challenging infectious pathologies in traditional, but also intensive raising of small ruminants, due to its health, welfare and also economic impact (decreased milk production, movement and thus, feeding difficulties, arthritis and ocular lesions in all other categories besides lactating ewes) (Almeida et al., 1992; Cottew and Yeats,1982; DaMassa et al., 1992). The etiology involves mainly *Mycoplasma agalactiae* in most of the flocks either ovine or caprine. Still, *M. capricolum capricolum* and *M. putrefaciens* along with *M. mycoides capri* could be also isolated in animals with either agalactia or arthritis (Fox et al., 2003).

The use of non-specific humoral response as indicator for the general immunity in vaccinated animals, besides providing standardization means concerning the immune status of the sheep and further, its efficacy to protect against mycoplasma disease, could bring considerable support to defining some peculiarities linked to the profile of mycoplasmosis in this species (Avramidis et al., 2002). Assessment of the functional level of different non-specific effectors during the evolution of the disease allows the interpretation of some features and phases connected to the post vaccination response, probably linked to the re-emergence of the disease in spite of the anti-agalactia constant vaccination in the areas of high epidemiological risk (Rodriguez et al., 2002; Szeredi et al., 2003).

The experiment aimed at clarifying and better understanding the humoral reactivity, which may also contribute to the establishment of some enhanced measures towards the control of the disease. The study also sought to evaluate the efficacy of different mycoplasma antigens in immunizing against contagious agalactia, by use of two commercial vaccines.

MATERIALS AND METHODS

Biological material and the experimental protocol. The investigations were conducted on two sheep flocks of 95 (n1) and 220 (n2) animals each, including different age categories of animals, such as ewes but also lambs, and rams to avoid orchitis and arthritis in the latter.

The animals were Merino crosses with the local Turcana breed, raised in a semi-intensive technology, periodically following the transhumance routes in NW Romania.

The animals were vaccinated with two types of vaccines, as a primary vaccination, following the producer's protocol.

Vaccines. This research monitored the influence of *M. agalactiae* S/94 and AG6, two strains included in two types of commercial vaccines for sheep, by looking at the systemic humoral immunity developed post-vaccination.

Mycoplasma agalactiae, strain S/94 induces an increase in agglutinating antibodies of at least 2 ln, evidenced by the slow tube agglutination reaction (RSAL), min 5 UE. This inactivated vaccine is recommended for the active immunization against the contagious agalactia of clinically healthy sheep and goats, both in the free herds and in those in which the disease progresses. The vaccine was administered in a dose of 1 ml/animal, regardless of the size of the animals, subcutaneously, at the base of the tail or in the fold. In ewes, the first vaccination was performed in the second part of the pregnancy (month 3), and the booster after 21 days. In youngsters and rams, the vaccination was carried out along with the other animals. Immunity is complete 21 days after the booster vaccination and lasts for 6 month post-vaccination, according to the manufacturer's instructions.

The AG 6 strain vaccine is a Mycoplasma agalactiae suspension inactivated by formalin and heat, adsorbed with aluminum hydroxide gel (min 7-10 UE/dose). This vaccine was used in the second flock following the same instructions as for S/94.

Blood samples were taken twice, before the vaccination and one month after the second dose, in both flocks. The blood was collected on a clotting gel for sera, which were separated after 2 hours by centrifugation (at 1308·g for 10·min) before processing by 24‰ zinc sulphate precipitation test (total Ig) and 4.2% polyethylene glycol precipitation test (circulating immune complexes, CIC) (Ghergariu et al., 2000).

Methods. Immunoglobulin measurements (Khokhlova et al., 2004). Total immune globulins, known as opsonins, play an important role in the 'first line of defence', that is innate immunity, against aggressors. At a pH·7.4, the electric charge and colloidal stability of gamma globulins are lower than those of serum albumins. Thus, concentrations as low as 24·mg/l/ of metal salts precipitate the immunoglobulin. A volume of 6.6·ml of serum was mixed with 193.4·ml of a 0.024% barbital buffer zinc sulphate solution and allowed to precipitate for 30·min at room temperature (22-23°C). Optical density (ODU) then was read spectrophotometrically (l = 475·nm, d = 0.5·cm) and transformed in Vernes degrees, by x 100 multiplication.

Circulating immune complexes' measurements (CIC) (Khokhlova et al., 2004). Quantifying the level of circulating immune complexes (CIC) allows evaluation of the molecular clearance capacity at a particular moment. A 4.2% polyethylene glycol (PEG) solution in borate buffer was used as the precipitating agent, while buffer-treated samples served as controls for borate-induced precipitation. The reaction was performed in a 96-well-plate to enhance spectophotometrical readings. Volumes of 196.7·ml of borate buffer and PEG solution, respectively, were mixed with 3.3·ml samples of the serum, for each sample, in parallel wells. The samples were allowed to precipitate at room temperature (22-23°C) for 60·min, then read spectrophotometrically at a wavelength of 450·nm in the test plate (d = 0.5·cm) (multichannel spectrophotometer SUMAL PE2, Karl Zeiss, Jena, Germany). CIC concentrations, expressed in optical density units (ODU) were calculated by subtracting the value of the control (serum + buffer) from that of the PEG precipitate and further expressed in units (U) by multiplication 10^3 times.

RESULTS AND DISCUSSIONS

The use of the zinc sulphate precipitation assay for the dosage of gammaglobulins provides a

supplement to the immunological profile in vaccinated animals, even without the identification of the classes (IgG versus IgM) of immunoglobulins involved. Following the results obtained, it is observed that the mean values of serum concentrations of gammaglobulins in animals vaccinated against contagious agalactia with S/96 strain are higher than the values of the concentrations recorded before vaccination. Nevertheless, in the flock vaccinated with the AG 6 strains, the increase did not differ significantly from the flock vaccinated with S/96, but the increase was lesser than in the first flock (164.47% versus 107.85%). The statistical significance of the increase in total Ig levels was high in both flocks ($p<0.01$-0.001).

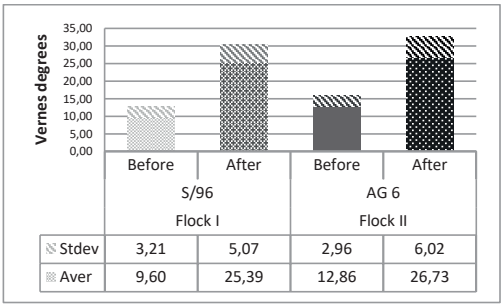

Figure 1. Changes in total Ig during the immunisation (Vernes degrees, x ± s)

Immune complexes arise continuously in the case of Ag-Ac coupling and are efficiently removed by the reticuloendothelial system. In some cases, the formation of immune complexes leads to hypersensitivity reactions.

The formation of immune complexes occurs in different cases: long-term infections, autoimmune diseases, on surfaces, for example in the lung, after repeated inhalation of antigens of different nature, and their presence indicates either an effective way to remove the antigen, or especially in the case of their chronic presence in the body, the ineffectiveness of removing antigens (Moraru 1984). The structure of immune complexes depends on the nature of the antigen and antibody that are involved in their synthesis, the antibodies involved are usually IgG-type (Roitt, 2001).

Frequently, CIC levels provide a measure of the body's reactivity, on one hand, and the severeness of the disease, on the other hand; in microbial diseases, excessive formation of CIC can lead to their deposition in various organs, trigerring the membrane attack complex of the complement and the consequent destruction of the tissue, release of new antigens and the induction of antibodies against the modified self.

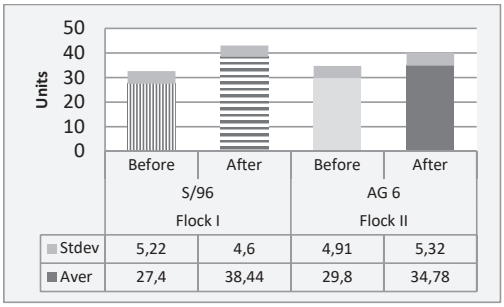

Figure 2. Changes in CIC during the immunisation (units, x ± s)

In Figure 2, the levels of immune complexes registered higher average values in the case of vaccinated sheep, while in unvaccinated sheep the serum concentration of immune complexes was lower.

The values obtained show that the total Ig and CIC levels are little influenced by AGAVAC vaccination, the differences not being statistically significant.

The role of vaccines lies in stimulating the immune system against pathogens, because it guarantees that a new contact of the body with the pathogen does not cause the disease. In order to improve the effectiveness of vaccines, it is important to preserve the characteristics of the pathogen without causing any harm to the animals. It is important that the inactivated substances in the vaccine do not greatly alter the properties of the antigenic proteins. Adjuvants are added to the preparation of inactivated vaccines to indirectly enhance the immunogenic properties of the antigen (Stewart-Tull, 1996).

The literature mentions that the high temperature used to inactivate the *M. agalactiae* vaccine antigen has a destructive effect on its surface proteins (Tola et al., 1999).

It should be noted that AG6 vaccine was inactivated with formalin and heat, and S/96 anti-galactic vaccine was only inactivated with formalin. Inactivation with sodium hypochlorite has the same altering effects on immunoproteins. By switching from the use of

formalin to the use of phenol, then from phenol to saponins as adjuvants, the surface proteins remain with a structure as close as possible to the intact mycoplasma. Tola et al. (1999) show that vaccines inactivated with saponins and phenol induce the formation of a high level of antibodies. The effectiveness of saponins is also noted by other authors (Rurangirwa et al., 1987). Saponin acts as an adjuvant and inactivator at the same time. This eliminates the problems of dose mixing and toxicity caused by various adjuvants.

The use of inactivated phenol and saponin vaccines limits economic losses, especially in areas that are economically dependent on sheep farming.

In regions where contagious agalaxia develops endemic, economic losses result from decreased milk production and shortened lactation, and the prophylaxis of this disease is not fully elucidated due to incomplete knowledge of the pathogenesis of the infection and the fact that highly effective vaccines have limited accessibility.

Table 1. Statistical significance of the total Ig and CIC variations

Antigen	Statistical significance		T test	
	Total Ig	CIC	Total Ig	CIC
S/96	p<0.01	NS	3.7	0.5
AG 6	p<0.001	NS	5.95	1.98

In previous studies, immunoprophylaxis of contagious agalactia was based on udder preparations from infected animals. These vaccines have not been particularly effective and at the same time have been vectors of other pathogens, such as scrapie agent (Caramelli et al., 2001). Recently, a number of studies have been conducted on the development of new anti-galactic vaccines with higher efficacy and safety (Leon et al., 1995; Buonavoglia et al., 1998; Tola et al., 1999; Greco et al., 2002). Experimental vaccines combined with aluminum hydroxide (Al(OH)3) or mineral oils as adjuvants have been shown to be effective (Buonavoglia et al., 1998; Tola et al., 1999; Greco et al., 2002), but the study their safety and immunogenicity require further investigation. Although aluminum hydroxide adjuvanted vaccines have been shown to be safe, they do induce the synthesis of a short-lived antibody titer that persists for a short period of time. In contrast, mineral oil adjuvant vaccines have a high immunogenic capacity, with high levels of antibodies, which persist for a long time and cause only a mild inflammatory reaction around the point of inoculation without being associated with systematic adverse reactions. Vaccines with mineral oil adjuvants are able to prevent the clinical manifestations of contagious agalaxis of animals affected by *M. agalactiae,* but sometimes there is a small increase in the retro-mammary lymph nodes (Buonavoglia et al., 2008).

Both tested strains, S/96 and AG6 induced a significant post-vaccination increases in total immunoglobulin titers (Table 1). No such differences were observed in the case of circulating immune complexes, where the differences between pre- and post-vaccine levels were non-significant for both flocks.

CONCLUSIONS

Comparing the two anti-galactic vaccines, one (S/96) containing formalin-inactivated antigen and the other (AG6) inactivated by heat and formalin, it can be seen that the latter enhances a more pronounced immunoglobulin increase (higher statistical significance of differences) than first, which may lead us to the conclusion that this booster with this vaccine is the cause of more active immune synthesis, because the antigen is practically more distorted than in the first vaccine.

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MICROBIOLOGICAL RESEARCHES OF THE VIRULENT SPECIES OF *Staphylococcus aureus* IN PATHOLOGICAL ABCESSES AT SWINES

Rita GOLBAN, Nicu BERZOI

State Agrarian University of Moldova, 42 Mircesti Street, Chisinau, Republic of Moldova,
Email: golbanrita@gmail.com

Corresponding author email: golbanrita@gmail.com

Abstract

Current researches are important due to the frequency of cases caused by some pathologies of abscesses recorded at pigs slaughtered in slaughterhouse conditions. In this context, samples from various internal abscesses of slaughtered pigs and the assessment of virulence and incidence of identified bacterial microflora species were studied. Microbiological researches on abscesses of different origins at slaughtered pigs has demonstrated the presence of the important bacterial microflora consisting of species of Staphylococcus aureus in all cases of microbiological laboratory investigation. S.aureus strains isolated from various sources of pathological abscesses at slaughtered pigs in slaughterhouse conditions expressed a higher proportion of quantitative bacterioscopic and bacteriological microbiological values in heart abscesses, followed by samples invaded with staphylococci in the chest, leg, jaw and so on. The Staphylococcus aureus strain expressed high virulence factors sampled by a phenotypic mechanism, and it was found that this strain showed the gene encoding the collagen binding protein and at the same time mediates the binding of Staphylococcus aureus to fibrinogen.

Key words*: abscesses, Staphylococcus aureus, pigs, pathologies, microbial cultures.*

INTRODUCTION

Staphylococcus aureus is one of the main colonizers of the human and animal body. In some cases this strain can become a pathogen, producing localized or systemic infections, less often staphylococcal infections occur as a result of exogenous contamination. Colonization with staphylococcal microorganisms can persist for months and even years, until they cause an infection, and colonized animals can contaminate other animals, as they are the most important reservoir of staphylococcal germs (Dall, 2018; Golban, 2015).

Staphylococcal infections may be unfavorable due to increased aggression of the bacterial strain, reduced ability of the animal body to defend itself against infection, or difficulty in treating an infected animal with an antibiotic-resistant strain (Brown, 2013; Carp-Carare, 2015; Dan, 2012). It has been found that approximately 15-40% of animals colonized with *S. aureus* can develop an infection with this germ at various times, the most common being abscesses of various etiologies, and colonized animals are a source of infection for other animals (Carp-Carare, 2014; Colobatiu, 2014; Enne, 2012).

Infections caused by *Staphylococcus aureus* are a major problem for swine abscesses lately due to their high share of infection and potential for severe evolution. Some factors are associated with the onset and spread of abscess infections favored by *Staphylococcus aureus* due to animal overload factors in maintenance rooms; failure to test for the presence of *Staphylococcus aureus*; ignoring the need to group swine-infected/colonized pigs in a demarcated area of the maintenance rooms; deficiencies in the application of contact precautions (Fiț, 2015; Ulea, 2011; Wang, 2013).

According to bibliographic studies, the statement that the most persistent bacterium present is the species *Staphylococcus aureus*, followed by other no less important microbial species, is interesting. Parasites can rarely cause abscesses, and they are more common in developing countries. In terms of the etiological agent of the nature of abscesses, they can be suggested by their location and their predisposing cause (Stevens, 2014).

For this reason, the main objectives of this research are the microbiological investigation of the virulent species *Staphylococcus aureus* in the pathological abscesses of different regions of pigs in slaughterhouses and the interpretation of data obtained from quantitative microbiological aspects.

MATERIALS AND METHODS

The microbiological researches were carried out from samples from different sources of slaughtered pig abscesses from the slaughterhouse of the Porco Bello LLC, Cimișeni, Criuleni district.

Microbiological investigations to identify the species *Staphylococcus aureus* were performed from various samples of slaughtered pigs. Bacteriological research has been subjected to a classical laboratory microbiological conduct, where the bacterial microflora characteristic of the species *Staphylococcus aureus* has been found, regarding the bacterioscopic and cultural characters and the differentiation of quantitative aspects in various samples of investigated abscesses of body regions in slaughtered pigs. For research, samples were taken from abscesses and investigated in the microbiology laboratory of the Faculty of Veterinary Medicine of SAUM, where the bacterial microflora was determined, the differentiation criteria, the morphological and cultural characteristics of the predominant *Staphylococcus aureus* species, the number of microorganisms in microscopy and microbial colonies specific to this species on common and special culture media. The microbial preparations made from the pathological samples were stained according to the Gram method, stained with gentian violet and fuchsia dyes, then microscopized at the immersion objective 90.

RESULTS AND DISCUSSIONS

Microbiological investigations of slaughtered pig abscesses from different regions show microbiological assessments in the following tables, which represent the quantitative study of bacterial microbiological investigation of the number of isolated microorganisms of *Staphylococcus aureus*, bacterial colonies found on common and special culture media.

The data from Figure 1 demonstrate the characteristics of the study of bacterial bacterioscopic microflora of the isolated species of *Staphylococcus aureus* from various regions of pigs slaughtered in slaughterhouse conditions. Thus, following the values of the microorganisms of this species visualized under microscopy from various samples in comparative aspect, a higher number of staphylococcal microorganisms is observed, evidenced by the abscess of the pathological sample, the highlighted values of *Staphylococcus aureus* with a number of 36 microbial cells determined in the sample with chest abscess in slaughtered pigs and other values of 28; 18; 16; 14; 12 to other samples with abscesses identified in slaughtered pigs.

Analyzing the microflora characteristic of the species *Staphylococcus aureus*, a higher number of microbial cells is observed under microscopy, however, in samples with characteristic abscesses, where the number of cases was more frequent: cord and swine chest, compared to with abscesses recorded in the ribs, leg, muscles, jaw and ears (Figure 2).

The data from Figures 3 and 4 reveal the characteristics of the study of bacterial bacteriological microflora from various abscesses taken after examination of microbiological passages on various common culture media: agar / broth on plates / test tubes and special: blood agar / plaque. Observing the indices of the colonies obtained from various samples in comparative aspect, a higher number of bacterial colonies is observed on the plaque agar medium 24 in number from the heart abscess sample of the identified species *Staphylococcus aureus*, compared to the abscesses from the mandible, chest, rib, leg etc., where the number of colonies on the agar / plate medium was 20; 19; 16; 14 and 12 microbial colonies. Hemolysis, characteristic to the species *S. aureus*, was determined in all cases on the blood agar medium.

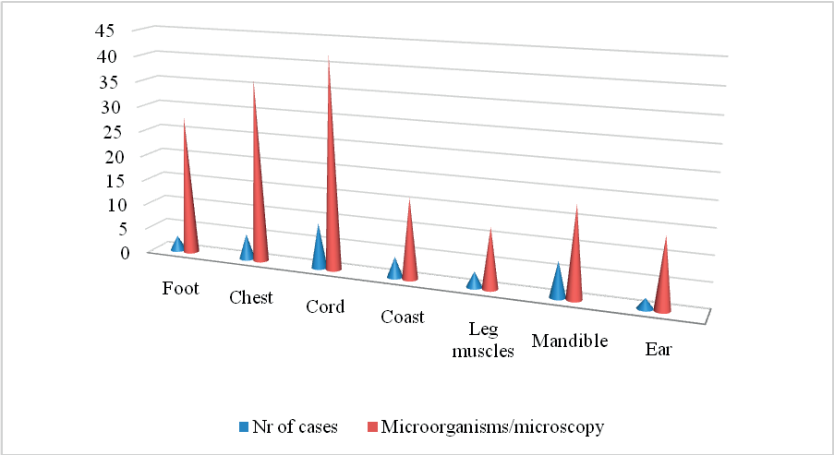

Figure 1. Bacterioscopic values of *Staphylococcus aureus* microflora in abscesses of slaughtered pigs

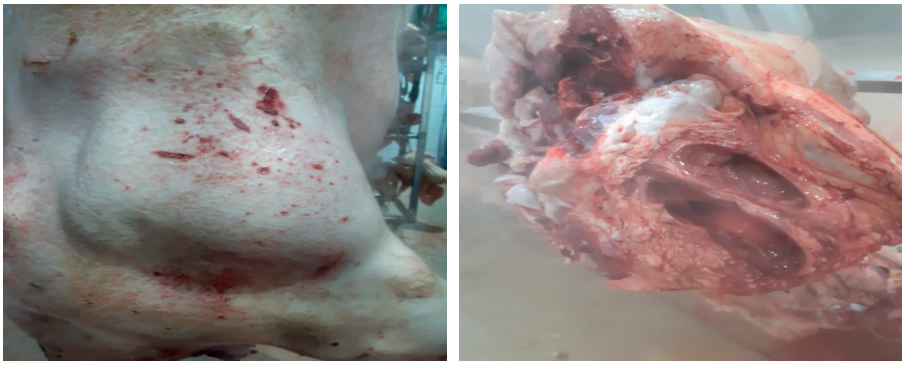

Figure 2. Abscesses

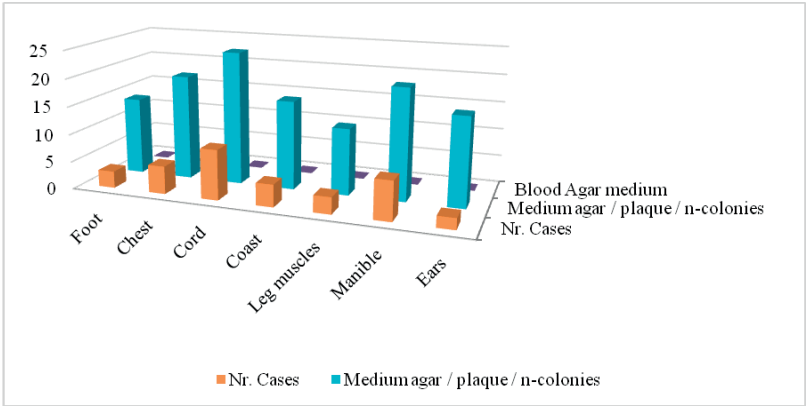

Figure 3. Bacteriological values of *S. aureus* microflora on agar/plaque in slaughtered pig abscesses

Observing the indices of the colonies obtained from various samples in comparative aspect, a higher number of bacterial colonies is observed on the plaque agar medium 24 in number from the heart abscess sample of the identified species *Staphylococcus aureus*, compared to the abscesses from the mandible, chest, rib, leg etc., where the number of colonies on the agar / plate medium was 20; 19; 16; 14 and 12 microbial colonies.

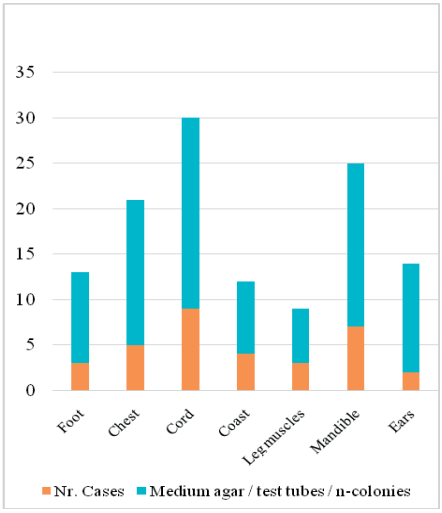

Figure 4. Bacteriological values of *S. aureus* microflora on agar / test tubes in slaughtered swine abscesses

Analyzing the results obtained regarding the values of the identified colonies from the abscesses of the investigated samples from the pigs slaughtered in slaughterhouse conditions compared to the number of registered cases of samples with identified abscesses, a number of colonies of 24 is observed; 20 and 19 higher correlated with the higher number of cases detected by abscesses in slaughtered pigs.

Therefore, these findings suggest that Staphylococcus aureus is present in a higher concentration in cor abscess samples; chest; mandible etc. On the special agar / blood medium in all cases of research through passages in the sampled abscesses investigated, hemolysis areas were observed, characteristic of the *Staphylococcus aureus* species.

At the same time, the values of the microbial colonies in Figure 5 developed on the jelly and broth culture media in test tubes also determined aspects characteristic of the species identified from the samples with investigated abscesses. Thus, as a result of the differentiation of the number of colonies, the highest number of colonies was found on the agar culture medium -21, identified from the sampling with heart abscess, followed by the sampling with abscess from the mandible, where the number of colonies determined 18 colonies and other samples investigated with a number of colonies of 18; 16, 12 and 10 microbial colonies of Staphylococcus aureus. The cultural aspects of the species *Staphylococcus aureus* identified in the liquid medium agar in all pathological samples with abscesses caused turbidity in test tubes.

Therefore, the bacterial microflora of the *Staphyloccoccus aureus* species has important aspects in the pathology of abscesses in various samples taken from pigs slaughtered in slaughterhouse conditions. These aspects indicate that the microflora of the abscesses of the porcine heart, chest, leg and sacrificed mandible are invaded by the species *Staphylococcus aureus* in a higher amount compared to other pathological specimens.

In these cases, according to our studies and reports, we conclude that the Staphylococcus aureus strain expressed higher virulence factors in these samples with a phenotypic mechanism, and that this strain showed the gene encoding the *Staphylococcus aureus* virus. collagen binding and at the same time mediates the binding of *Staphylococcus aureus* to fibrinogen.

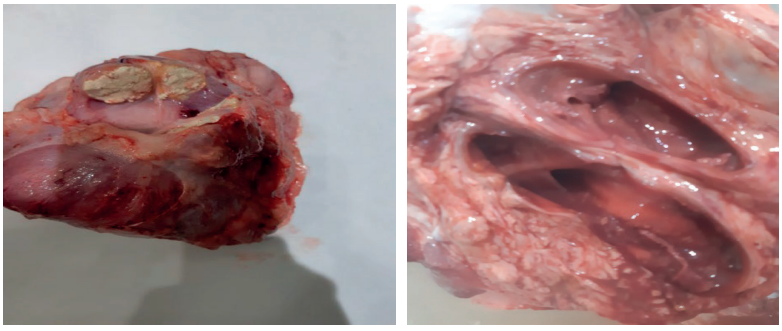

Figure 5. Abscesses investigated from sample

CONCLUSIONS

1. *S. aureus* strains isolated from various sources of pathological abscesses at pigs slaughtered in slaughterhouse conditions expressed a higher proportion of bacterioscopic and bacteriological quantitative microbiological values in heart abscesses, followed by samples invaded with staphylococci in the chest, leg , mandible, etc.
2. The *Staphylococcus aureus* strain expressed high levels of virulence factors in a phenotypic mechanism, and it was found that this strain showed the gene encoding the collagen binding protein and at the same time mediated the binding of *Staphylococcus aureus* to fibrinogen.
3. This study demonstrates the need to avoid damaging the skin and observing body hygiene in animals, which is the cause of the migration of bacteria that trigger microbial infections and abscesses.
4. In the context of the prevention of abscess pathologies, the timely administration of anti-inflammatory drugs to suspicious pigs is recommended.

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INCIDENCE OF CUTANEOUS *Staphylococcus* species IN EXTENSIVELY RAISED SWINE

Cristina Ioana LAZĂR[1], Gheorghița DUCA[2], Carmen Dana ȘANDRU[1, 2], Diana OLAH[1], Marina SPÎNU[1, 2], Emoke PALL[1, 2], Constantin CERBU[1], Adrian POTARNICHE[1], Aurel VASIU[1]

[1]University of Agricultural Sciences and Veterinary Medicine, Faculty of Veterinary Medicine of Cluj-Napoca, 3-5 Calea Mănăștur, 400372, Cluj-Napoca, România
[2]Institute of Research and Development for Montanology Cristian, Sibiu, Romania

Corresponding author email: cristina.lazar@usamvcluj.ro

Abstract

The commensal/opportunistic bacteriome in various animal species includes potentially pathogenic S. aureus. Wide-spread in humans, on the skin or mucosae, it induces a highly variable pathology, depending on its toxins and virulence, on the host and also on the environment. Much less is known about other staphylococci and their involvement in swine pathology. This research envisaged the incidence of Staphylococcus spp., in samples collected from extensively raised clinically healthy pigs, of the same age, during the period 2019-2020. The samples (n=49) were provided from farms of different sizes and in different years. Cotton swabs were used to sample secretions from anterior nares of individual pigs, and from the skin behind the ears of the animals. Classical microbiology methods were used to isolate Staphylococcus spp. and the isolates were identified using biochemical tests (API Staph, BioMerieux). Forty species of Staphylococcus were identified: Staphylococcus xylosus (47,5%), Staphylococcus lentus (30%), and Staphylococcus sciuri (22,5%). These bacterias seemed often present as a commensal animal-associated bacteria, but în some cases they could become pathologic în some diseases like bovine mastitis, and exudative epidermitis.

Key words: *swine, extensive raising, skin, staphylococci, pathogenicity.*

INTRODUCTION

MRSA represents lately an increasing problem in humans as well as in livestock. Nevertheless, the bacterial co-colonization of the skin in MRSA carriers has been poorly investigated (Strube et al., 2018).

The genus *Staphylococcus* currently comprises 81 species and subspecies, and most members of the genus are mammalian commensals or opportunistic pathogens that colonize niches such as skin, nares, and diverse mucosal membranes (Haag, et. Al 2019).

The distribution of the normal flora of *Staphylococcus* spp. is an important factor to understand the epidemiology of skin diseases in humans and animals (Nagase et al., 2001).

Despite the abundance of literature characterizing staphylococcal pathogenesis in humans, *S. aureus*, a major cause of infection and disease in a plethora of animal hosts, leading to a significant impact on public health and agriculture. Infections in animals are deleterious to animal health, and animals can act as a reservoir for staphylococcal transmission to humans. While about 20 to 30% of the human population carries *S. aureus*, the prevalence of the bacteria varies from host to host in animal species, the percentage of carriers reaching 90% in chickens, 42% in pigs, 29% in sheep, and between 14 and 35% in cows and heifers ((Haag, et. Al 2019).

Methicillin-resistant *S. aureus* (MRSA) emerged by the integration of resistance mechanisms in methicillin-susceptible *S. aureus* (MSSA). The acquisition of mecA or mecC is a public health concern due to limited options for treatment. Moreover, MRSA infections are related to longer hospitalization periods and higher mortality (Porerro et al., 2014).

Staphylococcus sciuri is an important pathogen for humans, because it is responsible for endocarditis, peritonitis, septic shock, urinary tract infection, pelvic inflammatory disease,

and wound infection. However, little information is known regarding the pathogenicity of *Staphylococcus sciuri* in animals (Chen et al., 2007).
However, those species have been isolated also from infections, both în veterinary and human medicine. More investigation into the role of the *S. sciuri* species group as commensal and pathogenic bacteria is required to fully assess its medical and veterinary importance (Nemeghaire et al., 2014).
Staphylococcus xylosus, Staphylococcus lentus, Staphylococcus equorum, Enterococcus faecalis, and *Pantoea agglomerans* were identified as pathogens in bovine mastitis (Da-Cheng Hao, 2018).

MATERIALS AND METHODS

Sampling. The samples were collected during the period 2019-2020, from extensively raised clinically healthy pigs, of the same age. The samples (n = 49) were provided from farms (n = 5) of different sizes and in different years. Cotton swabs were used to sample secretions from anterior nares of individual pigs (n = 34), and from the skin behind the ears of the animals (n = 15).
The envisaged farms were non-professional exploitations, with fattening pigs which are subject to commercial activities, namely, the sale of live pigs and meat in Romania, and were classified as biosecurity level II or III. The biosecurity level II included the general biosecurity levels: introduction to the exploitation register provided by the by the Order of the President of the National Sanitary Veterinary and Food Safety Authority no. 40/2010 on the approval of the Veterinary Sanitary Norm for the implementation of the process of identification and registration of pigs; keeping pigs in fenced areas, without the possibility of coming into contact with domestic pigs from other holdings or wild boars; prohibition of access to exploitation of foreign persons and the use of brief protective equipment, namely overalls or a work gown (https://www.meat-milk.ro/ansvsa-proiect-privind-regulile-de-biosecuritate-exploatatiile-de-porcine/).
Sampling procedures. The samples were directly inoculated into simple broth supplemented with serum, and were incubated in aerobic conditions for 24 h at 37ºC. After mixing gently the broth, sub-cultures from the broth were performed by streaking one loop of 10 µl over the surface of the Chapman agar and incubating the plate at 37ºC for 24 h in aerobic conditions.
Based on the morphology of the staphylococcus colonies on Chapman agar (yellow colonies) (Figure 1), they were individually subcultured on the Columbia Agar with 5% Sheep Blood agar, and were incubated at 37ºC for 24 h.

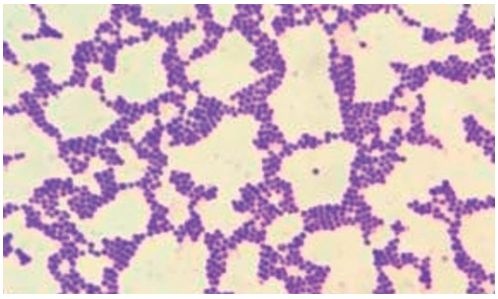

Figure 1. Coccoid bacteria - Gram-stain, x 100

Once the identity of the genus *Staphylococcus* was confirmed and to avoid the contamination, a colony from the subculture was picked and streaked on a new plate on simple agar, and incubated 37ºC for 24 h. These re-sub-cultured bacterial isolates were stored in 40% glycerol at -20ºC.
Staphylococcus spp. were then identified based on the colony characteristics, morphology, Gram stain, and colony pigmentation. Further, the identification procedure was continued by use of biochemical tests (API Staph, BioMerieux) (Figure 2).

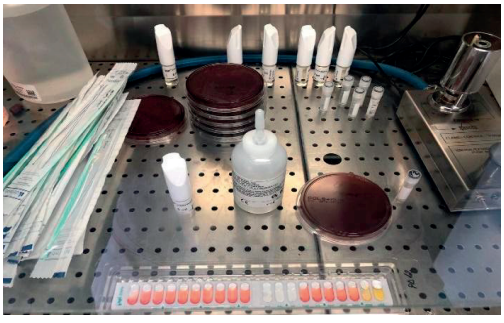

Figure 2. Api Staph kit (BioMerieux)

RESULTS AND DISCUSSIONS

The dominant species were *Staphylococcus xylosus* (47.5%), *Staphylococcus lentus* (30%), and *Staphylococcus sciuri* (22.5%).
The rates of detection of the *Staphylococcus* spp. on the skin samples, from the five different farms are shown in the Table 1. *Staphylococcus* spp., were detected on the skin of pigs 81.63%.

Table 1. Farm based positivity for *Staphylococcus* from the skin of the experimental pigs

Sampling	No. of samples	No. of positive (%)
A Farm	5	3 (60%)
B Farm	5	5 (100%)
C Farm	7	6 (85.71%)
D Farm	14	12 (85.71%)
E Farm	18	14 (77.77%)

The distribution of the *Staphylococcus* spp. according to the sampling area (the anterior nares of individual pigs, or from the skin behind the ears) are shown in Table 2.
The dominant species of *Staphylococcus* spp., were mostly similar in both sampling locations, except *Staphylococcus sciuri*, which was more frequently isolated from the anterior nares (23.52%), compared with skin behind the ears (6.66%). Similarly, *Staphylococcus xylosus* was found in a higher percentage (53.33%), on the skin behind the ears.

Table 2. The predominant species of *Staphylococcus* according the sampling area

	No. of positive from anterior nares = 34 (%)	No. of positive from skin behind the ears = 15 (%)
S. xylosus	11 (32.35 %)	8 (53.33%)
S. lentus	8 (23.52 %)	4 (26.66%)
S. sciuri	8 (23.52%)	1 (6.66%)

The prevalence of *Staphylococcus* spp. was different depending on the farm where the bacteria was isolated, as indicated in Figure 3.
Knowing that the five farms were located in different areas, and data shown us that two species of the three isolated are dominant species represented by *Staphylococcus xylosus* (Farm B = 60%, Farm D = 57.14%, and farm E = 38.88%) followed by *Staphylococcus sciuri* (Farm A = 66.66%, and Farm C = 57.14%), and *Staphylococcus lentus* (Farm C, and D = 28.57%, Farm E = 27.77%, and Farm B = 20%).

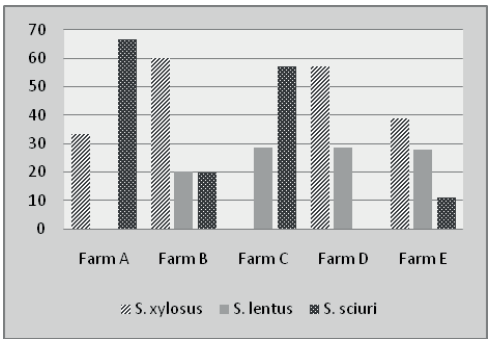

Figure 3. The dominant species of *Staphylococcus* isolated by the farm

Staphylococcus xylosus was isolated most frequently from the skin behind the ears (53.33%) and only 32.35% of isolated from anterior nares, *Staphylococcus lentus* was isolated in approximately equal percentages from the anterior nares (23.52%), and the skin behind the ears (26.66%), and the last one, *Staphylococcus sciuri* was mostly isolates from the anterior nares (23.52%).These results suggest that *Staphylococcus xylosus* and *Staphylococcus lentus* were well-adapted to the skin environment of healthy pigs.
Staphylococcus xylosus is a commensal of the skin of animals and humans. This last species was virtually defined as a non-pathogenic staphylococcus, but a few strains of *S. xylosus* were related to animal and human opportunistic infections, and murine dermatitis (Frisoni et al., 2007), as well as the most frequently isolated species associated with cow mastitis (Kot et al., 2012).
Currently little information is available regarding the pathogenicity of *S. sciuri* în animals (Chen et al., 2007). *S. sciuri* is mostly recovered from skin and mucous membranes of animals and has long been considered a non-pathogenic commensal bacterium. During the last decade this has been associated with several cases of bovine mastitis, as well as with goats infected with peste des petite ruminants virus, and also from cases of canine dermatitis, as well as from several outbreaks of fatal

exudative epidermitis in piglets (Beims et al, 2016).

S. sciuri group has also been found to carry multiple virulence and resistance genes. Indeed, genes involved in biofilm formation or coding for toxins responsible for toxic shock syndrome and multi-resistance, similar to those carried by *Staphylococcus aureus*, were detected (Nemeghaire et al., 2014). The presence of resistance and virulence genes similar to those found in *S. aureus* enhances the hypothesis that *S. sciuri* might be an important reservoir for these genes (Nemeghaire et al., 2014) for more pathogenic *Staphylococcus* species (Harrison et al., 2014).

CONCLUSIONS

The present study was conducted to reveal the incidence of cutaneous Staphylococcus species in extensively raised healthy swine. The high isolation rate of *Staphylococcus* spp. And the presence of several species: *Staphylococcus xylosus*, *Staphylococcus lentus* and *Staphylococcus sciuri* indicated an important pathogenic potential of the bacteriome in healthy animals.

These bacteria, although seemed often present as commensal animal-associated bacteria, in frequent and close contact between species on the farms may jump over species and cause pathologies bovine (mastitis), other pigs (exudative epidermitis), and could also preserve the reservoir of antibiotic resistance genes.

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ANTIMICROBIAL RESISTANCE ISOLATES FROM OVINE NECROTIC PODODERMATITIS: A REVIEW

Florina MARIAN[1], Gheorghița DUCA[2], Carmen Dana ȘANDRU[1, 2], Diana OLAH[1], Constantin CERBU[1], Marina SPÎNU[1, 2], Emoke PALL[1, 2], Aurel VASIU[1]

[1]University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, Faculty of Veterinary Medicine, 3-5 Calea Mănăştur, 400372, Cluj-Napoca, Romania
[2]Institute of Research and Development for Montanology Cristian, Sibiu, Romania

Corresponding author email: ghitaduca@yahoo.com

Abstract

Infectious pododermatitis is a common condition in sheep with initial lameness, with a variation in severeness over time. Independently on aetiology, the administration of antibiotics has proven to be ineffective, the disease often being exacerbated in a short time, the bacteria being refractory to antimicrobials. The use of antibiotics is common in sheep for disease treatment, health protection and as growth promoters. The main antibiotics used in farmed animals are tetracyclines, penicillin, quinolones and cephalosporins. According to the latest studies, E. coli, an ubiquitous bacterium often isolated from pododermatitis lesions, is unresponsive to all these antibiotics. Studies on the antibiotic resistance of the main pathogens of necrobacillary pododermatitis, Dichelobacter nodosus and Fusobacterium necrophorum, are not found in the literature, but the ineffectiveness of antibiotics on these bacteria directs towards a possible antibiotic resistance. Currently, multiple health organisations have identified proliferation of antimicrobial resistance as a global crisis. The decline in bacterial susceptibility to common antibiotics calls for global efforts on the rational use of antibiotics in veterinary medicine as a response to the spread of antimicrobial resistance.

Key words*: sheep, necrotic pododermatitis, Dichelobacter nodosus, Fusobacterium necrophorum, antibiotic resistance.*

INTRODUCTION

Footrot is an infectious disease caused by an interaction of two anaerobic bacteria, *Dichelobacter nodosus* and *Fusobacterium necrophorum,* and the bacterial community of the hoof (Beveridge, 1941) which in favorable environmental conditions multiply and destroy the foot tissue. This pathology develops more frequently in sheep but can also be diagnosed in other ruminants such as cattle, goats and South American camelids (Laing and Egerton, 1978; Ghimire et al., 1999).

The impact it has on animal welfare and the economic losses caused by the rapid spread within the herd, affecting a significant number of animals, gives the diseases more attention to the practices of preventing the introduction of this pathology in the flocks (Kennan et al., 2011).

Agricultural practices for fertilising the soil with manure, the content of which may be rich in antibiotic residues, are the major contributor to the emergence of antibiotic resistant bacteria, especially among sheep raised in the traditional system, which involves grazing on agricultural land (Khachatourians G.G., 1998). Unfounded administration of antimicrobial substances in various pathologies as well as their use as prophylaxis methods also participate in the emergence of antibiotic resistance; chlortetracycline, tylosin, neomycin, oxytetracycline are just some of the most commonly used antibiotics for this purpose.

This review concentrates data on the aetiology and epidemiology of footrot, the clinical evolution, the main methods of therapy as well as the resistance of bacterial strains to antimicrobial substances.

ETIOLOGY AND EPIDEMIOLOGY

Footrot is a sporadic-enzootic disease, with acute evolution that affects sheep of all ages, but the lesion picture is different depending on the age, in young animals more serious lesions can be detected. The breed also influences the evolution of the disease, in a study conducted by Beveridge in 1941, in Australia, there is a higher susceptibility of the merino breed compared to other breeds in which the disease has a milder evolution (Beveridge, 1941).

This pathology is highly contagious and can be spread by sheep that do not show clinical signs of disease and is easily transmitted from sheep to sheep via bedding, pasture or handling pens (S.C. Bishop and C.A. Morris, 2007).

Ovine footrot is caused by a Gram-negative bacillus, *Dichelobacter nodosus,* strictly anaerobic bacteria, immobile but with type IV pili, morphological aspect that gives it motility in damaged tissues (Bennett et al., 2009). This pathogen is a proteobacteria that belongs to the family *Cardiobacteriaceae*, genus *Dichelobacter*. Bacterial species belonging to this family are bacilli in the form of straight or curved stems, with round and thickened heads having a diameter between 0.5-1-7 μm and a length of 1-6 μm, usually staining gram negative.

Another anaerobic bacterium, *Fusobacterium necrophorum*, plays a secondary role in the pathogenesis of this pathology, being considered responsible for the necrosis of the tissues it populates (J.R. Egerton et al., 1969). It is a gram-negative, non-sporulated, immobile, necessarily anaerobic and fermentative spindle bacterium, belonging to the family *Fusobacteriaceae*, genus *Fusobacterium* (Roberts D.S., 1969). This bacteria is a normal inhabitant of the ruminant digestive tract and in conditions of high humidity interact with another bacteria, *Corynebacterium pyogenes,* to produce an infection of the skin between the toes. The synergistic action of the main pathogens causes lesions at the hoof which provides a portal of entry for organisms with habitat al soil, such as staphylococci and streptococci, *Escherichia coli*, *Clostridium perfringens* and *Actinomyces pyogenes* (M. A. Hurtado et al., 1998).

Under favourable environmental condition with mild air temperatures and high rainfall, the multiplication of these bacteria takes place, on the background of which the inflammation of the interdigital tissue takes place, a process that favours the invasion of the main pathogen, *Dichelobacter nodosus* (Angela Lacombe-Antoneli et al., 2007).

Compared to *Fusobacterium necrophorum,* which is ubiquitous in the soil or present in sheep faeces, *Dichelobacter nodosus* can survive in the environment for 7-10 days and for up to 6 weeks in hoof horn clippings, dry heat and cold weather significantly reducing the lifespan of this bacterium (Whittington, 1995).

CLINIC PICTURE

The benign and virulent are the two forms of the disease that are well-studied and described. In the benign footrot the strains of *D. nodosus* do not have virulence factors and from a lesional point of view only the interdigital skin is inflamed, comparative with virulent strains, underrunning of the hoof horn occurs (J.R. Egerton et al., 1969). The virulence of *Dichelobacter nodosus* is due to the number of members that are responsible for the secretion of thermostable proteins that allow this bacterium to digest the connective tissue between the horn and the flesh of the hoof (K.J. Smith et al., 2021).

Infectious pododermatitis suddenly begins with a lameness caused by pain in the foot, the animal avoiding using his affected limb accompanied with decrease appetite (John F. Currin et al., 2005). The skin and soft tissues of the interdigital space become erythematous and congested, pathological changes that in the absence of therapy will progress to erosion. The cracks created at the level of the hoof constitute a path of penetration of pathogens that will colonise the affected tissue and the activity of bacterial enzymes causes their destruction, the infection spreading to the sole of the hoof, undermining and causing the separation of the horny tissues (Whittier W.D. & Umberger S.H., 2010). Moderate to severe fever with significant increase of heart and respiratory rates are general changes in health (Wessam Monther Mohammed Saleh et al., 2019).

ANTIBIOTIC THERAPY

Antibiotics, substances capable of inhibiting the growth or destruction of microorganisms are widely used as bacteriostatic or bactericidal drugs used in the infection (Pomorska-Mól & Z., 2012). The rational use of antibiotics against pathogens is conditioned by the accurate diagnosis and knowledge of bacterial strains involved in the pathogenesis of the disease by sampling lesions, culture of bacterial species and their identification by specific

methods. It is most important that antibiograms be performed after the bacteria have been identified to test for bacterial susceptibility to the antibiotics tested. It is also important to know the pharmacological properties of the antibiotic and evaluating host factors (Gürdal Y. et al., 2018).

In the therapy of footrot there are two important limitations in the use of antibiotics. First of all it has a high efficacy only if the animals under treatment are held in dry conditions for 24 h following treatment. Second, the rapid elimination of the antibiotic from the body does not provide protection against reinfection (Abbott & Lewis, 2005). Despite these limitations, the administration of antibiotics in the case of this pathology remains the main form of therapy used in current practice.

ANTIBIOTIC RESISTANCE

Antibiotics are certainly the most widely used form of chemotherapy of all time. The beneficial effect of bread on which filamentous mushrooms were grown has been known since ancient Egypt and has been used to treat wounds and burns (Pecanac M. et al., 2013).

Penicillin was accidentally discovered in 1928 by Alexander Fleming, who forgot about hys staphylococcal culture and later found it in the laboratory and noticed how their growth was inhibited by a bluish-green mould of the genus *Penicillium,* which he later called penicillin.

Starting with penicillin, between the years 1940-1970, up to 23 classes of antibiotics were identified and obtained by the pharmaceutical industry, which were and are successfully used by clinicians. After this time, the rate of emergence of new classes of antibiotics is slowed down in contrast to the increasing rate of emergence of antibiotic resistance.

Many bacterial strains have become resistant to antibiotics, a large percentage of which are resistant to several classes of antibiotics, a process that has led to the phenomenon of multidrug resistance (Martinez J.L., 2014).

Antimicrobial resistance is old and is the expected result of the interaction of several microorganisms with the environment. Most antimicrobial compounds are naturally occurring molecules and such co-occurring bacteria have developed antibiotic defence mechanisms to survive these organisms being often considered intrinsically resistant to one or more antimicrobials (Khachatourians, G.G., 1998).

Although some species show a natural resistance to different classes of antibiotics, most species acquire this resistance through mutations in cell genes that lead to cross-resistance and the transfer of genes from one microorganism to another through plasmids, transposons, integrons and bacteriophages. Once bacteria gain resistance genes that help protect them from microbial agents, they can use various biochemical resistance mechanisms, the most common of which are enzymatic inactivation, altering target receptors and actively expelling the antibiotic through efflux cell pumps (Georgina Cox & Gerard D. Wright, 2013).

Repeated use of antibiotics increases bacterial adaptability to used antibiotics, some microbes exhibit cross selection and co-selection to different antibiotics along with direct selection (O'Brien, 2002).

Cross-resistance is in the situation in which resistance to one drug is associated with resistance to another drug and due to a single mechanism. Cross resistance can be limited to some antimicrobial class, can occur between all members or involve antimicrobials belonging to different classes. Co-resistance is due to the coexistence of genes or mutations in the same strain, each conferring resistance to a different class of drugs (Luca Guardabassi & Patrice Courvalin, 2006).

In a study of 97 strains of *Dichelobacter nodosus* bacteria isolated from small ruminants with lesions in which antibiotic treatment was attempted, 50% of the tested strains were resistant to penicillin and 70% were found to be resistant to the tested aminoglycosides (kanamycin, streptomycin, gentamicin and neomycin) (Angela Lacombe-Antoneli et al., 2007). In the same study, *Fusobacterium necrophorum*, the other bacterial strain isolated from pododermatitis lesions, is sensitive to penicillin and aminoglycosides.

CONCLUSIONS

Lameness is a common cause of well-being and economic concerns in most sheep-keeping

countries. Making a correct diagnosis is essential in setting up treatment and controlling lameness (A.C. Winter, 2008).

Footrot remains a difficult to treat infectious disease and the use of antibiotics as the first line of treatment in various diseases increases antibiotic resistance among bacterial strains (Georgina Cox & Gerard D. Wright, 2013).

Continuing the misuse of antibiotics will hamper our ability to effectively treat infectious diseases in the future.

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TOPICAL AUTOLOGOUS PLATELET-RICH PLASMA (PRP) IN MANAGEMENT OF PERIANAL FISTULAS IN A GERMAN SHEPHERD DOG

Eleonora MONTI[1], Giulia MORETTI[1], Lisa GAROFANINI[1], Rolando ARCELLI[1], Alexandra PETEOACA[2], Elisabetta CHIARADIA[1], Alessia TOGNOLONI[1], Antonello BUFALARI[1]

[1]Department of Veterinary Medicine, University of Perugia - Via S. Costanzo, 4, Perugia, Italy

[2]University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, 105 Splaiul Independentei, District 5, Bucharest, Romania

Corresponding author email: eleonoramonti94@gmail.com

Abstract

Platelet-rich plasma (PRP) is the processed liquid fraction of autologous peripheral blood with high concentration of platelets. Thanks to its ability to speed up the healing process, PRP is used in the treatment of many diseases in which tissue regeneration is required. Canine perianal fistulas disease (PAF) is a painful and chronic disease of the perianal tissues that affects medium to large breed dogs, predominantly German shepherd dogs.
The aim of this report is to describe the clinical efficacy of autologous PRP as an adjuvant therapy in the treatment of multiple perianal fistulas in a nine year old German shepherd dog.
Autologous PRP (3.5 mL) containing $8x10^5$ platets/ml was administered directly into fistulas by 2 injections at weekly intervals. Complete healing of the lesions occurred four weeks after the first treatment with PRP.
This case report hypothesizes that autologous PRP could be considered as an excellent adjuvant to conventional therapies for the treatment of canine perianal fistulas.

Key words: *Platelet-rich plasma, perianal fistulas, dog.*

INTRODUCTION

Platelet-Rich Plasma is a product made of autologous plasma with a higher concentration of platelets (PLT) than is found in normal whole blood (Marx, 2001).
Platelets contain storage pool of growth factors which promote tissue repair and influence the reactivity of vascular and other blood cells in angiogenesis and inflammation. In recent years the application of PRP has been widely extended in diverse medical and surgical procedures (Anitua et al., 2004). Thanks to its regenerative features, in human medicine autologous PRP is used predominantly in the fields of maxillofacial surgery (Marx et al., 1998), orthopedic surgery (Savarino et al., 2006), periodontic surgery (Hanna et al., 2004), plastic surgery (Powell et al., 2001), thoracic surgery (Englert et al., 2005), vascular surgery (Crovetti et al., 2005) and ophtalmology (Korobelnic et al., 1996).
Interest in the use of PRP is also growing in veterinary medicine, as shown by several studies conducted on its therapeutic use expecially in musculoskeletal, tendon (Torricelli et al., 2011; Bosch et al., 2011; Sample et al., 2018) and soft tissue (Farghali et al., 2017; Farghali et al., 2019; Perego et al., 2021) injures in horses and dogs.
Canine perianal fistula disease (PAF), also known as anal furunculosis, is a painful and chronic disease in which sinus tracts or ulcers spontaneously occur in the skin and soft tissues around the anus (Cain, 2019).
The disease affects predominantly German shepherd dogs (Budsberg et al., 1985), however other purebred and mixed-breed dogs also are affected (Day et al., 1992).
Inflammation and ulcerations around the anal region lead to tenesmus, constipation, hematochezia, self-mutilation, anal stenosis and severe discomfort. Dogs can show systemic signs like anorexia, weight loss, diarrhea and lethargy (Jamieson et al., 2002).
PAF must be distinguished from other conditions that can lead to perianal fistulization

such as anal sacs rupture, perianal ulcerated neoplasms (perianal adenoma or adenocarcinoma) or mucocutaneous lupus erythematosus (MCLE). Furthermore, glandular tissue left behind after an anal sacculectomy can result in chronic perianal draining tracts that can be misdiagnosed as PAF.
Usually the diagnosis of PAF is based on anamnesis, clinical signs and findings of the physical examination, but, in doubtful cases, performing histopathology can be necessary to exclude neoplasia or MCLE (Cain, 2019).
Although development of PAF was once believed related to anatomic conformation, the condition is now recognized as immune mediated, although the pathogenesis has not been fully delineated. Medical management based on immunosuppressive agents is the current standard of care for dogs with perianal fistulas (Cain, 2019). Few data are available on the possible clinical use of PRP (Perego et al., 2017), although its regenerative properties may be extremely helpful in treating the disease.
This report wants to describe the therapeutic efficacy of autologous PRP as an adjuvant therapy in the treatment of multiple PAF in a German shepherd dog.

CASE DESCRIPTION

A nine years old German shepherd dog, neutered female, with multiple perianal fistulas was treated for at least 9 months with cycles of prednisolone (Deltacortene®, Bruno Farmaceutici S.p.A, Italy) and various antibiotics orally. No significant improvement was noted after this period of time, so the dog was referred for clinical evaluation at the University Veterinary Teaching Hospital (OVUD) of the University of Perugia.
The dog was fully vaccinated against canine distemper virus (CDV), canine parvovirus CPV, leptospirosis, and infectious canine hepatitis (ICH) and received regular prophylaxis against ectoparasites.
On physical examination the dog presented poor coat and nutrition (3/9 BCS). Examination of perianal region revealed multiple fistulas in the dorsal and right lateral portion of perineum (Figure 1). These lesions were accompanied by erythema, serous/blood exudate, anal itching, pain on defecation leading to dyschezia.
A complete blood count (CBC) and biochemical profile revealed no abnormalities.

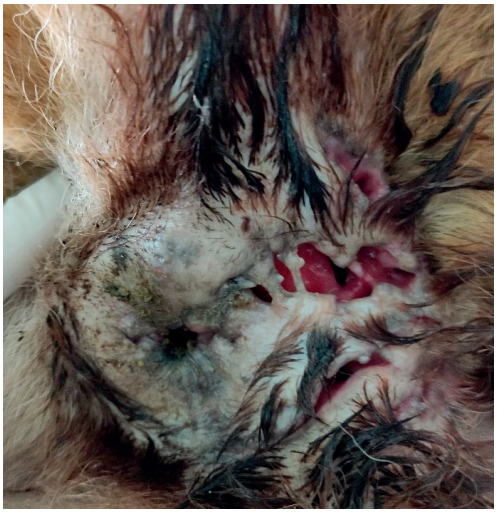

Figure 1. Multiple perianal fistulas on first physical examination

Due to the refractoriness to medical management, treatment with PRP infiltrations was instituted to promote tissue healing and regeneration.
At day 0 (D0) autologous PRP was obtained from whole blood collected from the jugular vein in citrate-dextrose solution vacutainer (Becton–Dickinson-Vacutainer®) according to Bianchini (2016). The platelet pellet, obtained after two centrifugations, was resuspended at final concentration of 8×10^5 PLT/µL and immediately injected into fistulas.
After placing the animal under general anesthesia, trichotomy, cleaning and washing of the perianal region was performed (Figure 2). During the cleaning of the lesions, a small and fresh vegetal foreign body (grass awn) was removed from the path of a fistula. A swab specimen of the fistulas was collected sterile for microbiological culture. With the help of a probe, 8 fistulas with a depth between 1.5 and 5 cm were recognized. A total amount of 0.5 ml of PRP were administrated in for fistulas longer than 2.5 cm whereas 0.25 ml were used for fistulas less than 2.5 cm in length (Figure 3).

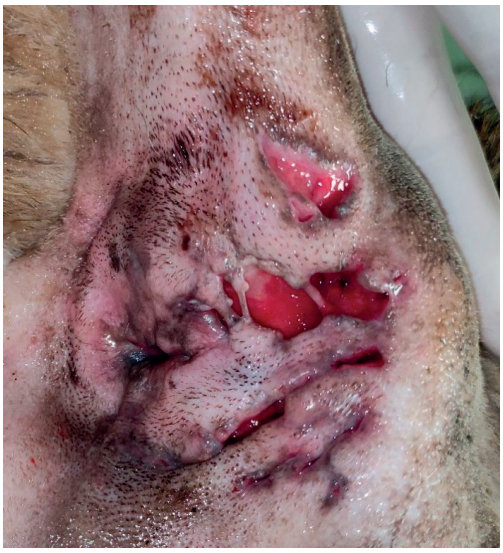

Figure 2. Perianal region after trichotomy, cleaning and washing at D0

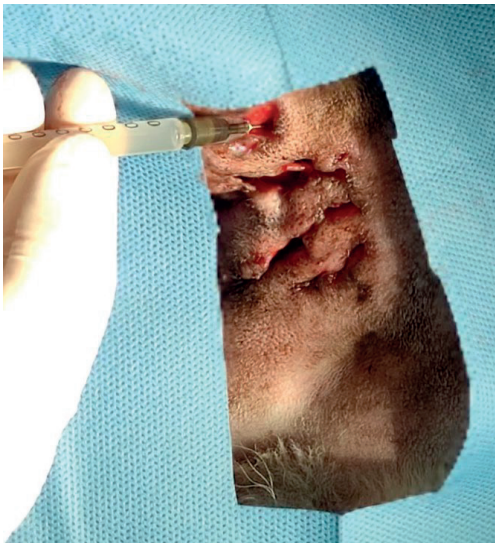

Figure 3. Perianal region during treatment with PRP (D0)

Seven days later (D7) the dog was checked and only three fistulas were found, ranging in depth from 0.7 to 1.5 cm (Figure 4). Therefore, the treatment with PRP (8×10^5 PLT/µL) was repeated by administering 0.25 mL in each fistula. In the meantime, the result of the microbiological culture had given a negative result.

At day 14 (D14) follow up no fistulas were found. Only a small superficial wound remained that was not treated with PRP (Figure 5).

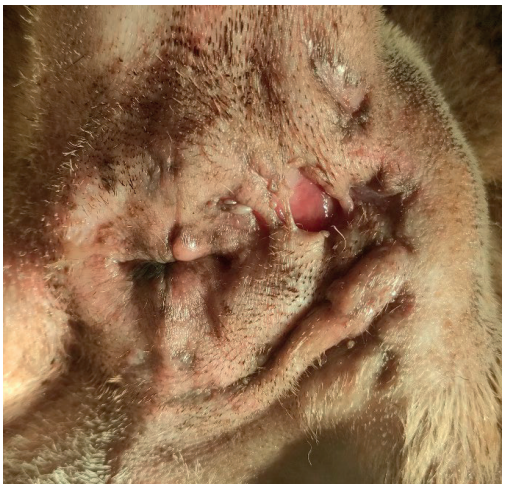

Figure 4. Clinical stage after 7 days (D7). A marked reduction of the fistula openings is observed

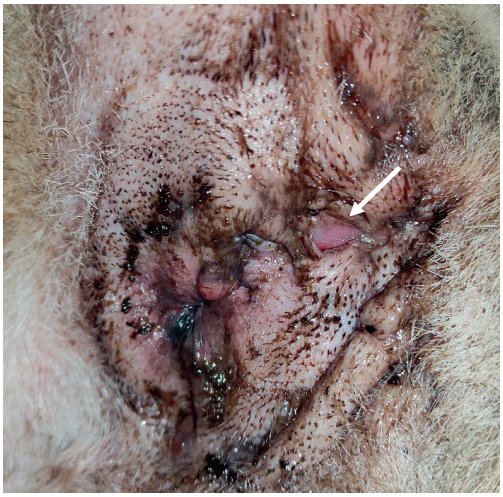

Figure 5. Clinical stage after 14 days (D14). Only a small superficial wound is left (white arrow)

The dog was then checked every two weeks in the following two months to evaluate the clinical improvement.

Medical therapy, starting from D0, involved the administration of 20 mg/kg metronidazole (Metrobactin®, Le Vet Beheer B.V., Italy) for 14 days, 1mg/kg prednisone (Deltacortene®, Bruno Farmaceutici S.p.A, Italy) for 1 week, then reduced to 0.75 mg/kg for five weeks and to 0.5 mg/kg for two weeks.

Tacrolimus ointment was applied topically for 4 weeks (PROTOPIC® 0,1%, Leo Pharma, Italy). From D0 the diet of the dog was restricted to a novel-protein (fish) diet.

Lesions improved significantly, with a marked reduction of the fistula openings observed starting one week after the first PRP application. Total disappearance of anal pruritus, serum exudate and dyschezia occurred in 14 days, while complete healing of the lesions occurred 4 weeks from the first treatment with PRP (Figure 6).

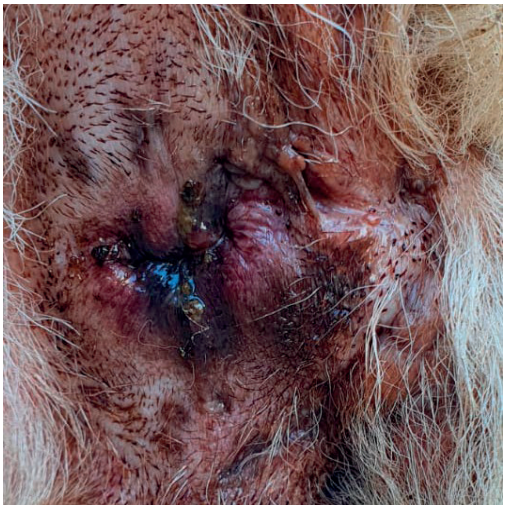

Figure 6. Complete healing of the lesions 4 weeks after the first application of PRP (D28)

DISCUSSIONS

Canine perianal fistulas disease is an extremely debilitating condition in affected dogs and can result in severe illness, up to euthanasia, if not effectively managed.

Treatment of PAF is challenging due to the lack of a complete awareness about its etiopathogenesis.

It was once believed that the development of PAF in the German shepherd dog was linked to anatomic features, such as low tail carriage and a higher density of perianal apocrine sweat glands (Budsberg et al., 1985; Killingsworth et al., 1988), but the consciousness that several breeds with different tail carriage can develop the condition and the lack of clinical response to antimicrobial therapy alone, led to investigate new theories (Killingsworth et al., 1988).

In the case of German shepherd dog, the strong association between the breed and perianal fistulas suggested a genetic susceptibility. In recent years few studies have explored potential genetic risk factors for the disease in German shepherd breed with interesting preliminary results (Kennedy et al., 2008; Barnes et al., 2009; Massey et al., 2014). A potential shared pathogenesis has been suggested for human Crohn's disease (CD) and ulcerative colitis and canine perianal fistulas, due to a particular genetic region found in both diseases (Massey et al., 2014). Furthermore clinical (Sandborn et al. 2003; Galandiuk et al. 2005) and histological (Day et al., 1992) similarities have been recognized between PAF and perianal CD in man.

This led to think that PAF probably has an immune-mediated pathogenesis, as many studies have tried to demonstrate (Day and Weaver, 1992; Day et al., 1993; Harkin et al. 1996; Mathews et al. 1997; Mathews and Sukhiani 1997; House et al. 2003; Tivers et al. 2008; Kennedy et al. 2008), and may be related to an inflammatory colitis or other chronic inflammatory bowel conditions (Jamieson et al.,2002), some of which can be related to food sensitivity, but, in the case of PAF, it is rarely the only contributing factor (Proverbio et al., 2010).

Due to these considerations, over the years there has been a paradigm shift from surgical management to long-term medical management of canine perianal fistulas. Surgical intervention and correction of anatomic factors were once the mainstays of therapy, with varying recurrence rates and a high prevalence of complications (Vasseur, 1984; Milner et al., 2006). Today immunosuppressive drugs are the most commonly used therapies for management of canine perianal fistulas, with the best evidence of efficacy for calcineurin inhibitors (cyclosporine A or tacrolimus) (Harkin et al., 1996; Mathews and Sukhiani 1997; Mathews et al., 1997; Tisdall et al., 1999; Griffiths et al., 1999; Misseghers et al., 2000; Patricelli et al., 2002; Mouatt, 2002; Doust et al., 2003; O'Neill et al., 2004; Hardie et al., 2005; Klein et al., 2006; House et al., 2006; Harkin et al., 2007; Stanley and Hauptman, 2009).

Despite their effectiveness, finding alternative treatment strategies, which allow at least to reduce the dosages or treatment duration of immunomodulatory molecules, is very important due to the many side effects of these agents and very high costs of some of them

(Mathews and Sukhiani 1997; Tisdal et al., 1999).

On purpose, PRP is increasingly used in regenerative medicine, as evidenced by several published and experimental reports in human and veterinary medicine, and could represent a new resource in the treatment of PAF.

Autologous PRP is a cost-effective and readily available therapeutic blood derivative. It is rich in growth factors, expecially platelet-derived growth factors (PDGF) and transforming growth factor-β (TGF-β) (Kim et al., 2009), which influence cellular recruitment, proliferation and differentiation enhancing wound healing and tissue regeneration (Anitua et al., 2004).

In this clinical case, PRP was combined with prednisone, tacrolimus and metronidazole, which are conventionally used alone or combined for the treatment of PAF (Harkin et al., 1996; Tisdal et al., 1999; Misseghers et al., 2000; Stanley and Hauptman, 2009).

Tacrolimus and prednisone are both immunomodulatory agents.

Tacrolimus, such as cyclosporine, is a calcineurin inhibitor. They cause decreased growth and activation of T lymphocytes (Palmeiro et al., 2013) but, unlike cyclosporine, which is most effective when administered orally, tacrolimus is effective topically, minimizing the risk of systemic adverse effects (Lauerma et al., 1997).

Its topical application inhibits T-lymphocyte activation and cytokine elaboration in the skin and draining lymph nodes (Homey et al., 1998) and appears to be safe when applied for long periods in humans, with no systemic accumulation and minimal adverse effects (Reitamo et al., 2000). It brings considerable benefits in humans with psoriasis, atopic dermatitis, pyoderma gangrenosum, oral and perineal Crohn's disease, and vulvar lichen sclerosis (Assmann et al., 2003; Assmann et al., 2000; Assmann et al., 2001; Casson et al., 2000; Ruzicka et al., 1999; Ruzicka et al., 2003; Ruzicka et al., 1997; Schuppe et al., 1998).

To authors knowledge, there are few studies involving topical application of tacrolimus ointment in dogs with perianal fistulas (Misseghers et al., 2000; Stanley and Hauptman, 2009).

Tacrolimus and prednisone used individually for the treatment of perianal fistulas have provided encouraging results. In 1996 Harkin et al. observed that 67% of patients with PAF, treated with oral prednisone and a commercially available novel-protein diet for up to 16 weeks, improved but only the 33,3% of dogs achieved complete wound healing (Harkin et al., 1996).

In 2000 Misseghers et al. evaluated the effect of 16 weeks topical application of 0.1% tacrolimus ointment as the sole treatment for perianal sinuses in 10 dogs. In that study, 5 dogs had complete resolution of lesions, 4 dogs had a partial response and 1 dog did not improve.

A better outcome resulted from the combination of the two molecules, in association with a novel-protein diet and metronidazole, administered for 16 weeks (Stanley and Hauptman, 2009). At the end of that period, perianal sinuses resolved completely in 15 of 19 dogs treated.

For this study, the authors chose to combine them, in order to provide initial systemic immunosuppression with prednisone and to inhibit local T-lymphocyte activation with tacrolimus.

Despite the negative outcome of the microbiological culture, metronidazole was administered due to its antimicrobial activity against faecal anaerobes and immunomodulatory effects (Killingsworth et al., 1988).

Finally, a strict diet based on a new protein (fish) was included with the intent of avoiding allergens in case the perianal sinuses or any concomitant colitis may have been caused or exacerbated by a food antigen.

This drugs association led to complete wound healing after only 4 weeks from the starting of the treatment.

We can not determine to what extent every drug was responsible for the healing of the fistulas, but, based on the results of the studies conducted on their use, we can hypothesize that PRP could have reduced healing time.

The observation of the dog is still in progress, as only 8 weeks have passed from the first application of the PRP to the drafting of this case report, but in this period no recurrences of lesions and no adverse effects were observed.

Unfortunately, recurrence of disease is a common problem following cessation of immunosuppressive treatment for immuno-mediated pathologies, therefore it will be

interesting to observe if there will be a recurrence of the lesions, once the low dose of prednisolone-based therapy is stopped.

CONCLUSIONS

This case report hypothesizes that the autologous PRP obtained with a double centrifugation method could be considered as an excellent adjuvant to conventional therapies for the treatment of canine perianal fistulas.
Associating PRP with therapeutic protocols already note may speed the healing of perianal fistulas.
For this purpose, new large-scale studies are needed in order to elucidate a clear mechanism of action and identify negative effects of use.

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CHARACTERIZATION OF A *Clostridium chauvoei* STRAIN, CANDIDATE AS A VACCINE STRAIN

Elena NEGRU [1], Anca BULGARU[1], Horia DINU[1], Dragoş LUPU[1], Mihai DANEŞ[2], Maria-Rodica GURAU[1], Doina DANEŞ[1]

[1]University of Agronomic Sciences and Veterinary Medicine, Faculty of Veterinary Medicine, 105, Splaiul Independentei, 5th district, Bucharest, Romania
[2]Faculty of Veterinary Medicine/Spiru Haret University, 256 Basarabia Avenue, 2nd district, Bucharest, Romania

Corresponding author email: elenanegru2003@gmail.com

Abstract

Clostridium chauvoei is the etiological agent of blackleg, a severe disease of cattle and small ruminants, characterized by necro-hemorrhagic myositis and a superacute evolution. The superacute evolution of the disease does not allow antibiotic therapy to be effective; therefore vaccination remains the main prophylactic measure. The aim of the present study was characterize a C. chauvoei isolate, with the purpose of producing a vaccine against blackleg for small ruminants. The bacterial strain, isolated from a case of blackleg, was identified based on morphological, cultural, biochemical characteristics and PCR. The alpha toxin of the 12 hour anaerobically cultured strain provided a hemolytic titer of 1/1024, and the biological value assessed in vivo on Balb/C mice, calculated as lethal dose 50%, of 16 LD50. The inactivated whole-cell culture absorbed onto aluminum hydroxide was successful in protecting 100% of vaccinated guinea pigs, against death and clinical disease, in the challenge test. The results of the experiments recommend the isolated strain as a candidate for blackleg vaccine production.

Key words: *alpha toxin, blackleg, immunogenic, Clostridium chauvoei, vaccine.*

INTRODUCTION

Anaerobic pathology, determined by members of the Clostridium genus, is a major cause of economic loss, especially in small ruminant herds, where other pathogens, such as retroviruses and mycoplasmas, often evolve simultaneously (Enache et al., 2017; Turcu et al., 2010). Blackleg is a worldwide disease of cattle and small ruminants, severe and fatal, caused by *Clostridium chauvoei,* a spore-forming, Gram-positive, anaerobic bacterium. The rapid progression and high mortality of the disease cause major losses in livestock production (Groseth et al., 2011; Frey and Falquet, 2015). Cattle aged 6 to 24 months are more susceptible to blackleg, while in sheep, the disease can occur at all ages. *C. chauvoei* spores persist in the environment in the soil of pastures, manure and perished animals. Animals can become infected via the digestive or the respiratory tract, and in the case of small ruminants, infections can occur through skin lesions, caused by shearing or castration (Hatheway, 1990). The spores are transported to muscle tissue, where they remain dormant until anaerobic conditions are generated via tissue trauma, promoting the germination, multiplication and toxin production of the bacteria. The *C. chauvoei* toxins cause necrosis and edema, fever and lameness (Uzal, 2012; Frey and Falquet, 2015). The disease progresses rapidly and the death of the affected animal occurs within 48 hours of clinical onset. Due to the brief evolution of blackleg, generated mainly by bacterial toxins, antibiotic therapy is generally not effective; therefore, vaccination represents the main tool of controlling the disease (Rychener et al., 2017). Vaccination against blackleg has been documented since 1882, and remains the safest course of action for disease control (Useh et al., 2006). The aim of this study was to obtain an effective vaccine against blackleg, using a field *C. chauvoei* strain , isolated from a case of blackleg in sheep.

MATERIALS AND METHODS

Bacterial culture and toxicity assessment. The *C. chauvoei* strain was previously isolated from

a case of blackleg case in a traditional flock of sheep.

The strain was multiplied using a medium prepared based on: 1% liver meat glucose cysteine broth, 1% cooked meat medium broth, 1.5% *C. perfringens* spore broth, 1.5% casein yeast peptone, 0.7% liver hydrolysate, 0.5% yeast extract, 0.5% glucose, 0.5% L-cysteine, 0.05% fresh liver extract. Incubation was carried out 12 hours, at 37°C, 200 rpm, at a pH of 7.2 (± 0.2). Samples were collected during incubation and before inactivation for bio-molecular analysis and potency tests.

The toxin was obtained by centrifugation at 4000 rpm, for 30 minutes at 4°C, followed by filtration of supernatant through Millipore® filters of 0.8 µ 0.45 µ, 0.2 µ and finally 0.1 µ. The toxicity evaluation was performed on dilute samples (1/6, 1/8, 1/10) in peptone water. Each dilution was inoculated intravenously to Balb/C mice, 0.5 ml / mouse. The control group (5 Balb/C mice) was inoculated with sterile peptone water. All the animals were monitored during 72 hours post-inoculation.

Biochemical properties of the *C. chauvoei* strain. The biochemical characterization of the isolate was performed using conventional methods.

Nucleic acids and proteins extraction. The ZR Fungal/Bacterial DNA Miniprep Kit (Zymo Research) was used for genomic DNA extraction and two others - the Ambion™ TRIzol™ Plus RNA Purification Kit (Thermo Fischer Scientific) and Direct-zol Miniprep (Zymo Research) - for mRNA isolation and purification. The soluble proteins in the culture broth were concentrated at +4°C by ammonium sulfate precipitation (0.476% w/v) and centrifugation (20,000 g/1 h), and stored at –85°C in the 4× native buffer (40% (v/v) glycerol, 0.5 M Tris, pH 6.8).

Toxins genes detection. Amplification of the toxins sequences for *Clostridium* species were performed according to the literature (Table 1). Primers for highly conserved gene regions o C. chauvoei were designed using Vector NTI Advance 11 (Table 1). The GeneAmp® PCR 9600 system (Applied Biosystems) and Fast Start High/High Fidelity PCR System kits (Roche) were used for the amplification according to the manufacturer's recommendations, using 10 µm for each primer. The amplicons were visualized with ethidium bromide on agarose gels (Sigma-Aldrich), at appropriate concentration, and photographed with a ChemiDoc XRS+ imager (Bio-Rad Laboratories, Inc.).

Table 1. Primers and results of *Clostridium chauvoei* identification and gene expression detection

No.	Primers name	Primers sequence	Gene identification / toxotype (PCR)	Expression detection/ toxotype (RT-PCR)	Reference
1	cctACC-F	TCCATCAGGATTATCACGTGTTGG	α toxin – 687bp	α toxin – 687bp	This paper
2	cctACC-R	CCTGCATGCTCAACAGTATGGTTT			
3	ccfF	ATCGGAAACATGAGTGCTGC	flagellin C/fliC – 460bp	flagellin C/fliC – 460bp	
4	ccfR	AGTCTTTATGCTTCCGCTAG			
5	nanACC-F	ATCAGCAATAGATACATC	sialidase/nanA – 438bp	sialidase/nanA – 438bp	Vilei et al., 2011
6	nanACC-R	TGACCTCTTCCTGGTCCTGT			

Gene expression. An analysis of gene expression at the mRNA level was performed for the main virulence factors according to the literature (Table 1) using a GeneAmp® PCR 9600 system (Applied Biosystems) and Titan One RT-PCR system and Transcript One Step RT-PCR kit (Roche), as recommended by the manufacturer. The amplification products were visualized with ethidium bromide on agarose gels (Sigma-Aldrich), at appropriate concentration, and images were taken with a ChemiDoc XRS+ imager (Bio-Rad Laboratories, Inc.).

Hemolytic activity. Hemolytic activity was assessed of two fold serial dilution (up to 1:2048) of clostridial filtrate incubated sheep red cells (with PBS prewashed). The reaction was developed after 1h at +37ºC and 12 h at +4ºC. The experiments were performed in triplicates of 2 replicates.

Culture inactivation and vaccine formulation. The bacterial culture inactivation was performed when the pH showed no more variations of the preset value using formaldehyde (solution 37%), added up to the final concentration of 0.6%. The inactivation process took place over

the course of 7 days at 37°C. The addition of formaldehyde caused a pH drop of 1 unit. To check the efficacy of the inactivation process, 1 ml of the inactivated culture was inoculated i.m. to guinea pigs in 10 replicates.

The vaccine against blackleg was formulated using an aluminum-hydroxide based adjuvant. The pH was adjusted to 7.2 with sodium hydroxide solution, and the final product was tested for sterility by propagation on 10% sheep blood agar, incubated at 37°C, for 72 hours.

Vaccine efficacy test. To investigate the efficacy of the *C. chauvoei* bacterin vaccine, a challenge test was performed on guinea pigs. Each of the 10 guinea pigs was inoculated subcutaneously with 2 ml of the vaccine. After 14 days, the animals received a booster inoculation, using the same dose of vaccine. The 10 guinea pigs used as negative controls, were inoculated with sterile 0.9% saline solution, via the same route. The animals were monitored for 5 days after each inoculation, in order to note and record any systemic or local side effects. At 21 days after the booster inoculation, the vaccinated and the control animals were challenged with 0.5 ml i.m. of *C. chauvoei* live culture. The guinea pigs were monitored for 72 hours post inoculation.

RESULTS AND DISCUSSIONS

Based on PCRs results, it was confirmed that the *C. chauvoei* strain belonged to the known species or toxotype (Table 2).

Toxin expression was identified at the transcriptional level too (Figure 1, Table 2).

Table 2. Analysis results for *Clostridium chauvoei*

Toxin/ techniques	PCR	RT-PCR	Hemolytic activity
α toxin/ chauvoei	poz.	poz.	poz.
fliC/chauvoei	poz.	poz.	n.a.
nanA/ chauvoei	poz.	poz.	n.a.

Legend: poz. - positive signal; n.a. - not applied

Worldwide virulent *C. chauvoei* strains have the same toxigenic pattern represented by cytotoxin A, aggressive (Frey et al., 2012) and immunogenic (Nicholson et al., 2019). This *C. chauvoei* isolate is no exception. The expression of the main antigens of the isolate was highlighted at translational (Figure 1, Table 2) and functional levels (Table 3).

Hemolytic activity of the *C. chauvoei* isolate toxin reached the highest titer at a dilution of 1:1024 (Table 3).

The biochemical properties of the *C. chauvoei* isolate are summarized in Table 4.

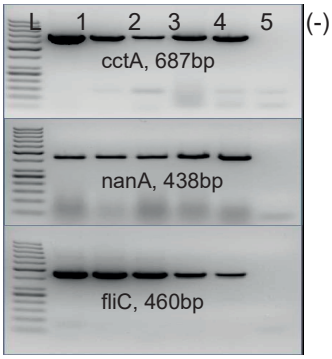

Figure 1. RT-PCR experiment/toxins expressions: *C. chauvoei:* 1 - 8 h 30' pi; 2 - 9 h 30' pi; 3 - 10 h 30' pi; 4 - 12 h pi; 5 - 13 h pi; RNA of strain was extracted from bacterial culture and amplified in order to detect specific genes expression. L - weight 50bp DNA ladder; numbers: - harvest time; (-) negative control

Table 3. Hemolytic activity of alpha toxin for *C. chauvoei* strain

Toxins dilution	**1/2**	**1/4**	**1/8**	**1/16**	**1/32**	**1/64**	**1/128**	**1/256**	**1/512**	**1/1024**	**1/2048**
C. chauvoei sample 1	+	+	+	+	+	+	+	+	+	+	-
C. chauvoei sample 2	+	+	+	+	+	+	+	+	+	+	-

Legend: +, red cells hemolysis; -, lack of hemolysis.

Table 4. Biochemical properties of the isolate

Glu	Fru	Gal	Mal	Lac	Inu	Sal	Man	Gli	Dex	Ind	Gel
+	+	+	+	+	-	-	-	-	-	-	+

Legend: GLU - glucose, Fru – fructose, Gal – galactose, Mal – maltose, , Lac-lactose, Inu – inulin, Sal – salicilin, Man- mannitol, Gli – glicerol, Dex – dextrose, Ind – indole formation, Gel - gelatin hydrolysis, + = positive result, - = negative result.

The toxicity test for the *C. chauvoei* isolate was performed on *BALB/c* male mice of 21-24 grams. The LD_{50} (lethal dose 50) was calculated for culture supernatant by a non-linear logistic regression with specific confidence intervals, depending on the minimal value necessary for the vaccine formulation. Each dilution was inoculated by caudal intravenous route 0.5 ml/mouse, in 5 replicates. The control group was represented by 5 mice inoculated with sterile peptone water. The animals were monitored for 72 hours after inoculation; moribund animals were humanely euthanized and considered as positive results (Table 5). The experiments were performed in duplicates. The biological value of the *C. chauvoei* toxins assessed *in vivo* was 16 LD_{50}.

Table 5. Toxicity tests design and results

Species/ Toxotypes	Toxin dilutions	Dead mice		
		24 h pi	48 h pi	72 h pi
C. chauvoei	1/6	5	-	-
	1/8	5	-	-
	1/10	**0**	**1**	**1**

Legend: numbers - media of duplicate experiments; bold letters - highest dilution inflicting death.

Culture inactivation tests demonstrated the efficacy of the formalin treatment. All 10 guinea pigs inoculated with the inactivated culture survived, and showed no local or systemic reactions following inoculation.

The efficacy test of the formulated blackleg vaccine was performed on guinea pigs. Post-vaccination side effects were limited to small local reactions at the site of inoculation, in the form of subcutaneous nodules, sized 0.2-0.4 cm. The animals in the control group showed no local or systemic reactions to the saline solution. All the animals in the vaccinated group survived the challenge with the virulent *C. chauvoei* culture, and none of the vaccinated animals developed clinical disease. Within the control group, all the guinea pigs died within 48 hours following the challenge. The results of the current study have demonstrated that the locally isolated strain of *C. chauvoei* can be an adequate candidate for vaccine production.

Anaerobes belonging to the *Clostridium* genus can cause numerous severe diseases, and their ability to sporulate cancels the eradication attempt. Clostridial bacterins are of high importance in livestock production, as immunization is the only option to avoid animal clostridiosis (Zaragoza et al., 2019). The selection, characterization and improvement of *Clostridium* strains are essential the development of an effective vaccine to control this burdensome pathology (Negru et al., 2021). Due to the complex antigenic structure of *C. chauvoei*, some researches recommend the use of local isolates to secure the vaccine's efficacy (Araujo et al., 2010). Other important factors are the use of appropriate culture media to ensure a positive impact on the antigenicity capacity of the *Clostridium* strain (Cortinas et al., 1994), and the stabilization / induction of *in vitro* multiplication capacity in large scale setting (Jabbari et al., 2012). Further research is required in order to determine the efficacy of *C. chauvoei* vaccines in protecting the target species against blackleg (Uzal, 2012).

CONCLUSIONS

A local *C. chauvoei* isolate was genetically and biochemically characterized, with the purpose of obtaining an effective vaccine against blackleg. Toxin potency value assessed *in vivo* for the *C. chauvoei* isolate was 16 LD_{50}. The alpha toxin of the isolate had a hemolytic titer of 1/1024. The vaccine prepared using the *C. chauvoei* strain was successful in protecting 100% of the vaccinated guinea pigs in the challenge test. The results of the experiments recommend the isolated strain as a candidate for blackleg vaccine production.

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A COMPARATIVE EPIDEMIOLOGICAL EVALUATION OF TWO SUBSEQUENT EPISODES OF MAREK'S DISEASE ON THE SAME FARM

Alexandra OPRESCU[1], Aurel VASIU[2], Carmen Dana ȘANDRU[2, 3], Diana OLAH[2], Florina MARIAN[2], Marina SPÎNU[2, 3], Emoke PALL[2, 3], Gheorghiță DUCA[3], Mariana RUSU[3], Ioan Liviu MITREA[1]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd, District 1, Bucharest, Romania
[2]University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, 3-5 Calea Manastur, Cluj-Napoca, Romania
[3]Institute of Research and Development for Montanology Cristian, Sibiu, Romania

Corresponding author email: aurel_vasiu@yahoo.com

Abstract

Marek's disease (MD) is highly economically impacting viral disease in chickens aged 4 weeks - 4 month. Various hypotheses were posed to explain its recurrent epidemiology and increasing virulence over the years. This study aimed at comparing the epidemiological indicators of two confirmed Marek's disease episodes (DIVA Real Time PCR) in two different series of Isa Brown pullets hatched one week apart on the same farm. The data indicated an increasing mortality due to MD from week 6 towards week 14, from 0.28 to 2.72% in the first series and 0.07 to 1.43% in the second one, with a cumulative mortality 3.26% and 2.28% respectively. There were no statistically significant differences between the weight gains of the two series on week 14 (peak of MD mortality), but it decreased in the first series (1398.7g versus 1478.00 g respectively) by week 16. Although the two episodes significantly differed in mortality ($p<0.05$), except a slight variation of the size of the flock, no other circumstances could have been identified as influential causes, the variability being attributed to differences in viral pathogenicity.

Key words: *Marek's disease, Isa Brown pullets, mortality, variability, viral pathogenicity.*

INTRODUCTION

Stressors and the evolution of infectious diseases have a negative effect on the health and welfare of birds raised in intensive farming systems. Hatching, vaccinations, microclimate factors of the halls, food deficiencies affect the innate and adaptive immunity. Infectious diseases resulting in immunosuppression (Gumboro disease, chicken infectious anemia Marek's disease), can be directly correlated with increased susceptibility to viral, bacterial and parasitic diseases thus influencing post-vaccination immunity (Hoerr, 2010) and increased morbidity and mortality with significant economic losses (Lütticken, 1997; Woolhouse, 2011).

Marek's disease (MD) is a disease induced by a virus of the Herpesviridae family (OIE, 2010) which affects chickens, associated with lympho-proliferative syndromes (Baigent and Davison, 2004; 2006) with polymorphic clinical manifestations, and from a lesional point of view characterized by the appearance lymphoid tumors in the spleen, liver and nerves (Calnek, 1979; 2001). Functionally altered lymphocytes can no longer truly support humoral or cell-mediated immunity. It is important to differentiate between enteric viruses, reoviruses, retroviruses, adenoviruses or avian pneumoviruses, which are associated with cell depletion or lymphoid organ atrophy, affecting their function.

In infectious diseases in chickens, the immunosuppressive potential of the etiological agent is poorly characterized (Gong et al., 2013). After establishing the diagnosis by applying specific methods, to control the outbreak, it is imperative to assess the degree of immunosuppression. The effective application of external and internal biosecurity measures, the reduction of stressors, the application of specific and non-specific prophylaxis, the increase of disease resistance could have a major impact on the elimination of these diseases. Moreover, in the case of MD through

genetic selection it would be possible to eliminate this pathology (Hoerr, 2010; Zhang et al., 2015). In this framework, the aim of our study focused on evaluating the developmental model of MD in Isa Brown replacement chicks, hatched at one week difference, coming from an intensive breeding farm.

MATERIALS AND METHODS

The research included two series of Isa Brown chickens, raised on the same intensive raising enterprise, in different chicken houses. The first series (A) included 109,350 birds while the second (B) was somewhat larger, of 110,625 birds. All birds shared the same rearing technology, while the anti-Marek's disease was performed on day 1 (Innovax ILT/HVT + Nobilis Rismavac + Cryomarex Rispens + HVT), in the incubation unit of the farm.

The birds were closely monitored during their technological cycle from week 1 to week 16. The body weight in g was measured and compared to the range limits. Similarly, the body weight range, the growth curve, the weekly mortality, cumulated mortality and feed consumption were recorded.

Marek's disease appeared on week 6 in both series of birds. At necropsy, samples were collected from the nerves, spleens, ovaries, livers, feather pulp and gizzard of the diseased chickens on FTA (Flinders Technology Associates) cards. An FTA card is a chemically treated filter paper designed for the collection, preservation and shipment of biological samples for subsequent DNA and RNA analysis. Special chemicals lyse and inactivate bacteria and viruses and preserve their DNA and RNA for detection by PCR. The swabs collected from various organs need to be pressed against the FTA card. If the procedure is correct after placing the samples on the card, you will notice changes in the color of the card, initially pink, and modifying it to white. The cards are dried at room temperature, heated in the microwave for 20 seconds at 900W. Subsequently, the samples were analyzed by DIVA RT-PCR in the CEVA Phylaxia laboratories in Deventer, the Netherlands, and the histopathological analysis of the samples was performed in the same laboratory.

When the disease episode started, the mortality caused by Marek's disease was also recorded separately, based on the pathological changes noticed at necropsy, while all the other parameters were continuously recorded.

The birds received balanced fodder according to their age, and were watered *ad libitum*.

Preventive and control measures were applied according to the layer replacement technology.

RESULTS AND DISCUSSIONS

Well-known as a viral neoplastic disease of chickens, Marek's disease (MD) is defined by the neoplastic changes at mainly T cell levels, which can reside in immune suppression and also neurological clinical disease. The virus is shed mainly at the feather pulp level, and afterwards is dispersed by the dust particles in the chicken house, the respiratory process leading to initiation of the pathogenesis. Vaccination against Marek's disease, although a wide-spread preventive procedure, does only inhibit the clinical expression, not the shedding of the virus by the infected birds (Boodhoo et al., 2016). Thus, by use of the Rispens (CVI988) vaccine, the infection decreased as clinical prevalence, but not the persistence of the virus on the farm.

Although some researchers believe no major problems appear to be uncontrolled with existing anti Marek's disease vaccination and non-specific preventive technologies worldwide (Morrow and Fehler, 2004), there are some episodes difficult to diagnose and prevent form re-emerging.

On the investigated enterprise, the clinical expression of the disease was recorded as an acute form with birds showing transient paralysis of the limbs. The FTA card results indicated in both episodes, the presence of the Rispens and MDV1 strains in all samples tested by PCR. The histopathology tests revealed an infiltration with small lymphocytes, some lymphoblasts and plasma cells at the level of the sciatic nerve. No changes were observed in the brain of the birds.

Tables 1 and 2 present the descriptive epidemiological indicators of the Marek's disease episodes compared during the research period.

Table 1. Descriptive indicators of the Marek's disease episode A

Age weeks	Morta-lity	Cumu-lated Morta-lity no.	Cumu-lated morta-lity %	Marek morta-lity no	Cumu-lated Marek morta-lity no	Marek morta-lity %
1	560	560	0.51			
2	163	723	0.66			
3	42	765	0.70			
4	48	813	0.74			
5	66	879	0.80			
6	225	1104	1.01	187	187	**0.28**
7	485	1589	1.45	389	576	**0.86**
8	521	2110	1.93	427	1003	**1.49**
9	276	2386	2.18	243	1246	**1.85**
10	362	2748	2.51	282	1528	**2.27**
11	207	2955	2.70	145	1673	**2.49**
12	337	3292	3.01	94	1767	**2.63**
13	221	3513	3.21	42	1809	**2.69**
14	57	3570	3.26	17	1826	**2.72**
15	38	3608	3.30			
16	21	3629	3.32			
Aver	226.81	2140.25	**1.96**	202.89	1290.56	**1.92**
St. dev.	182.65	1206.85	1.10	145.19	591.42	0.88

The data recorded indicated a non-significantly ($p = 0.08$) increased overall cumulative mortality in episode A versus B (1.96 ± 1.10 versus 1.37 ± 0.67, Tables 1 and 2), noting a significant decrease in mortality due to MD in the two episodes (A and B) ($p = 0.01308$), which may occur due to the accommodation of the second series birds to the existing infectious pressure.

Table 2. Descriptive indicators of the Marek's disease episode B

Age weeks	Morta-lity	Cumu-lated Morta-lity no.	Cumu-lated morta-lity %	Marek morta-lity no	Cumu-lated Marek morta-lity no	Marek morta-lity %
1	650	650	0.59			
2	114	764	0.69			
3	38	802	0.72			
4	39	841	0.76			
5	47	888	0.80			
6	81	969	0.88	46	46	**0.07**
7	211	1180	1.07	171	217	**0.32**
8	387	1567	1.42	235	452	**0.67**
9	214	1781	1.61	138	590	**0.88**
10	221	2002	1.81	185	775	**1.15**
11	187	2189	1.98	69	844	**1.26**
12	159	2348	2.12	59	903	**1.34**
13	129	2477	2.24	42	945	**1.41**
14	40	2517	2.28	17	962	**1.43**
15	26	2543	2.30			
16	650	650	0.59			
Aver	199.56	1510.50	**1.37**	106.89	637.11	**0.948**
St. dev.	200.10	742.58	0.67	76.91	334.52	0.50

But a significant decrease in mortality due to Marek's disease in one-week apart two episodes, A and B ($p = 0.01308$), which could stand for the accommodation, to some extent, of the birds of the second series to the infectious pressure existing on the farm. This assumption is supported by the decrease in total death (cumulative mortality due to all causes) by almost 50% from episode A to B.

When compared to the previous literature data (6.0 to 15.3%, Witter et al., 1970; 2005), the values obtained in this survey are significantly lower (0.948-1.92%), maybe due to continuous implementation of vaccination procedures, which diminished the clinical reflection of the disease (Biggs, 2001).

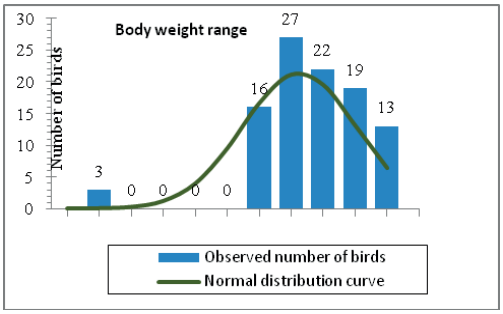

Figure 1. The body weight range recorded during episode A

The descriptors of weight range in the first series of birds are presented in Table 3.

The body weight range indictors were the same for both series, but there were slight differences in the normal distribution curve due to the disease episode.

Table 3. Variables considered to evaluate the body weight range in the first series of birds (A)

Number of birds weighed	100
Mean body weight	1153 g
Mean + 10%	1268 g
Mean – 10%	1038 g
Number of birds between 1,268.399 and 1,037.781 kg	3
Uniformity = ([100-3]/100) x 100 =	97%
Standard deviation	37 g
Coeficient of variation (CV), %	3.24%

In both series, the weight gain was only slightly altered, the difference between the two by the end of the episode in week 14 of the technology being of only 100 g. The more severe episode seemed to be self-limiting in a shorter time (15 weeks) as opposed to the milder one (16

weeks). No investigations on the further laying potential and the possible influence of the Marek's disease development in the two series were carried out.

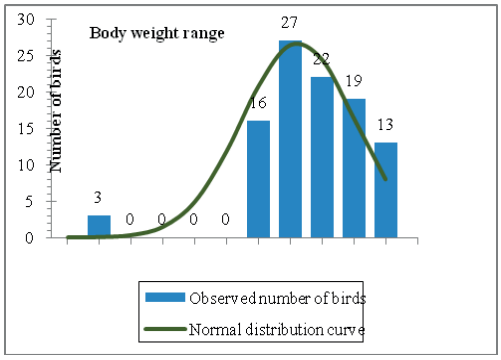

Figure 2. The body weight range recorded during episode B

In this enterprise, in the first six weeks, the infection spread, in spite of the vaccination program implemented on time and according to the recommendations of the vaccine producer, were sufficient to significantly ($p<0.05$) increase the mortality due to Marek's disease in series B, but not in series A, when comparing week 6 with week 7. In both series, the disease caused death for 5 weeks in a row, the number of birds dying of Marek's disease decreasing towards week 11. These observations are supported by the literature, according to which in commercial chicken houses virtually all birds become infected within the first few weeks of their life (CABI Datasheet, 2021). The results of the literature reveal the outbreak of MD disease from 7 to 31 weeks (Bercea, 1981). However, there are other studies that show an increased incidence of MD cases between 3-5 months and unlikely after the age of 8 weeks. The results of the literature reveal the outbreak of MD disease from 7 to 31 weeks (Bercea, 1981). However, there are other studies that show an increased incidence of MD cases between 3-5 months and unlikely after the age of 8 weeks.

More than one MDV strains were observed, including the vaccine Rispens strain in both A and B series. The incidence of non-pathogenic viruses seems to become higher with the increase in age of the birds (CABI Datasheet, 2021).

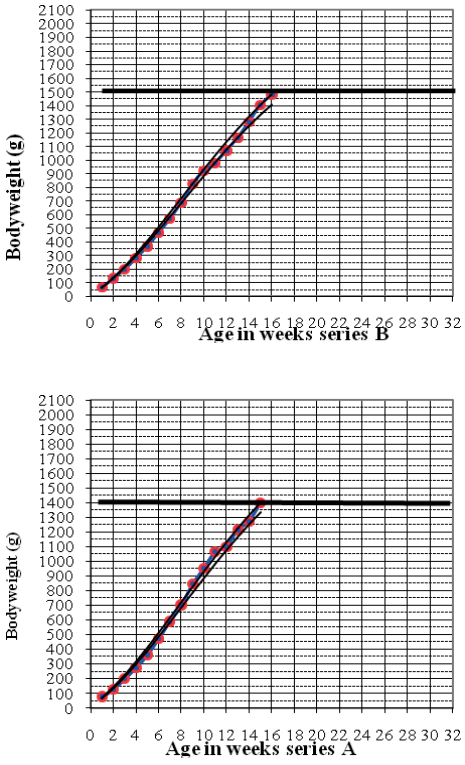

Figure 3. Growth curves for the two series which indicate the impact of the Marek's disease on weight gain in A and B

The etiological agent incriminated in the production of MD is an alpha-herpesvirus, it is a virus with oncogenic potential, it is highly contagious (Beigent et al., 2006). The incidence of this disease together with MD lymphoid leukosis of retroviral nature showed a significant increase between 1930 - 1950, with a negative effect on the birds health and welfare and implicitly with significant economic losses, globally the losses were estimated at 2 billion USD (Morrow and Fehler, 2004).The epithelial cells loaded with virus serve, by respiratory route, to transmit the disease to susceptible birds but also to contaminate the environment, the organic dust remaining infectious for several months. Affected birds could shed the virus throughout their lives, therefore precoucious diagnosis and early measures are of utmost importance. Sometimes transfer via various beetles in the bedding (*Alphitobius diaperinus,* classified in

the *Tenebrionidae* family), could also intervene in transmission (CABI Datasheets).

Due to the transformation into an intensive industry, in the case of poultry farming we are witnessing a reduction in genetic diversity and an increase in susceptibility to various diseases (Nair, 2005). The application of specific immunoprophylaxis in the case of MD is considered a relatively effective method in preventing the occurrence of this pathology (Atkins et al., 2004).Viral infections in chickens with immunosuppressive effects have a major impact on health and the economy. Although the application of specific immunoprophylaxis measures can successfully prevent the occurrence of epidemics, the emerging variants of the virus still cause increasing difficulties in controlling the disease. Because there are differences in susceptibility from a genetic point of view, such an increased level of genetic resistance could provide true means of preventing this disease. The development of genetic maps and the identification of genes that are responsible for resistance can contribute to the development of chicken lines resistant to this virus. Also, the identification of chicken lines based on the post-vaccine response may represent new possibilities for the appropriate selection of vaccination protocols (Bumstead, 1998). For effective prevention of MD, in-depth knowledge of the epidemiology and pathogenesis of the disease is a priority (Atkins et al., 2004). The immune response to MD can be regulated by the haplotype, so selection of the vaccine based on haplotype B is important. It is also important to identify and effectively eliminate stressors, which has a negative impact on the post-vaccination response (Atkins et al., 2004). For the effective control of the disease it is important to combine specific immunoprophylaxis with the increase of genetic resistance by applying appropriate selection programs (Nair, 2005). The results obtained from the study can be partially correlated with data from the literature (Adameşteanu et al., 1980).

Vaccination alone does not manage to control MD, especially if the management on the farm is poor. Infectious MDV present in dander, feathers and litter from infected flocks stays infectious for many months. Correct implementation of biosecurity measures including adequate disposal of cadavers and filthy litter, followed by appropriate disinfection of the chicken houses, strict control of bird and personnel movements help in avoiding the emergence of pathogens with increased virulence. The birds can easily get infected in a contaminated environment when being placed there while their immune system is immature (CABI Datasheet). Furthermore, the vaccination of such birds proves to be inefficient.

The increasing virulence of MDV strains worldwide requires for new specific preventive strategies. The possibility exists, supported by past and current research, for an increasing percentage of changes in virulence due to appearance of new variants. Lack of scientifically sound information for each and every episode of MD and its epidemiology dynamics can lead to further spread and further spatial expansion (Lopez et al., 2015; Lopez et al., 2019).

CONCLUSIONS

Based on the result of this study, it can be concluded that the increased incidence of MD cases is due to the lack of technological gap between different series of chickens. Effective control of MD cases can be ensured by the correct combination of technological and veterinary measures. Techniques that identify the viral pathotypes and allow the consequent monitoring of the vaccination efficacy along with personalized sanitary programs and establish more rigorous vaccination program against MDV and other viral pathogens.

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ETHIO-EPIDEMIOLOGICAL ANALYSIS OF AN ABORTIGENIC OUTBREAK OF SALMONELLOSIS IN SHEEP

Emoke PALL [1], Diana OLAH[1], Adela CHIRA[1], Gheorghiță DUCA[2], Marina SPÎNU [1], Carmen Dana ȘANDRU[1], Mihai CENARIU[1], Emilia UNGUREANU[1], Aurel VASIU[1]

[1]Department of Infectious Diseases, Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, 400372, Cluj-Napoca, Romania
[2]Institute of Research and Development for Montanology Cristian, Sibiu, Romania

Corresponding author email: emoke.pall@usamvcluj.ro

Abstract

Abortion is one of the economically important pathological implications of salmonellosis in sheep. Salmonella enterica subspecies enterica serotype abortusovis is the sheep-specific abortive serotype. Regional endemic Salmonella abortions also lead to calving of dead offspring and reduced viability, diseases in lambs from infected dams. The present study focused on the description of two outbreaks of S. abortusovis infection, with special reference to the epidemiological situation in Northwestern Romania, in the winter of 2020-2021. Two herds in which the morbidity rate (abortion) in the last period of gestation was between 13.92% - 16.66% were studied. Fetal parenchymal organs and gastric contents were harvested and processed using classical microbiological methods. Bacterial strains were confirmed by biochemical and serological methods and were identified as Salmonella abortusovis serogroup B, serotype BO. Antimicrobial susceptibility was evaluated using agar diffusion method. Multidrug resistance was found in six of these strains; all were resistant to sulfatrimethoprim *and doxycycline, one to erythromycin and one to ciprofloxacin. In order to avoid economic damage in regions known to have enzootic potential, immunoprophylaxis in dams is strongly recommended.*

Key words*: Salmonella abortusovis, serotype, multidrug resistance, sheep abortions.*

INTRODUCTION

Sheep farming in the current territory of our country is a tradition of millennia, bringing substantial economic benefits to local communities. According to Eurostat data, the sheep population in Romania is constantly growing, with an increase of approximately 482.600 animals in the period 2017-2020 and thus reaching approximately 10.4 million sheep. Thus, Romania is on the third place among the member states of the Union European (INSSE, EUROSTAT). Sheep pathology, including diseases with zoonotic potential, is widespread, therefore, given the large number of herds in our country, it is important to know the possible dangers to public health and their implications, as well as the causes that could lead to important economic losses following the evolution of infectious diseases such as salmonella abortion. The increase of sheep herds in Romania is not in line with European trends, the risk of evolution of various pathologies being implicitly higher in countries where the number of animals is higher. According to the OIE, the notifiable diseases belonging to the chapter 14 - which includes the main communicable diseases considered important from a socio-economic point of view and / or for public health and international trade in animals and animal products are: goat and ovine brucellosis (excluding *Brucella ovis*), contagious agalaxia of sheep and goats, contagious pleuropneumonia of goats and sheep, enzootic abortion of sheep (ovine chlamydiosis), Maedi-visna, Nairobi's disease, infectious orchiepididymitis of rams (*Brucella ovis*), ad ovine, salmonellosis (*Salmonella abortusovis*), scrapie (OIE).

Of particular economic importance, by affecting adults, but also the next generations of offspring, are the abotrive diseases such as: chlamydial abortion, brucellosis, salmonellosis (*S. abortusovis*), listeriosis, Q fever, ovine genital campylobacteriosis and toxoplasmosis (Menzies 2011, Holler 2012, Alemayehu et al., 2021). Bacteria belonging to the genus *Salmonella* are pathogens that can infect a wide

range of hosts, including humans (Besser, 2018; Popa and Papa, 2021). The growing number of *Salmonella* infections reported in recent decades reveals a problem worthy of consideration by the medical and veterinary services, with a considerable socio-economic impact (Kerr et al., 2022). *Salmonella* spp. infections are becoming more common in animals. In sheep, salmonella serotypes other than those specific to group B may be isolated, especially *S. typhimurium* and *S. dublin*, with a wide ubiquity, but the specific abortion-inducing agent is *S. abortusovis* (Amagliani et al., 2021).

Unlike other *Salmonella* species, *S. enterica* subsp. *enterica* serovar *abortussovis* is adapted to sheep and has host specificity (Jack 1971, Lamas et al., 2018). Considered as a zoonotic pathogen, its importance lies in the economic losses that occur in the production systems of sheep in regions that depend on grazing (Pardon et al., 1988; Sojka et al., 1983). Ovine salmonellosis has been most commonly associated with sheep herds in Europe and the Middle East, causing abortions, stillbirths, and diseases in infected lambs at birth (Alemayehu et al., 2021). These are mainly due to the epidemic nature of the disease, which is most recognized when the pathogen is newly introduced into a herd, as mass abortions occur. Endemic scenarios also cause abortions of up to 50% in sheep herds in newly introduced individuals, usually during the first gestation (Clune et al., 2021). *Salmonella abortusovis* is a pathogen belonging to the *Enterobacteriaceae* family genus *Salmonella*, species *Salmonella enterica* subspecies *enterica* serovar (serotype) *abortusovis*, commonly named *S. enterica* serovar *abortusovis* or *S. abortusovis*, is a Gram- negative bacterium (Jajere et al., 2019). *Salmonella abortusovis*, is not the only one associated with salmonella abortion in sheep. Abortions can also occur after infections with other serotypes (eg *Salmonella dublin*, *S. typhimurium, S. montevideo, S. brandenburg, S. indiana*) which can also cause reproductive losses in this species (Spickler, 2017).

Epidemiological data suggested that *S. abortusovis* serotype is one of the leading causes of sheep abortions in Europe and Western Asia, where it is a major pathological and economic problem in countries with a sheep-based economy (Valdezate et al., 2007). In this context, the purpose of this study was to investigate an outbreak of disease characterized by abortions and mortality in young sheep, which occurred in two different herds of non-professional farms belonging to households in northwestern Maramures, Romania. To achieve this goal, information was collected regarding the circumstances in which the outbreak apaired, followed by a description of the herds, the area of origin, the veterinary actions regularly applied to determine the occurrence of the infectious disease. The outbreak was characterized by performing analyzes to diagnose the incriminated agent/agents in the occurrence of infectious processes with the management and control of the disease.

MATERIALS AND METHODS

A. Study area and animals. For this study, two private households, located in the northwest of Transylvania, belonging to a commune within the Maramureș County, were considered. The households are free of infectious diseases and are not in a restricted zone due to a eradication program for a notifiable disease. The first household (A) has a total number of 359 animals (n = 359, 11 adult rams, 45 sheeps (aged beetwen 3-5 years) and 250 young animals). The ewes with lambs are housed in a 500 sq m shelter with paddock, without mechanization and without indoor installations. The rest of the sheep, the young animals and the rams are housed in an another 100 sq m shelter. The maintenance of sheep is done freely on permanent bedding, and the shelter is divided into rest and movement boxes. The evacuation of the bedding (garbage) is done after the sheep go out to the pasture, using a tractor equipped with a blade. Milking is done manually in a separate space. In the second household (B), the animals are housed in a shelter of about 250 square meters. The herd consists of 120 animals (n = 120, 102 ewes with lambs, 3 rams and 15 young sheep). During the indoor housing period, the basic food is fibrous, coarse (hay and corn), succulents and small amounts of concentrates (0.3-0.5 kg/day/female) during the period of late gestation and early lactation. The watering system have constant level and are connected to

the water pipe. During the summer, the sheep are kept in the pasture, the food being exclusively made of green mass, and the watering is done from the valleys around the pasture or from the well in the gutters. In herd A at the beginning of the indoor housing period, the pregnant sheep are sheltered in two herds of 150 ewes each. Immediately after lambing, the sheep were moved into common compartments with an average capacity of 50 sheep and lambs. One week after the start of lambing, a space is created that allows the formation of common compartments. Gradually, as the sheep lamb, the resized space allows the construction of other compartments, so that at the end of lambing in the stall there will be six common compartments with 50 sheep each.
After weaning the lambs, by removing the dividing walls between the individual lambing boxes, a common box is created for the weaned lambs. This model ensures good supervision of ewes and lambs and increased comfort by allowing access of ewes and lambs in the paddock during good weather.
In herd B the shelter is compartmentalized with dividers and is provided with paddock. Pregnant ewes and lambs have separate spaces made with dividers, while rams and lambs each have separate compartments.

B. Description of the outbreak and collection of samples. Abortion was preceded by some general symptoms that consisted of fever, restlessness, loss of appetite, vaginal discharge, and was followed by placental retention and metritis. Full-term lambs showed hyperthermia, loss of appetite, dyspnea, diarrhea and death within a few days. In order to perform the laboratory tests, two abortion samples were collected from the herd A and one sample from the herd B. After performing the necropsy the samples were collected from gastric contents and liver.

C. Sample processing. The samples collected were processed according to *ISO 6579: 2002/ Amd 1: 2007* method for the detection of *Salmonella*. The samples was preenriched with peptone water (Oxoid) (24 h/37°C), enriched in Rappaport Vassiliadis *Salmonella* broth (Oxoid), and later incubated in xylose lysine desoxicholate agar (XLD, Oxoid) for 48 hr. The RapidOne system (ThermoFischer Scientific, Remel) was used to identify and taxonomically classify isolated strains. *Salmonella* spp. strains were further confirmed serologically by a slide agglutination test at IDSA-LNR.

D. Antimicrobial sensitivity evaluation. The antimicrobial sensitivity patterns of the isolated strains were evaluated using the standard Kirby-Bauer disk diffusion method according to the CLSI guidelines. The strains were tested towards 11 antimicrobials: amoxicillin (10 µg) (Oxoid), cephazolin (30 µg) (Oxoid), doxycycline (30 µg) (Oxoid), enrofloxacin (ENR, 5 µg) (KRKA), gentamicin (10 µg) (Oxoid), oxytetracycline (OT, 30 µg) (Oxoid), ciprofloxacin (30 µg) (Oxoid), polimixin B (30 µg), (Oxoid), sulfatrimethoprim (1.25 µg) (Oxoid), cloramfenicol (10 µg), (Oxoid), erytromicin (10 µg), (Oxoid).
Based on the growth inhibition zone diameters (mm), the bacterial strains were recorded as resistant (R), intermediate (I) and susceptible (S). For further analysis, intermediate and resistant pattern isolates were grouped as resistant. The multiple antibiotic resistance index was recorded according to the procedure described by Krumperman (Krumperman 1983), so for the calculation of the MAR index the total number of antibiotics to which the isolate was resistant / the total number of antibiotics tested was taken into account. According to Kruperman, values lower than 0.2 are considered low risk, while values higher than 0.2 indicate a high risk (Krumperman 1983).

RESULTS AND DISCUSSIONS

Two flocks of sheep were examined due to frequent abortion reports. The abortion rate varied between 13.92% and 16.66%, mainly affecting the young ewes. Abortions have occurred in the last weeks of pregnancy, full-term lambs have been unviable and have died within the first few days of lambing.
Following the evolution of the epidemiological indicators in the two investigated herds, it was observed that, although there were differences in the size of the herds (animals: 359 - A

compared to 120 - B), the evolution of the indicators was not proportional to the total number of animals. Thus, the number of diseases in herd A was higher than in herd B, the differences between herds leading to lower morbidity rates in farm A than in B. The evolution of mortality was similar, the percentage being double in B, while the percentage of lethality in the case of herd B represents 138.9% compared to A (Figure 1).

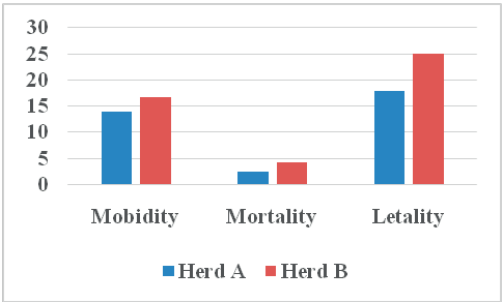

Figure 1. Epidemiological indicators calculated for the abortion episode diagnosed in herd A and B

The morbidity and mortality rates assesed during the evolution of the investigated episode suggest the absence of antibodies, motivated by the previous absence of infection in the two outbreaks as well as the absence of endemic disease. The owners did not implement the vaccination as a control measure until after the installation of the losses from the investigated episode. The increased lethality of 25% in the case of herd B suggests either the lack of adequate intervention or late intervention for the treatment of sick animals, or the possibility, especially in outbreak B, of the presence of predisposing factors that could have harmed the immune status of the animals. Bacteriological examination of the samples revealed the presence of *Salmonella* spp. The bacterial strains were subsequently characterized serologically. TSI agar culture was used for serotyping of somatic antigen O and semi-solid agar culture for flagellar antigen serotyping H. Belonging to somatic group B (somatic antigens 4,5) and possession of flagellar antigens and 1,6 were demonstrated by serotyping with the respective antisera, which confirmed the *Salmonella abortusovis* serotype. After the first abortions, the pregnant ewes from flock A, characterized by the lowest abortion rate (13.92%), were treated with enrofloxacin for 5 days s.c., after which no other cases were registered.

In an attempt to mitigate the harmful effects of *S. abortusovis* by antimicrobial therapy, the antimicrobial susceptibility of isolates was assessed by the diffusimetric method. The results showed a favorable antimicrobial sensitivity of *S. abortusovis* strain in herd A, where the multiple antibiotic resistance (MAR) index was lower (Table 1).

Table 1. The antimicrobial sensitivity patterns of the isolated strains

Antibicrobials	Herd A	Herd B
Cephazolin	S	S
Enrofloxacin	S	S
Oxytetracyclin	S	I
Amoxacillin	S	S
Cloramfenicol	S	I
Ciprofoxacin	I	R
Gentamicin	I	R
Polimixin B	I	R
Doxycyclin	R	R
Sulfatrimethoprim	R	R
Erytromicin	I	R
MAR	0.54	0.72

However, diffusion method showed that the isolate is resistant to sulphatrimetroprim and doxycycline and moderately sensitive to ciprofloxacin, gentamicin, polymyxin B and erytromicin. In contrast, the isolate from herd B has a low sensitivity, being resistant to ciprofloxacin, doxycycline, erythromycin, sulfatrimethoprim, polimixin B and moderately sensitive to chloramphenicol and o oxytetracyclin with a MAR index which is worrying - multiple antibiotic resistance may be the result of frequent use of antibiotics. A higher sensitivity in herd A compared to herd B was obtained by the less frequent use of antimicrobial agents in the studied flocks.

Salmonella infections in animals is for most countries with intensive animal husbandry, one of the most important veterinary health problems due to economic losses and their implications for human health by triggering food poisoning following the consumption of contaminated products (Heredia et al., 2018). Among the species involved, in the first place, in terms of significant losses, are the birds, followed by pigs, cattle and sheep. Bacterio-logical tests performed in this study confirmed

the presence of *Salmonella* serovar *abortusovis*, based on biochemical characteristics. Serological confirmation includes isolated agents in the BO group, typical of abortive salmonella in sheep. However, for the effectiveness of eradication measures, regular serological controls should be required, after identifying the etiological agent and at the same time its potential for antibiotic resistance by PCR and multiplex PCR (Geresu et al., 2021). This serotype maintains its virulence for fetuses and newborn lambs, but has reduced virulence in adult sheep, the pathogen spreading in the environment during abortion, infecting new hosts, which could quickly lead to abortions. Factors that could lead to the spread and increase of the prevalence of the infection can be directly correlated with unbalanced rations, disturbances of the intestinal flora, intestinal and hepatic parasites and individual factors associated with some breeds and genetic susceptibility. Other factors are related to the seasonal migration of animals, the nomadic lifestyle of sheep herds, insufficient management in the application of sanitation measures.

CONCLUSIONS

The presence of *Salmonella* serotypes that induce abortion and the death of newborn lambs in flocks is a major economic problem in geographical areas with a sheep-based economy and raises a number of questions regarding the implementation of preventive measures. Higher antimicrobial resistance in herd B compared to herd A, can be explained by the abusive use of antimicrobials in the prophylactic treatment of various pathologies (metritis, mastitis, diarrhea, pneumonia, necrobacillosis or coccidiosis).

In order to avoid economic loss in regions known to have enzootic potential, immunoprophylaxis in ewes is recommended.

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CLINICAL-DIAGNOSTIC COORDINATES IN PROSTATIC AND PARAPROSTATIC CYSTS IN DOGS

Valerica PREDA (CONSTANTINESCU), Maria ROȘCA
Iuliana CODREANU, Alexandra Mihaela CRISTIAN, Mario CODREANU

University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Mărăşti Blvd, District 1, 011464, Bucharest, Romania, Phone: +4021.318.25.64, Fax: + 4021.318.25.67, Email: alexandrapopa613@gmail.com

Corresponding author email: alexandrapopa613@gmail.com

Abstract

Prostatic cysts are fluid-filled structures located adjacent to the prostate gland. Clinical expression is often asymptomatic, rarely tenesmus, lethargy, anorexia, and hematuria occur.Paraprostatic cysts are more common in uncastrated dogs over 8 years of age, without a predisposition to breed. These fluid-filled cysts are often localized or extend from the outer edges of the prostate.The research took place between 2017-2020 on 23 dogs of different breed and age, within the Clinic of the Faculty of Veterinary Medicine Bucharest and within the private veterinary practices.
The diagnosis of paraprostatic cysts was established in 7 dogs age 1-5 years (n = 1), 5-10 years (n = 2), 10-15 years (n = 4), of different breeds.The reason for the presentation at the clinic was the observation by the owners of the tendency to constipation (n = 5), dysuria (n = 2) and a urination with blood (n = 3). Ultrasound identification of cystic formations with paraprostatic localization highlighted the existence of cystic formations with dimensions of 20 mm (n = 4), 70 mm (n = 2) and 3.8 cm (n = 1). In the mass of the prostate glandular parenchyma, cystic dilatations were identified, with an anechoic content with rare corpuscular elements in suspension (n = 3) and cellularity (n = 4), accompanied by ultrasound specific artifact distal enhancenment. Intraprostatic cysts were found in 16 dogs age 1-5 years (n = 3), 5-10 years (n = 5), 11-15 (n = 5) and 16-20 years (n = 3), common breed (n = 6), German Shepherd (n = 3), pointer (n = 1), English Bulldog (n = 1) Dachshund (n = 1), Afghan Greyhound (n = 1) and West Highland White Terrier (n = 3).
The reason for presenting to the doctor was dysuria (n = 5) and hematuria (n = 7), or routine ultrasound examination. Ultrasonography detected single, multiple or scattered cystic formations of round / ovoid type with a fine echogenic wall clearly delimited by the rest of the parenchyma of infracentimetric dimensions (n = 12) centimeters in 4 dogs, with clearly homogeneous and anechoic content, accompanied by the distal enhancement.

Key words: *prostatic cysts, dogs, paraprostatic cysts.*

INTRODUCTION

Prostatic cysts are fluid-filled structures located adjacent to the prostate gland. (Codreanu and Nae, 2016).
Prostate cysts are described as an increase in prostate secretions that exert greater pressure on the excretory ducts (Khadidja and Adel, 2017). Consequently, they are retention cysts secondary to excretory canal obstruction due to prostatic squamous metaplasia (Johnston et al., 2000).
Clinical expression is often asymptomatic, unless the size of the cyst significantly increases prostate volume, when tenesmus, lethargy, anorexia, and hematuria occur (Krawiec and Heflin, 1999).
Paraprostatic cysts are more common in uncastrated dogs over 8 years of age, without a predisposition to breed. These fluid-filled cysts are often localized or extend from the outer edges of the prostate, but their exact origin is uncertain (Rajan, 2014).
Research has suggested that their origin is secondary to ductal occlusion of the prostate following squamous metaplasia or a type of prostate hematoma in the final stage or secondary to the remains of the uterus masculinus or the Mullerian duct. They can increase to significant size causing tenesmus and dysuria with hematuria following direct compression of the colon and bladder (Vititoe, 2017).

MATERIALS AND METHODS

The research took place between 2017-2020 on 23 dogs of different breed and age, within the Clinic of the Faculty of Veterinary Medicine

Bucharest and within the private veterinary practices.
The studies and investigations were carried based upon the suspicion and confirmation of the diagnosis of prostatic cysts or paraprostatic cysts which involved performing the clinical examination and paraclinical analysis consisting in urine analysis and imaging evaluations.
Preliminary urinary biochemical examination was performed using Urispec Plus urine strips or diagnostic strips or interpreted using a respective automated analyzer, IDEXX VetLab UA Analyzer, and urinary density was determined using a refractometer. REC-300ATC. The assessment of the urinary sediment was performed with the Optika microscope.
Imaging was performed using the ESAOTE Veterinary MyLab 60, Sonoscape DW-F5 with microconvex probe.

RESULTS AND DISCUSSIONS

The diagnosis of paraprostatic cysts was established in 7 dogs age between 5 years (n = 1), 5-10 years (n = 2), 10-15 years (n = 4), of different breeds.
The reason for the presentation at the clinic was the observation by the owners of the tendency to constipation (n = 5), dysuria (n = 2) and a urination with blood (n = 3) (Table 1).

Table 1.Total patients with paraprostatic cyts

TOTAL PATIENTS WITH PARAPROSTATIC CYSTS *(n = 7)*				
BREED		AGE		
		1-5 years	6-10 years	11-15 years
German Shepherd	*(n=3)*	*(n=3)*	*(n=2)*	*(n=2)*
West Highland White Terrier	*(n=3)*			
Bichon Maltese	*(n=1)*			

The animals were examined clinically and the data obtained were recorded in the clinical observation sheet, recording a body temperature with variations in normal parameters, a good general condition with water and food appetite present, and heart and respiratory rate within physiological limits.
The data collected from the clinical examination on the frequency of urination revealed a normal frequency and quantity, with the observation of a yellow color in 4 patients and red in 3 individuals being interpreted as a suspicion of initial hematuria.
Palpation of the urinary and genital tract indicated a lack of obvious physical changes in the kidneys or bladder, but with the detection of a soft consistency in the genital area (n = 2).
Urine samples collected by the owners were subjected to the biochemical analysis examination where the presence of leukocytes ++ (n = 4), urobilinogen + (n = 3), proteins + (n = 3), +++ (n = 1), blood + (n = 4), +++ (n = 3), with a urinary pH of 5.5, 6.5 (n = 4), 7, 7.5, urinary density of 1.030 (n = 5), 1.020, 1.040, were identified, and on microscopic examination the presence of erythrocytes and flat epithelial cells. (n = 7).
Ultrasound identification of cystic formations with paraprostatic localization highlighted the existence of cystic formations with dimensions of 20 mm (n = 4), 70 mm (n = 2) and 3.8 cm (n = 1) contributing vital to establishing the diagnosis of certainty. In the mass of the prostate glandular parenchyma, cystic dilatations were identified with a irregular wall (n = 3) and distinct (n = 4) with an anechoic content with rare corpuscular elements in suspension (n = 3) and cellularity (n = 4), accompanied by ultrasound specific artifact represented by the phenomenon of posterior enhancenment (Figures 1, 2).

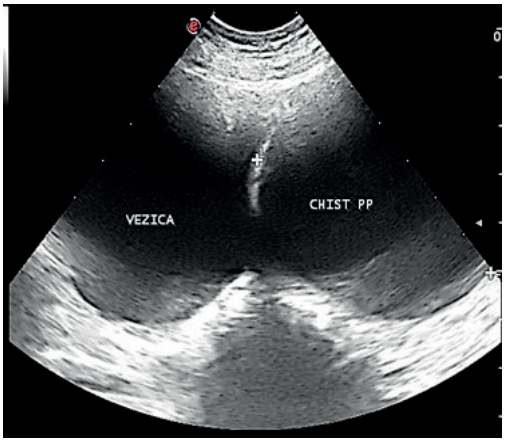

Figure 1. Paraprostatic cyst

Paraprostatic cysts located deep in the bladder neck were also accompanied by obvious dysuric phenomena (n = 2) with a tendency to mechanical urinary retention.

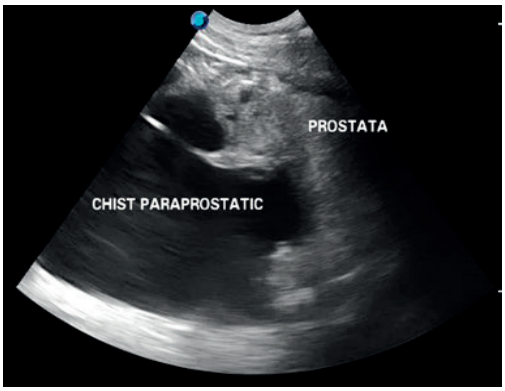

Figure 2. Paraprostatic cyst

Intraprostatic cysts (Figures 4-7) were found in 16 dogs with age between 1-5 years (n = 3), 5-10 years (n = 5), 11-15 years (n = 5) and 16-20 years (n = 3), breed - common breed (n = 6), German Shepherd (n = 3), Pointer (n = 1), English Bulldog (n = 1), Dachshund (n = 1), Afghan Greyhound (n = 1) and West Highland White Terrier (n = 3) (Figure 3).

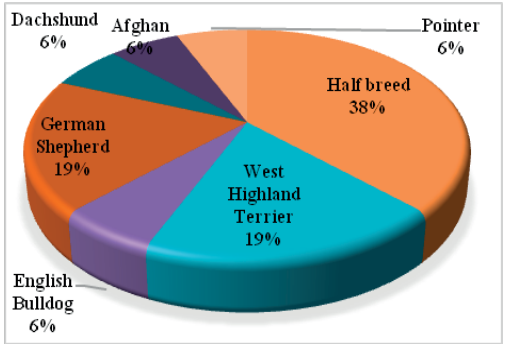

Figure 3. Distribution by breeds in dogs with intraprostatic cysts

The reason for presenting to the doctor was dysuria (n = 5) and hematuria (n = 7), in the rest of the patients their identification at the routine ultrasound examination was a diagnostic surprise. Body temperature was generally within the range of range of the species (n = 14). The frequency of urination was generally normal (n = 12) and pollakisuric in 4 individuals, with a yellow (n = 11) and red (n = 7) color being observed.

Physical examination of the genitourinary system did not reveal any obvious physical changes, and digital rectal examination revealed a prostatomegaly (n = 12) with painful sensitivity present in one patients.

The urine samples collected were chemically analyzed:- leukocytes ++ (n = 4), protein + (n = 2), ++ (n = 2), blood + (n = 3), ++ (n = 2), +++ (n = 4) , ++++ (n = 7), pH of 5.6 (n = 4), 6.5 (n = 8), 7 (n = 3) and urinary density of 1.030 (n = 10), 1025, (n = 6), and when evaluating the urinary sediment, the presence of red blood cells and ammonia-magnesium phosphate crystals was observed (n = 1).

The ultrasound imaging detected single, multiple or scattered cystic formations of round/ovoid type with a fine echogenic wall, clearly delimited by the rest of the parenchyma, with infracentimetric dimensions (n = 12) and centimeters size in 4 dogs, with clearly homogeneous content, anechogen and accompanied by the phenomenon of distal enhancement.

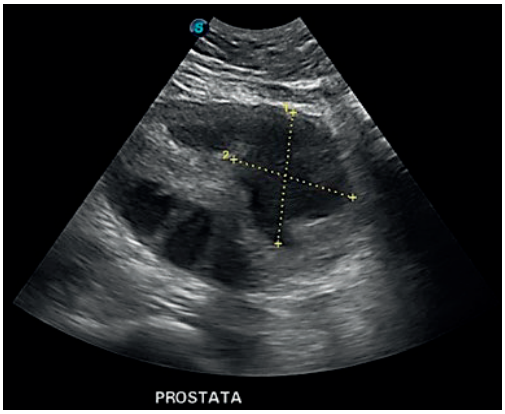

Figure 4. Intraprostatic cyst

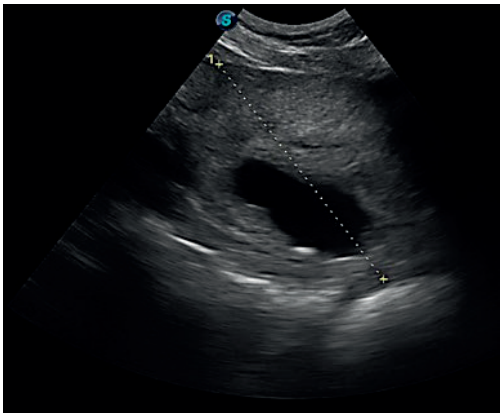

Figure 5. Intraprostatic cyst

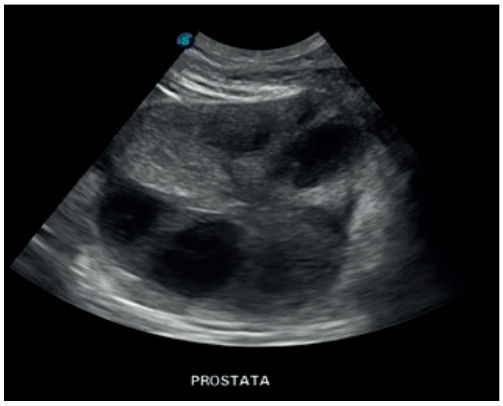

Figure 6. Intraprostatic cyst

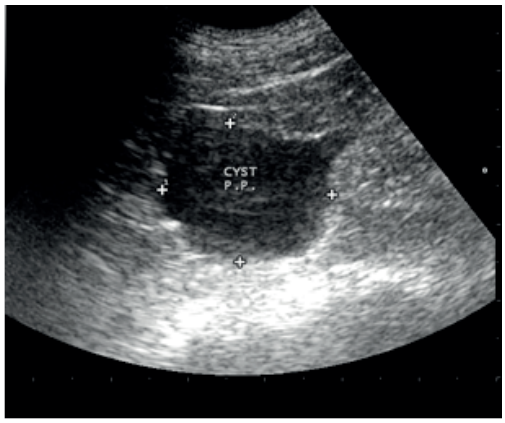

Figure 7. Intraprostatic cyst

CONCLUSIONS

Intraprostatic and paraprostatic cysts can vary in severity, with clinical signs that are often non-specific leading to common diagnoses of prostatic syndrome.

The present study indicates that a full diagnosis will be established after an complete physical examination with transrectal digital palpation and with ultrasound exam which has a great value for diagnosis.

Prostatic ultrasound includes visualization of the prostatic gland, its urethra, the bladder and the locoregional lymph nodes.

The research included in the study offers clinical and diagnosis coordinates which, altogether, and in a correlative way, form a complete diagnosis, suggesting a prognosis and most important the orientation of the therapeutic intervention.

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CASE STUDIES REGARDING THE HEMATOLOGICAL PARAMETERS IN POLYCYSTIC KIDNEY DISEASE IN CATS

Maria ROȘCA, Alexandra Mihaela CRISTIAN, Liliana HUȘTEA (DECEI), Valerica PREDA (CONSTANTINESCU), Ramiro SIMION, Mario CODREANU

University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Mărăști Blvd, District 1, 011464, Bucharest, Romania, Phone: +4021.318.25.64, Fax: + 4021.318.25.67, Email: alexandrapopa613@gmail.com

Corresponding author email: alexandrapopa613@gmail.com

Abstract

Anemia is a common and potentially fatal complication of chronic kidney failure caused by polycystic kidney disease, leading to faster disease progression and increased mortality. Anemia is due to the loss of erythropoietin-producing cells in the kidneys or due to an inflammatory condition resulting in iron sequestration, bleeding from the gastrointestinal mucosa, reduced red blood cell survival due to uremia or consequent adverse drug effects and poor nutritional status.

The present investigative study was conducted on a number of 7 cats, of Persian, British Shorthair, and European breeds, with different sexes and ages.

In this study, hematological paraclinical investigations were performed in 7 cats, in order to detect a normochromic anemia, the resulting data being grouped in normal results in parameters in one patient, results with minor deficits in 4 cats and severe results in 2 individuals.

Key words: *anemia, PKD, cats.*

INTRODUCTION

Anemia is a common and potentially fatal complication of chronic kidney failure caused by polycystic kidney disease, leading to a faster progression of the disease and increased mortality (Codreanu, 2020).

Anemia is due to the loss of erythropoietin-producing cells in the kidneys or due to an inflammatory condition resulting in iron sequestration, bleeding from the gastrointestinal mucosa, reduced red blood cell survival due to uremia or due to adverse drug effects and nutritional status 2016.

Importantly, symptoms related to anemia, including reduced physical functioning and fatigue, have been identified as high priorities by patients with CKD (Urquhart-Secord, 2016).

Polycystic Kidney Disease (PKD) is a genetic kidney disease that has been found in Persian cats, affecting 37-49% (Lee et al., 2010), being one of the leading causes of insufficiency. renal impairment in cats and the most common feline genetic disease. (Lyon et al., 2004), characterized by the appearance of cysts smaller than 1 mm to more than 1 cm in the renal parenchyma and occasionally in the liver (Bosje et al., 1998), pancreas and spleen (Scalon et al., 2014), sporadically recorded in the literature since 1967 (Guerra et al., 2020).

Hematologic consequences of PKD-induced uremia are dominated by normochromic normocytic anemia following erythropoietin deficiency, which results in reduced erythrocyte lifespan. Anemia also contributes to the exacerbation of lethargy and loss of appetite in the patient (Codreanu, 2020).

MATERIALS AND METHODS

The present investigative study was conducted on a number of 7 cats belonging to the Persian, British Shorthair and European breeds, of different sexes and ages, for the period 2018-2022, in the Clinic of the Faculty of Veterinary Medicine and in the veterinary units Canivet and VetMedical Consulting SRL.

The hematological paraclinical investigations were performed in 6 cats, in order to detect a normochromic anemia, the resulting data being grouped with minor deficits in 4 cats and severe results in 2 individuals.

Table 1. Total number of patients included in the study

TOTAL PATIENTS OF THE STUDY (n=6)					
BREED	AGE			SEX	
Persian *(n=4)*	1-5 years	6-10 years	11-17 years	M	F
European *(n=1)*	*(n=1)*	*(n=5)*	*(n=1)*	2	5
British Shorthair *(n=2)*					

Determination of hematological parameters was performed with IDEXX VetAutoread Hematology Analyzer (Figure 1) and Scil Vet Abc Plus and Genrui - 5-Part Auto Hematology Analyzer KT-6610 (Figure 2).

Figure 1. IDEXX VetAutoread Hematology Analyzer

Figure 2. Hematology - Scil Vet Abc Plus

Imaging for the diagnosis of Polycystic kidney disease was established by using the Esaote Veterinary MyLab 60 ultrasonography (Figure 3).

Figure 3. Esaote Veterinary MyLab 60

RESULTS AND DISCUSSIONS

In this study group were included felines in which renal cysts suggestive of polycystic kidney disease were detected in terms of correlation with hematological investigations.

Case 1 - Persian, 11 years, Male

The anamnesis taken from the owner indicates a state of apathy, fatigue, refuses food, has lost significant body weight in the last 2 months before the consultation, occasional vomiting and prefers places withdrawn for about a week.
The detailed clinical examination is detailed and presented below, additionally being discovered an increase in the volume of the kidneys and with an irregular shape noticed at the physical examination of the urinary tract, pallor of the mucous membranes present.

Table 2. Results of the haematological exam case 1

Persian, *11 years, Male*	**Hematological values**	**References values**
RBC *(M/µL)*	**3.59**	**5.00-10.00**
Hemoglobin *(g/dL)*	**7.0**	**9.00-15.1**
Hematocrit *(%)*	**25.1**	**30.0-45.0**
WBC *(K/µL)*	**5.3**	**5.5-19.5**
MCV *(fL)*	**44.9**	41.0-58.0
MCH *(Pg)*	**16.8**	12.0-20.0
MCHC *(g/dL)*	**37.4**	29.0-37.5
PLT *(10^9/L)*	**254**	100-514

Case 2 - Persian, 6 years, Female

During the clinical interrogation, the owner complained of severe changes in the general condition of the animal, noting a considerable decrease in body weight and a deteriorating general condition.
The clinical examination revealed a symptomatology suggestive for the clinical diagnosis of chronic renal failure, with oral ulceration accompanied by halitosis, mucosal pallor and a blood pressure of 151/72 mmHg.

Table 3. Results of the haematological exam in case 2

Persian, *6 years, Female*	**Hematological values**	**References values**
RBC *(M/µL)*	**4.22**	**5.00-10.00**
Hemoglobin *(g/dL)*	**5.9**	**9.00-15.1**
Hematocrit *(%)*	**19**	**30.0-45.0**
WBC *(K/µL)*	**12.4**	5.5-19.5
MCV *(fL)*	**45**	41.0-58.0
MCH *(Pg)*	**14**	12.0-20.0
MCHC *(g/dL)*	**32**	29.0-37.5
PLT *(10^9/L)*	**178**	100-514

Case 3 - **Persian, 6 years, Female**

The owner came to the clinic with a 6-year-old Persian cat of a Persian state, where he observed a state of drowsiness manifested for several days, along with apathy, a reduced and selective appetite, the presence of a characteristic polyuria syndrome. polydispyness, rapid breathing and lethargy.

Table 4. Results of the haematological exam in case 3

Persian, *6 years, Female*	**Hematological values**	**References values**
RBC *(M/µL)*	**2.82**	5.00-10.00
Hemoglobin *(g/dl)*	**8.4**	**9.00-15.1**
Hematocrit *(%)*	**18**	**30.0-45.0**
WBC *(K/µL)*	**6.1**	5.5-19.5
MCV *(fL)*	**48.2**	41.0-58.0
MCH *(Pg)*	**17.6**	12.0-20.0
MCHC *(g/dL)*	**36.7**	29.0-37.5
PLT *(10^9/L)*	**321**	100-514

Case 4 - British Shorthair, 10 years, Female

The owner of the 10-year-old British Shorthair feline cat noticed a state of apathy lately, the constant presence of gastrointestinal disorders translated by occasional vomiting, accompanied by a selective appetite and nausea, syndrome diarrhea with the expression of yellow diarrhea stools with increased frequency and a weakening of the animal by highlighting the chest and urinary disorders externalized by urinating in impermissible places and with an appreciable amount and excessive thirst.

Table 5. Results of the haematological exam in case 4

British Shorthair, *10 years, Female*	**Hematological values**	**References values**
RBC *(M/µL)*	**3.9**	**5.00-10.00**
Hemoglobin *(g/dL)*	**10.3**	**9.00-15.1**
Hematocrit *(%)*	**32**	30.0-45.0
WBC *(K/µL)*	**7.2**	5.5-19.5
MCV *(fL)*	**45**	41.0-58.0
MCH *(Pg)*	**17**	12.0-20.0
MCHC *(g/dL)*	**32**	29.0-37.5
PLT *(10^9/L)*	**368**	100-514

Case 5 - Persană, 4 years, Female

The clinical observation sheet summed up anamnestic data revealing a patient with a reduced appetite, with a state of accentuated drowsiness, along with a progressive weakening observed by the owner recently.

The non-specific evaluated symptoms are presented in detail below, and paraclinical investigations are imperative in order to establish a diagnosis.

Table 6 . Results of the hrematological exam in case 5

Persian, *4 years, Female*	**Hematological values**	**References values**
RBC *(M/µL)*	**4.0**	**5.00-10.00**
Hemoglobin *(g/dL)*	**8.9**	**9.00-15.1**
Hematocrit *(%)*	**25**	**30.0-45.0**
WBC *(K/µL)*	**17.2**	5.5-19.5
MCV *(fL)*	**45**	41.0-58.0
MCH *(Pg)*	**11**	**12.0-20.0**
MCHC *(g/dL)*	**35**	29.0-37.5
PLT *(10^9/L)*	**450**	100-514

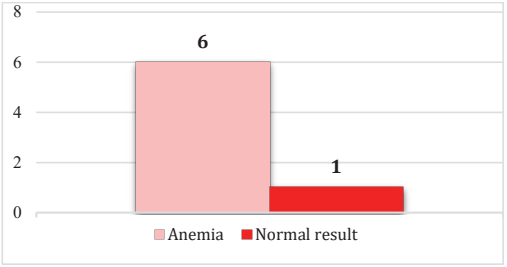
Case 6 - European, 7 years, Male

The owner came to the clinic with a 7-year-old European male cat and a male, following the finding of an altered general condition expressed by depression, anorexia and polyuria-polydipsia syndrome.

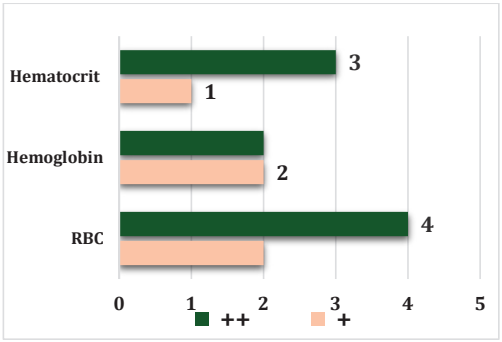
Table 7. Results of the haematological exam case 6

European, *7 years, Male*	**Hematological values**	**References values**
RBC *(M/µL)*	**4.6**	**5.00-10.00**
Hemoglobin *(g/dL)*	**9.9**	9.00-15.1
Hematocrit *(%)*	**39**	30.0-45.0
WBC *(K/µL)*	**11.2**	5.5-19.5
MCV *(fL)*	**50**	41.0-58.0
MCH *(Pg)*	**19**	12.0-20.0
MCHC *(g/dL)*	**35**	29.0-37.5
PLT *(10^9/L)*	**250**	100-514

The hematological paraclinical diagnosis of anemia was established in 6 cats based on the RBC *(M/µL)* value, which was below the normal range (5.00-10.00), accompanied by additional data provided by the parameters of Hemoglobin (g/dl) that was below the limit of 9.00-15.1 in 4 cats, and on the Hematocrit (%) percentage registered below the lower limit in 4 cats.



Graphic 1. Frequency of diagnosis of anemia in patients with PKD



Graphic 2. Classification of the intensity with which the investigated parameters were affected in patients with PKD

CONCLUSIONS

Anemia, diagnosed in 6 cats, with polycystic kidney disease is an indicator and prognostic factor in relation to the degree of kidney damage. Depending on the degree of substitution and compression of the parenchymal index of cystic lesions, positive correlations can be made between the severity of the medium-long term process in terms of erythropoietin deficiency, the size of the number and the progression of cystic lesions, relation to the correlation between hematinic medication (plastic and catalytic) and hormone support medication (erythropoiesis).

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HISTOPATHOLOGICAL ASPECTS OF AGONAL THROMBUS AND ITS ROLE IN AGONAL DEATH DIAGNOSIS - PRELIMINARY STUDY

Raluca-Elena TIU, Raluca Ioana RIZAC, Raluca Mihaela TURBATU, Adina Mihaela PÎRVU, Manuella MILITARU, Emilia CIOBOTARU-PÎRVU

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

Corresponding author email: ralucatiu@yahoo.ro

Abstract

The mechanisms of blood coagulation in cases of agonal death are rarely studied in veterinary and human medicine. Agonal thrombi are considered as being formed antemortem. Nevertheless, those are misdiagnosed as cruors because of some gross common features. This preliminary study aims to highlight histopathological differences between agonal thrombi and post-mortem clots. Thirteen cases were included: ten domestic carnivores and three chickens. Agonal death diagnosis was established based on the clinical history and followed by standard necropsy and histopathological special staining techniques (Mallory and Masson Trichrome Stain) to highlight the fibrin deposition and pattern. Histological findings of intraventricular and intra-atrial agonal thrombi were similar in all cases and consist of: presence of Zahn's lines, layered display of fibrin deposition, intact or altered erythrocytes and mononuclear infiltration. One case displayed similar features in subepicardial veins. Histopathological examination of clots did not reveal the presence of fibrin deposition, nor Zahn's lines. Considering that in all cases of agonal thrombi the histopathological findings were different from the clots, the agonal thrombus can be associated with agonal death, ruling out the sudden death.

Key words*: agonal thrombus, agonal death, sudden death, cruors.*

INTRODUCTION

The mechanisms of blood coagulation are well studied nowadays, but the lack of research regarding agonal coagulation keeps an open door to this topic. One of the most encountered types of death is the agonal one. Most of the researchers discuss about thrombi (which are formed intra-vitae) or cruors (post-mortem coagulation), but few references about a third category: agonal thrombus, with no true separation between them (Hansma et al., 2015; Kondou et al., 2020; Malone et al., 2008).

It is well known that thrombosis is a pathology that takes place during the animal lifetime, usually associated with impaired mechanisms of coagulation, while cruors are referring to post-mortem clots (Di Fazio et al., 2021; Jackowski et al., 2006; Jackowski et al., 2011). Their gross examination has important differences: thrombi are pale-coloured (because of their high content of fibrin) usually found within the blood vessels with strong adherence to the vascular wall, while post-mortem clots are red-dark coloured, with soft consistency and non-adherent qualities (Kalubert et al., 1988; Kappler et al., 2017; Van Winkle & Bruce, 1993).

The gross examination of agonal thrombi reveals a yellow to red colour, soft to mild elasticity and can be found in the blood vessels, but mainly in the heart chambers with a mild adhesion to the endothelium (Ciobotaru, 2013; Hansma et al,. 2015; Michaud et al., 2013; Roberts et al., 2012).

Considering the previously published studies, there are no references regarding agonal thrombosis in animals. Consequently, the present study aims to establish the histopathological differences between agonal thrombi and cruors in animals and also to have the diagnostic standard in order to rule out sudden death.

MATERIALS AND METHODS

In this preliminary study were included thirteen cases: ten domestic carnivores and three

chickens who all suffered of agonal death. All the cases that took part of this study were animals which was well known that died with a period of agonal suffering. In order to take samples of the agonal thrombus, standard necropsy was performed in a period between one to twelve hours post-mortem. The gross examination of the agonal thrombus was followed by samplings for histopathological investigation, 10% formaldehyde fixation for 24 hours, routine triming, routine hematoxilin-eosine staining and special staining techniques: Mallory and Masson trichrome (Mansueto et al., 2019; Nagasaky et al., 2008; Van Winkle & Bruce, 1993).

Mallory and Masson trichrome are special staining techniques used for the visualization of fibrin (red stained). The aforementioned special stainings were used in order to confirm the presence of fibrin deposition in the agonal thrombus.

RESULTS AND DISCUSSIONS

The recent publications on agonal thrombus tend to describe separately this category with the main objective to associate it with the cases that had an extended agonal period. Usually, the agonal thrombus in humans is found in the right chambers of the heart, but some studies also involve the pulmonary vessels (Hansma et al., 2015).

The necropsy findings in domestic carnivores and chickens were similar to previously published studies in humans: all the agonal thrombus was found in the chambers of the heart, pale-reddish in colour with e soft-mild consistency and fine adherences to the endothelium (Figure 1). Another characteristic found in all cases is the intertwining of the agonal mass to the pectinate muscles and trabecula of the heart, proving that agonal thrombi are formed during the lifetime of the animal when the contractility of the heart is still present.

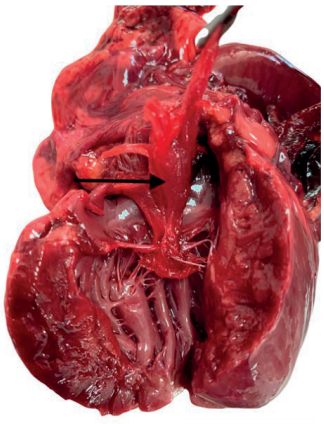

Figure 1. Agonal thrombus in the left heart chambers (dog)

One of the most important element of the intra-vitae thrombus is the presence of fibrin deposition arranged in a layered pattern (Zahn's lines) which are not found in the post-mortem clots (Mansueto et al., 2019). Also, the intra-vitae thrombus formation is based on the Virchow's Triad: impaired blood flow (turbulence or stasis), hypercoagulability and endothelial injury (Mansueto et al., 2019; Zachary, 2017,). These are the two main characteristics of a thrombus that are not associated with cruors. Cruors have no adherences to the endothelium, do not content fibrin depositions, being mainly formed out of erythrocytes and mononuclear infiltration (Fineschi et al., 2008).

Microscopical examination of agonal thrombi revealed the same elements for all cases, the most important being the presence of fibrin depositions, identified especially with Masson Trichrome staining. Alongside fibrin identification, a specific layering of it starts to form the pattern of Zahn's line (Figure 2 A, B)

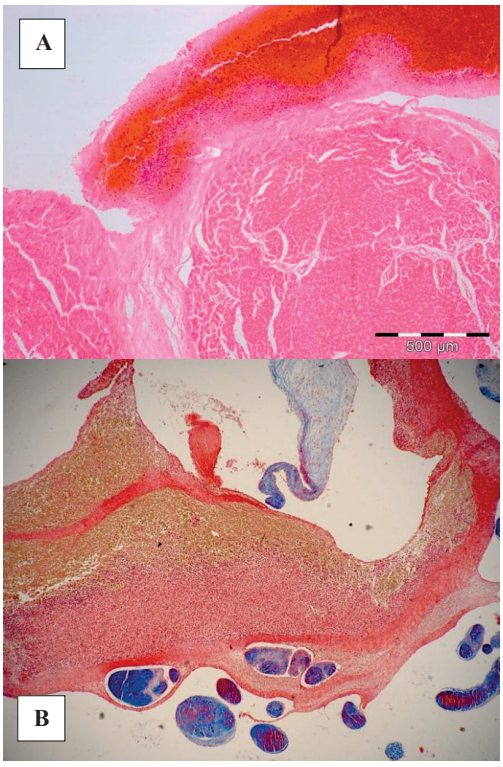

Figure 2**.** **A**-Agonal thrombus (dog), adhesion to the endothelium, HE stain, x50; **B**- Lines of Zahn (arrow) (dog), Masson Trichrome stain, x50

Fine, interrupted adherence between oxyphil fibrin network of agonal thrombus and endothelial cells were identified in histopathological investigation, as well. The cellular component of the agonal thrombus was represented by erythrocytes and clustered leukocytes. (Figure 3 A, B)

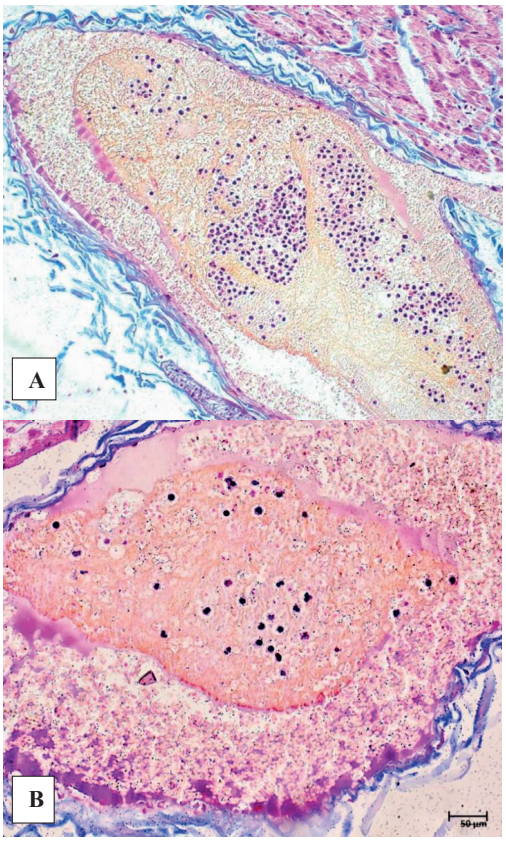

Figure 3. **A -** Agonal thrombus (dog), fibrin layering in the subepicardial veins, Mallory stain, x200; **B -** Clustered mononuclear infiltration in the agonal thrombus (dog), Mallory stain, x400

The microscopic view of wave-like fibrin layering was seen in the mass of the agonal thrombus in all cases presented, with random distribution (Figure 4).

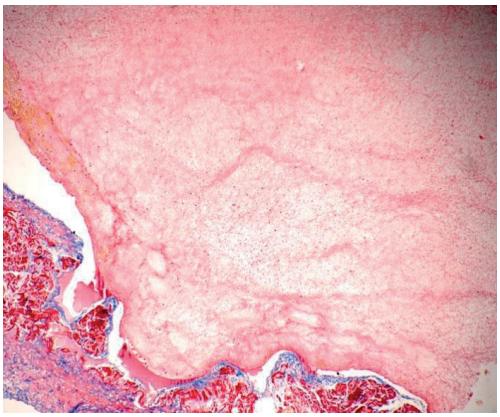

Figure 4. Agonal thrombus (dog), moderate fibrin deposition Masson Trichrome stain, x200

One particular aspect of agonal thrombosis was found in a carnivore where agonal thrombus was found also in the subepicardial veins. Agonal thrombus found in the small veins proves that agonal suffering probably alters the blood flow in all of the circulatory system (Figure 3A).

Even though the literature divides the coagulation of blood in ante-mortem or post-mortem, the agonal blood coagulation, that is to be associated with agonal death, needs to be studied for its importance to the forensic pathology field. All the gross and histopathologic differences of the agonal thrombi versus cruors tend to give us the explanation that this category exists and, even more, is the way to rule out sudden death scenarios in forensic pathology. Although they do not have a clinical significance, because of forming in agonal death (meaning the animal already suffered from other pathology that determine the agonal period), their importance to the pathology field is bigger. Knowing that agonal thrombi are structures that form in the *articulo-mortis* (the point of death), those are not to be misdiagnosed with thrombus vera nor with cruors as expression of the post-mortem coagulation of blood (Barkhausen et al., 2002; Hansma et al., 2015)

CONCLUSIONS

The differences between ante-mortem, post-mortem and agonal thrombi described previously lay the path for finding the ways to distinguish them at necropsy, those should be categorized differently giving the fact that do not form in the same way nor do they have the same characteristics. The agonal thrombus seems to have more common elements with the thrombus which is formed ante mortem then with the cruors, proving that they form strictly during the agonal period of death while the heart of the animal is still active.

ACKNOWLEDGEMENTS

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CLINICAL, NEUROLOGICAL AND IMAGING DIAGNOSIS ON CANINE AND FELINE INTRACRANIAL MASSES - A CASE SERIES REPORT

Raluca Mihaela TURBATU, Cristina FERNOAGĂ, Alexandru Gabriel NEAGU, Niculae TUDOR, Constantin VLĂGIOIU

University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, 105 Spl. Independentei, District 5, Bucharest, Romania

Corresponding author email: raluca.tbt@gmail.com

Abstract

Intracranial masses are a large group of malign and benign structures, that can affect any system of the body, including the nervous system. Since the incidence of this pathology is influenced by several factors, this paper aims to present the epidemiological data collected from 58 cases - 41 dogs (70.68%) and 17 cats (29.31%) diagnosed in the Clinic of the Faculty of Veterinary Medicine in Bucharest between 2018 and 2020. The reviewed factors included signalment data like species, breed, age, and sex. Also, for each case, clinical and neurological deficits were analysed and the results were used to localize the lesion within the nervous system and to establish the differential diagnoses according to "VITAMIND" acronym. The confirmation and the anatomical site of the mass were recorded based on the imagistic features obtained on MRI. This study is limited only to a provisional diagnosis, as the animals included in the database were not subjected to a post-mortem examination.

Key words: *neurological examination, brain mass, intracranial mass, nervous system MRI.*

INTRODUCTION

Intracranial masses are structures of a benign or malignant nature whose point of origin is represented by the anatomical structures of the nervous system. In veterinary medicine, they are an important cause of mortality, especially in geriatric dogs (Klopfleisch, 2016). Previous studies showed a predisposition of brachycephalic breeds to develop glial and pituitary tumors, while dolichocephalic breeds show a predisposition to meningioma. Top affected breeds include Boxer, Doberman, Golden Retriever, and Scottish Terrier (Kishimoto et al., 2020). Clinically, intracranial neoplasia will progress to chronic signs and slow progress. The most common manifestations in affected patients are seizures, changes in behaviour (walking in a circle, head pressing, disorientation), and discrete deficits in neurological examination that may express as a reduced response to menace or contralateral paresis (in advanced stages) (Schwartz et al., 2011). Although surgical techniques and antemortem biopsy are diagnostic procedures commonly used in developed countries, not all types of masses can be addressed in these ways, and high costs can be a limiting factor for pet owners (Dickinson, 2014). Therefore, advanced imaging techniques, and especially MRI, are a non-invasive alternative by which the clinician may suspect intracranial neoplasia and may choose between treatment or euthanasia, depending on the situation (Troxel et al., 2004; Bentley, 2015).

Starting from the previously discussed framework, this chapter aims to present the cardinal aspects of the clinical, neurological, and imaging data recorded in canine and feline patients diagnosed with cerebral masses within the clinic of the Faculty of Veterinary Medicine of Bucharest.

MATERIALS AND METHODS

The study was conducted between January 2018 and December 2020 and included 58 patients (41 dogs and 17 cats) diagnosed with intracerebral masses at the Clinic of the Faculty of veterinary medicine in Bucharest. For each case, the recording of the history and the performance of the clinical and neurological examination were mandatory steps, indispensable for establishing the neuroanatomical

diagnosis. Thus, depending on the specificity of the symptoms, the lesion was located in the forebrain, cerebellum, brainstem, or vestibular apparatus. Differential diagnosis and choice of paraclinical investigations were made based on the acronym "VITAMIND" (vascular/ inflammatory/trauma/anomaly/meta-bolic/ idiopathic/neoplastic/degenerative (Dewey & da Costa, 2016). The main inclusion criterion in this study was the confirmation of a mass within the brain by the MRI technique.

All data were manually collected from the Consultation Register and relevant information regarding signalment factors, symptomatology, and neurological deficits were analysed to establish their correlation with the intracranial mass development.

RESULTS AND DISCUSSIONS

Regarding the impact of species on the results obtained, the percentage of affected dogs (70.68%, n = 41) was higher compared to the number of affected patients within the feline population (29.31%, n = 17). As for the breed, 70.73% (n = 29) of the dogs were purebred and 29.26% (n = 12) were crossbreed. Purebreds included in the study belonged to: Maltese Frise (n = 8), French Bulldog (n = 3), Beagle (n = 3), Poodle (n = 2), Pug (n = 2), American Pitbull (n = 2), Boxer (n = 1), Basset Hound (n = 1), Bullterrier (n=1), Pinscher (n = 1), Siberian Husky (n = 1), Yorkshire terrier (n = 1), Spitz (n = 1), Labrador (n = 1) and Pekingese (n = 1).

In the feline population, the highest percentage was associated with the European breed (70.58%, n = 12), followed by the Burmese breed (23.52%, n =4) and the Persian breed (5.88%, n = 1).

Few previous studies provide data on the incidence of intracranial tumours in domestic carnivores, as there are many differences in homogeneity of populations and growth systems between countries (Kishimoto et al., 2020). However, there have been several reports who showed a higher incidence in Bulldog and Boxer (Miller et al., 2019) confirming the data obtained in this study.

The average age in the canine population was 10.36 years and the patients ranged from 6 to 18 years. The best-represented group included patients with an age higher than nine years (63.41%). Similar results were obtained in the feline population, in which the average age was 12 years, the examined cats belonging to a group ranging from 7 to 20 years old. In this species, the category of patients over 9 years was represented by over 94% of cases. The obtained results reiterate the data cited by the literature according to which for any patient over middle age who shows progressive clinical signs, the suspicion of neoplasia must be considered for every differential diagnosis performed (Song et al., 2013).

Regarding the gender distribution in the population, the number of males was significantly higher than the number of females in both species, the percentage obtained being 56.09% in dogs (n = 23) and 58.82% (n = 10) in the cat population.

The anamnesis revealed the presence of epileptiform seizures in 72.41% of patients (n = 42, of which 30 dogs and 12 cats). The seizures were generalized, tonic-clonic, and the patient's recovery to a normal mental status was prolonged. Owners have noticed changes in behaviour, compulsive walking in a circle, with a tendency to get stuck in corners. These deficits were accompanied by a series of non-specific clinical signs, with progressive evolution such as reduced body weight, decreased appetite, and a depressed mental status, with loss of interest in the owner or environmental stimuli.

Clinical examination revealed the presence of several pathologies associated with the geriatric age, such as the presence of heart murmurs, degenerative eye lesions - cataracts, glaucoma, or tumours of the skin or mucous membranes (Figure 1, A and B).

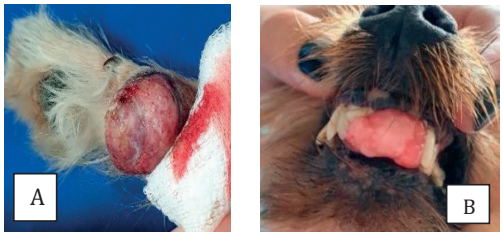

Figure 1. [A] Crossbreed dog, 11 years old, with mastocytoma in the carpal area of the right forelimb and [B] Yorkshire, 8 years old, with epulis in the oral cavity. Both cases showed epileptiform seizures and the MRI revealed the presence of intracranial masses

The aim of the neurological examination was the localization of the lesion within the central nervous system, by correlating the recorded deficits with the neuroanatomical diagnosis.
Thus, in 36 patients (28 dogs and eight cats), the neurological signs were compatible with a lesion of the cerebral hemispheres/cortex, being expressed as depressed mental status, head turn, circling, absent/reduced menace response unilaterally or bilaterally, contralateral proprioceptive deficits (Figure 2).

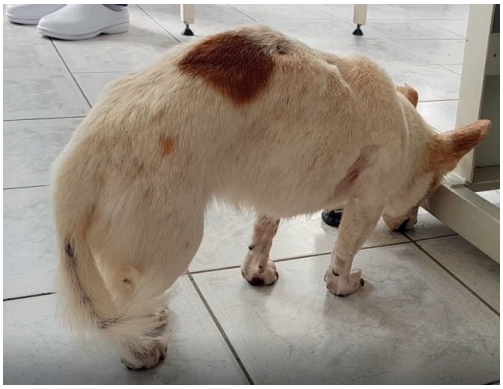

Figure 2. Crossbreed dog, 13 years old, with a mass in right cerebral hemisphere. The neurological examination revealed a gait characterized by circling on the right side, with the tendency to get stuck in corners. All proprioceptive tests were modified for the right thoracic and pelvic limbs

A total of eight patients (five dogs and three cats) had brainstem lesions, expressed by a severely depressed mental status (stupor), permanent decubitus, multiple cranial nerve deficits, associated with impaired respiratory and cardiac function (Figure 3).
Lesions with localization in the central vestibular system were identified in six patients (four dogs and two cats) who manifested head tilt, walking in small circles, nystagmus, and proprioceptive deficits ipsilateral to the direction of the head tilt.
Four other cases (three dogs and one cat) had neurological deficits compatible with cerebellar dysfunction, showing incoordination, hypermetropia, cerebellar ataxia, the inability of performing goal-targeted movements.

Figure 3. European cat, 16 years old, with a mass at the level of the brainstem. The neurological examination revealed a severely depressed mental status (stupor) accompanied by permanent decubitus and the head turn on the right side. The respiratory and cardiac functions were also affected

Also, for four patients (three dogs and one cat), the neurological expression was nonspecific, the deficits being associated with dysfunction of several brain segments, which led to a diffuse/multifocal neurolocalisation.
The chronic, progressive, focal evolution of the symptoms, corroborated with the average age of the population (10.36 years in dogs and 12 years in cats), were the main criteria in establishing the differential diagnosis. To exclude vascular and metabolic causes, the prioritization of paraclinical investigations included blood tests (biochemical and haematological profile), cardiological examination, abdominal ultra-sound, chest radiography, and MRI imaging technique.
Based on the results, patients with systemic disorders were excluded and we studied only those cases for which the MRI technique revealed aspects compatible with the presence of intracranial masses (well-delimited structures, with T1 isointense signal, T2/FLAIR hyperintense signal and T1 hyperintense contrast enhancement, with mass effect on adjacent structures).
For all cases, the imaging technique confirmed the neurolocalisation established in the previous stage. The highest percentage, 58.46% (n = 36, 28 dogs and eight cats), was attributed to patients with masses located within the cerebral hemispheres (Figure 4, A, B).

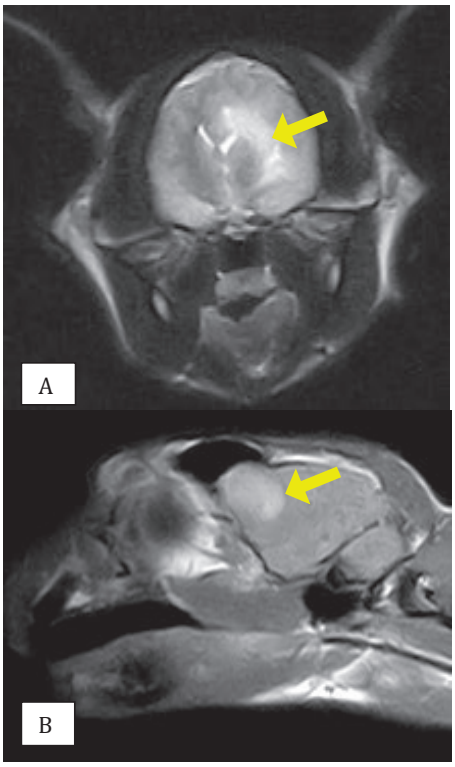

Figure 4. Feline, M, 9-years-old. [A] T2 Transversal and [B] T1 sagittal contrast enhancement MRI sections. Both sequences show a well delimited mass, of approximately 13x14 mm, with hyperintense signal T2, isointense signal T1 and hyperintense contrast enhancement T1, localized at the level of the frontal lobe of the left hemisphere (original)

The localisation of these structures in the brainstem was identified in 15.38% (n = 8, five dogs and three cats) of the patients (Figure 5, A, B), and at the level of the vestibular apparatus in 12.38% (n = 6, four dogs and two cats) of the patients (Figure 6 A, B).

The lowest percentage, 7.69% (n = 4, three dogs and one cat), was attributed to cerebellar localization. For other four patients (three dogs and one cat) the lesion was diffuse.

Although the MRI technique provides multiple useful aspects in formulating the differential diagnosis, we reiterate the idea that the establishment of a definite, etiological diagnosis, requires the corroboration of history information, with clinical signs and with the confirmation of histopathological examination.

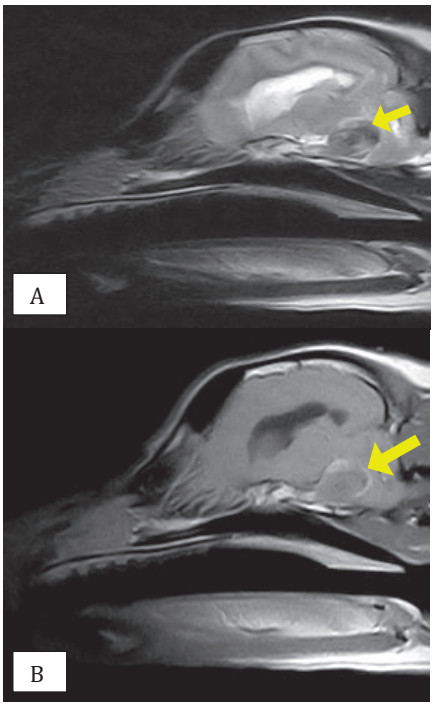

Figure 5. Mixed-breed dog, M, 12-years-old. [A] T2 and [B] T1 contrast enhancement sagittal MRI sections. All sequences show a mass with hypointense signal T2 and hyperintense contrast enhancement T1, localized at pontine level (original)

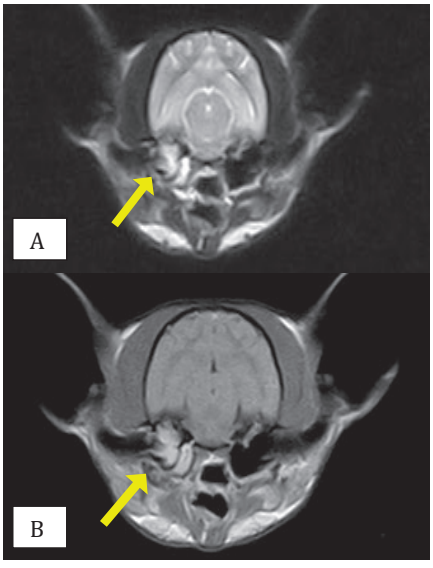

Figure 6. French-bulldog, M, 8-years-old. [A]T2 and [B] T1 contrast enhancement transversal MRI sections. All sequences show an area with hyperintense signal T2 and minimal hyperintense contrast enhancement T1 at the level of the tympanic bulla, compatible with otitis media and interna/mass - suspicion of cholesteatoma (original)

CONCLUSIONS

1. Intracranial formations were correlated with geriatric animals, the average age being 10.36 years in the canine population and 12 years in the feline population.
2. The anamnesis showed the presence of chronic, progressive clinical signs, and 72.41% (n = 42, of which 30 dogs and 12 cats) of the patients showed epileptic seizures.
3. Neurolocalisation was associated with lesions of the cerebral hemispheres for 36 cases (28 dogs and eight cats), of the brainstem for 8 cases (five dogs and three cats), of the vestibular apparatus for six cases (four dogs and two cats) and the cerebellum for four cases (three dogs and a cat).
4. In most patients, the confirmation of intracranial formations was based on MRI aspects, which revealed the presence of masses of variable size and localisations, with T2 hypersignal, T1 hyperintense contrast and mass effect on adjacent areas.
5. The etiological diagnosis implies the confirmation of the histological examination, which could not be achieved for the analysed patients.

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NANOTECHNOLOGY VERSUS OVERDOSING ANTIMICROBIAL SUBSTANCES

Emilia UNGUREANU, Sergiu Dan ZĂBLĂU, Marina SPÎNU, Diana Ioana OLAH, Adrian Valentin POTÂRNICHE, Monica Ioana SUĂTEAN, Florina MARIAN, Gheorghe Florinel BRUDAȘCĂ

University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca,
Faculty of Veterinary Medicine, 3-5 Calea Mănăştur, 400372, Cluj-Napoca, Romania

Corresponding author email: emilia.ungureanu@usamvcluj.ro

Abstract

In the last century, antimicrobial therapy evolved from being one of the most important achievements in the history of medicine to one of the major global health challenges. The overdosing of antimicrobial substances was held responsible for the appearance and the worsening of the antibiotic resistance phenomenon. Thus, scientists were called to design and implement new strategies surmounting the shortcomings of the antibiotic therapy. Nanotechnology has revealed promising solutions to the overdosing problem by use of tailored antibiotic administration schemes, both as dosage and administration route. The aim of this review is to highlight the role of nanotechnology in preventing and controlling antibiotic resistance, also emphasizing its advantages and limitations, for a better understanding of the current trends. The paper is based on scientific articles and systematic reviews identified on the Web of Science database, centralized and classified according to specific keywords. Due to their unique physico-chemical properties, the nano-based delivery systems described could become an important mean of avoiding irrational employment of antibiotics by usage of the minimal clinically active amount.

Key words*: antimicrobial resistance, nanoparticles, drug delivery systems.*

INTRODUCTION

Antimicrobial substances were one of the greatest discovery in modern medicine era and reported as one of the most successful forms of chemotherapy in the history of therapeutics and pharmacology (R. I. Aminov, 2010). The years 1935, 1941 and 1943 marked the discovery of sulphonamides, penicillin and streptomycin but also marked the lowest rate of mortality in infectious diseases (R. Aminov, 2017), highlighting the impact that antimicrobial theraphy had via prophylactic and metaphylactic use both in human and veterinary medicine. Nowadays, more and more antimicrobial substances are starting to lose their curative activity and even to harm by beeing used abusively without results (Gupta, 2018). Estimations are that antimicrobial resistance (AMR) will be held responsible by 2050 for the death of 10 milions of people (Shankar, 2016) and CoVID pandemic seems to be worsening and speeding up the process due to unauthorised prescriptions and high rates of biocides used (Lucien et al., 2021). In what concerns a *One Health* approach, it can be mentioned that the areas involved both in the originate of the AMR and also in finding alternative ways to reduce it are human, animal and environmental sectors (Palma et al., 2020). Regarding the fact that the development of new antimicrobial substances is happening at a relatively slow rate (Chebotar et al., 2021), some alternative methods can be mentioned such as genetical approaches, viral attacks, fecal microbiota transplants and last but not least nanotechnological approach (Gupta, 2018). Nanotechnology has become indispensable in almost every sector and presents an important research interest for biomedical applications (Castro et al., 2020). Defined as the understanding and restructuring of biomaterials to the order of nanometers, it has a remarcable role in responsible but most of all controlled drug administration, aiming to deliver the active compounds at predefined rates and targeting a specific group of cells (Safari & Zarnegar, 2014). The development of antimicrobial therapy through nanoparticle systems allows the achievement of the

therapeutic effect with the minimum dosage and it can be applied due to their unique physicochemical properties (Kalhapure et al., 2017). In this framework, the review aims to highlight the main mechanism of AMR, the role of overdosing the antimicrobial substances and also the implications of nanotechnology in combating this phenomenon. Moreover, our main focus is on describing the main drug delivery systems, their advantages and limitations and the medical applications possible for combating the overdosing and overusing antimicrobial substances.

MATERIALS AND METHODS

Succeeding a bibliographic study, scientific articles were selected from the *Web of Science Core Collection* database, they were centralised and classified according to specific key words. An ascendant trend was observed by searching the key words "antibiotic-loaded nanoparticles", and since their first citation (2010) and the current year (2022), an increase of 300% of the number of publication was observed, resulting a growing interest for researchers in varied areas of interest.

THE PHENOMENON OF ANTIMICROBIAL RESISTANCE

Microorganisms that are naturally sensitive to the action of an antibiotic may sometimes develop partial or total resistance either by destroying the antibiotic or by maintaining the bacterial growth even in the presence of the active substance which should inhibit the growth and development (Premlatha, 2019). Microbial resistance to antibiotics is a serious public health problem being largely caused by the inappropriate use of active substances and all aproaches, including the *One Health* principle, in order to analyse this phenomenon, should consider also that deaths are still ocurring in developing countries due to lack of access to appropriate antibiotic treatment (Ma et al., 2016). A rapid increase in the number of antibiotic-resistant microorganisms, together with the relatively slow development and late implementation of the new antimicrobials, poses a serious threat to humanity (Ma et al., 2016). International organizations have launched a series of complex activities aiming to limit the spread of bacterial resistance, including monitoring resistance, managing and analysing data obtained and also approving and inducing relevant administrative and legislative decisions and proposed novel approaches for combatting antimicrobial resistance (Eleraky et al., 2020). Therefore, antibiotic resistance can be defined as the ability of bacteria to reproduce in the presence of a certain concentration of drugs, which exceeds the normal concentration of the antibiotic used. The inherited ability of microorganisms to grow at high concentrations of an antibiotic, regardless of the duration of the treatment is quantified by the minimum (low) inhibitory concentration of that antibiotic, formula that is often used to evaluate the eficacity of an active substance (Brauner et al., 2016).

THE ROLE OF NANOMEDICINE

The appearance of highly pathogenic bacteria together with the limited production of new antibacterial substances have led to the inefficency of current antibiotic therapy with relevant risks in both human and veterinary medicine (Parisi et al., 2017). The availability of new antibacterial drugs is a very complex process, given the ability to produce and apply new effective and safe drugs, in addition to the high production costs and the time required for the approval of new drugs, which can take more than 10-15 years (Eleraky et al., 2020). Nanotechnology plays a vital role in increasing the efficacy of existing antimicrobial substances by increasing their physicochemical properties and stability, prolonging their release, ability to act directly at the site of the infection and improving systemic circulation, with a subsequent reduction of the associated side effects, compared to the classic medications used so far in therapy (Eleraky et al., 2020; Kalhapure et al., 2015). The physicochemical properties of nanosystems as particle size, surface charge and solubility are key factors that control vital processes, such as intracellular absorption or biodistribution. Nanometer-sized substances allow better loading efficiency of both hydrophilic and lipophilic drugs, thus improving the antibacterial effect (Bottaro, Larsen, 2008). In

addition, for a better penetration into the cells of nanosystems loaded with antibiotics, the transition to the reticuloendothelial system was performed, therefore improving cell biodistribution and absorbtion. Host cells, such as anionic macrophages, attract positively charged nanosystems compared to uncharged or negatively charged ones (Eleraky et al., 2020).

TYPES OF ANTIBIOTIC-LOADED NANOPARTICLES

Nanosystems can be classified according to the properties of the matrix and the material in which they are loaded, therefore being divided into inorganic and organic nanosystems (Baranwal et al., 2018). It can also be mentioned that certain NMs (nanomaterials) posses antimicrobial ability by themselves, while others require proper biochemical modifications including conjugations or loading with antimicrobial substances, acting as delivery agents (Eleraky et al., 2020; Parisi et al., 2017). Inorganic nanosystems come from inorganic oxides and their synthesis technique depends on the chemical reduction of metal salts with a reducing substance. Environmental parameters (e.g. temperature, pH) play a major role in determining the specificities of these capacity, but rather the improvement, optimization and bioavailability of the material used and also the ability to deliver patient benefits (Mcdonald et al., 2015). Therefore, polymeric nanoparticles are less than 100 nm in size, and are composed of biodegradable and biostable polymers, copolymers and drug molecules that can be entrapped, encapsulated, physically adsorbed on the surface or chemically linked to the surface of the particle (Safari & Zarnegar, 2014).

CONCLUSIONS AND DISCUSSIONS

Following the bibliographic study, the nanotechnology seems to be representing the most promising solution to the phenomenon of antimicrobial resistance caused mainly by overdosing the active substances (Skwarczynski et al., 2022). Therefore, antibiotics like ciprofloxacin (Ibrahim et al., 2015), gentamicin
(Huang et al., 2016), enrofloxacin (Paudel et al., 2019) and so many others can be improved in terms of performance by also minimising the dosage and reducing the risk of overdosing. The clinical application of nanomedicine is generally difficult to achieve due to limited time resources and high costs. The main challenges are related to clinical translation regarding the biological properties such as safety, biocompatibility, intellectual property, legal directives and also the release of in vitro drugs and therefore their antibacterial activity (Eleraky et al., 2020). Organic nanosystems, such as liposomes, lipid based nanoparticles, polymer micelles or polymer nanoparticles have better biodegradability and biocompatibility, thus constituting the main choice in clinical use (Parisi et al., 2017; Safari & Zarnegar, 2014). Known and mentioned in scientific literature since 1920, polymer science was able to provide synthetic chemistry options and also to initiate the first clinical applications two decades later (Mcdonald et al., 2015). Polymeric nanoparticles are highly used as nanocarriers due to their high structural integrity, stability during storage, method of synthesis and preparation and controlled release at the desired site (Alonso, 2004; Donelli, 2014). A general agreement has been formed that the size range is not as important, the overall advantages and profitability compared to the traditional way of treatment (Eleraky et al., 2020). These barriers limit the use of nanoparticles in the current protocols of treatment regardless of their effectiveness. Certain aspects need to be considered before the clinical approach of nanomedicine as follows: *the nanopharmaceutical design* (can influence the physical and chemical stability, biodegradability, way of administration and also the possibility of having a comercial product), *the preclinical evaluation* (the need to evaluate *in vitro* the cytotoxicity, the pharmacocinetics and pharmacodynamics and toxical evaluations) and *the clinical evaluation* (clinical trials in order to establish the practical applicabiliy of the antibiotic-loaded nanoparticles) (Eleraky et al., 2020).

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INTERDEPENDENCE OF ORAL MICROBIOME-HABITAL MICROBIOME AND ITS ANTIBIOTIC RESISTANCE IN HEALTHY DOGS

Aurel Vasiu[1], Diana OLAH[1], Cătălina CORNEA, Gheorghiță DUCA[2], Marina SPÎNU[1], Monica Ioana SUĂTEAN[1], Emoke PALL[1]

[1]Department of Infectious Diseases, Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, 400372, Cluj-Napoca, Romania
[2]Institute of Research and Development for Montanology Cristian, Sibiu, Romania

Corresponding author email: aurel.vasiu@usamvcluj.ro

Abstract

Antibiotic resistance is a growing phenomenon which involves a pronounced zoonotic risk. Healthy dogs can acquire antibiotic-resistant bacteria in their living environment, but also on occasional clinical examinations at the veterinary clinic where they are presented for consultation. In order to follow the way in which the microbiome transfer can be performed in a veterinary clinic to regular patients, saliva samples (n = 8) were collected from healthy dogs presented at a veterinary clinic in Cluj-Napoca. The bacterial population was also tested for resistance to antibiotics. The dogs were regular patients of the veterinary clinic, originating from different districts of Cluj-Napoca. Thus, the intersection between patients is performed only in the veterinary clinic. Samples were also collected from various surfaces in the consulting and waiting rooms. The samples were processed using classical microbiological methods and identified by rapid biochemical assays. The susceptibility to certain antibiotics was evaluated using agar diffusion method. In this study, bacteria of the same species were isolated from patients with different habitats, supporting the possible interchangeability of the microflora, probably in the case of repeated visits to the same office. The presence of a large number of strains involved in the oral microbiome associated with increased resistance to antimicrobials calls for the implementation of enhanced biosecurity measures.

Key words*: dogs, saliva, bacteria, veterinary clinic, antimicrobial resistance.*

INTRODUCTION

The subject of nosocomial infections in veterinary medicine is not very deeply studied, unlike human medicine. This is probably due to the fact that the animals do not have long-term contact with the environment of veterinary practices and veterinarians.

However, there are reasons why the possibility of transmitting bacteria in the veterinary office should not be ignored. One of these reasons, enough to study the subject, is the possibility of transmitting antibiotic-resistant bacteria. It is important to note that pets, especially dogs, have a specific research behavior. This increases the chances of the colonization of the skin and mucous membranes with bacteria. Also, one aspect that could increase the importance of the topic is the fact that in the veterinary office, unlike human hospitals, sick and healthy animals live in one space and are in direct contact. People, however, follow safety precautions to prevent the transmission of bacteria to other patients. The normal microflora of animals is directly proportional to environmental factors, so it is the main target of substances entering the body and is also involved in the transformation of natural and foreign substances to the body. This can lead to dysbiosis, changes in physiological, biochemical and immunological parameters, accumulation and selection of atypical strains and finally, the emergence of pathological processes (Yakshigulova, 2016).

The micro-environment of the veterinary office is considered a potential factor that can influence the composition of the oral microflora of pets. Direct contact between patients in waiting rooms, contact with surfaces and objects in the veterinary office, surgery and contact with the veterinarian are factors that lead to bacterial colonization and possibly to the appearance of nosocomial infections. In addition to these major risk factors are immunosuppression, antibiotic therapy and disease (Stull and Weese, 2015). The pathogens responsible for the occurrence of pathological processes, either transmitted from one patient to another or from staff to patient, may be

resistant to antimicrobials. Antibiotic-resistant bacteria that can be transmitted from veterinary clinics include *Escherichia coli*, *Clostridium* spp., *Enterobacter* spp., *Enterococcus* spp., *Staphylococcus* spp., *Acinetobacter baumannii*. These bacteria are a serious concern not only because of their virulence, but also because of their resistance to antibiotics. Infections associated with these bacteria are difficult to treat and can be associated with a serious prognosis (Weese, 2020).

The aim of this study was to highlight and characterize microorganisms isolated from the oral cavity of dogs and the environment where they intersect and to draw a conclusion about the possibility of bacteria transmission between dogs and the role of the environment in veterinary clinics on this transmission.

MATERIALS AND METHODS

Samples from the oral cavity were collected from 8 (n = 8), clinically healthy dogs aged between 5 months and 7 years. The purpose of the visit to the vet was routine internal and/or external deworming, vaccination. The dogs subjected to the study only intersect in the veterinary office where the samples were collected. The owners of each animal were informed accordingly about the details of the study and the agreement signed by them was obtained for the collection of samples from the oral cavity. Prior to sampling, each dog underwent objective clinical examination, and animals with various pathologies were excluded from the study. All dogs are constant patients of this veterinary office, which intersect with each other and have contact with the environment of the office (walls, floor, etc.).

In addition to the dog population, samples were taken from the veterinary office. Samples were collected from the angle of 2 walls (consultation room) and from the angle between a wall and the floor (waiting room). For initial microbiological analyses, the samples were inoculated in nutrient broth and nutrient agar (both from Oxoid) in aerobic conditions at 37°C for 24 hours. After obtaining the isolated colonies, the catalase test and the oxidase test were performed. Bacterial strains identification were performed by standard microbiological methods adopted from the Clinical and Laboratory Standards Institute (CLSI) guideline. The identification of microorganisms was performed using GP 24 (Diagnostics) for the identification of Gram-positive bacteria and GN 24 (Diagnostics) kits for the identification of Gram-negative bacteria (Figure 1).

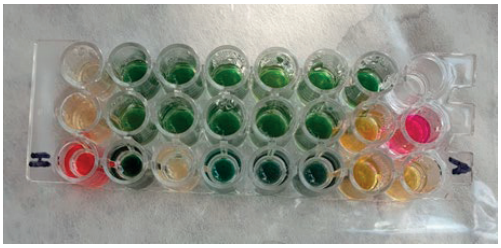

Figure 1. The kit used to characterize bacterial strains

The results obtained in the biochemical tests together with those obtained in the oxidase test were evaluated using the microID software.

The antimicrobial sensitivity patterns of the isolated strains were evaluated using the standard Kirby-Bauer disk diffusion method according to the CLSI guidelines. The strains were tested towards 15 antimicrobials: GEN-gentamicin; AK-amikacin; TE-tetracycline; DOX-doxycycline; CIP-ciprofloxacin; LEV-levofloxacin; TOC-trimethoprim; C-chloramphenicol; A/S-ampicillin/sulbactam; CRO-ceftriaxone; TOB-tobramycin; AMC-amoxicillin; CD-clindamycin and E-erythromycin; P-penicillin. The antibiotic assay discs were purchased from Oxoid. The results were evaluated based on the growth inhibition zone diameters (mm) and were classified as sensitive (S), Intermediate resistant (I) and resistant (R) (according to the standard values of CLSI 2020). The multiple antibiotic resistance index was also calculated according to Krumperman (Krumperman 1983).

RESULTS AND DISCUSSIONS

In this study, eight dogs were sampled from a private veterinary clinic in Cluj-Napoca. The dogs were of different breeds, sizes, sexes and ages. Following the objective clinical examination performed before collecting the samples, it was proved that all the dogs were clinically healthy. Samples were also collected from the veterinary office where these dogs are regularly checked.

After transferring bacterial culture sampes from nutrient broth to agar, several types of colonies developed. Thus, for each sample collected from dogs, a minimum of 2 and a maximum of 3 types of colonies were identified for each sample.
In the samples collected from the environment of the veterinary office, 1 or 2 types of colonies developed.
After examination of the pure cultures, the presence of 13 Gram-negative and 12 Gram-positive bacteria was observed. Out of a total of 9 Gram-positive bacteria, identified in 6 dogs, 3 are members of the genus *Staphylococcus* (*S. arlettae, S. epidermidis, S. acidominimus*), and 2 are members of the genus *Actinomyces* (*A. radingae, A. turicensis*).
In the case of a sample collected from the dog, bacterial strains were identified identical to those isolated from the samples collected from the veterinary clinic.
Of the 3 bacteria identified in the veterinary clinic samples, one is sporulated (*Clostridium difficile*) along with *Aerococcus viridans* and *Facklamia sourekii* strains (Table 1).

Table 1. Isolated Gram-positive bacteria

Bacterial strain ID	Bacterial strain
1.1	*Staphylococcus arlettae*
3.1	*Micrococcus spp.*
4.2	*Aerococcus viridans*
4.3	*Enterococcus hirae/dispar*
6.1	*Actinomyces radingae*
6.2	*Actinomycens turicensis*
6.3	*Staphylococcus epidermidis*
9.2	*Staphylococcus acidominimus*
10.1	*Bacillus mesentericus*
11	*Clostridium difficile (clinic)*
12.1	*Aerococcus viridans (clinic and patient)*
12.2	*Facklamia sourekii*

Among the isolated Gram-positive bacteria, some have zoonotic potential, such as Bacillus or *Clostridium* species, in which case the control is difficult, given their sporulated character. Due to the isolation of the genus *Clostridium* from the clinical microclimate, it is important that those responsible for mandatory disinfection re-evaluate the zoonotic risks posed by it and propose periodic disinfection using broad-spectrum disinfectants.
The results of the oxidase test showed that out of 13 Gram-negative bacteria, 10 are oxidase-positive and 3 are oxidase-negative (Table 2).

Table 2. Isolated Gram-negative bacteria

Bacterial stain ID	Bacterial strain
1.2	*Alcaligenes faecalis*
1.3	*Acinetobacter lwofii*
3.2	*Escherichia coli*
3.3	*Brevundimonas diminuta*
5.1	*Delftia acidovorans*
5.2	*Achromobacter xylosoxidans*
8.1	*Yersinia aldovae*
8.2	*Pseudomnas aeruginosa*
8.3	*Myroides odoratimimus*
9.1	*Escherichia coli*
9.3	*Brevundimonas diminuta*
10.2	*Aeromonas hydrofila*
10.3	*Bordetella bronchisepica*

The evaluation of antibiotic sensitivity/ resistance indicated very different patterns, depending on the tested strain.
Various classes of antibiotics have been used, including penicillins, aminoglycosides, tetracyclines, cephalosporins and fluoroquinolones.
After identifying the susceptibility of each bacterium, it was found that most bacterial strains are sensitive to the used antibiotimicrobials (Table 3).

Table 3. Susceptibility of bacterial strains to antibiotics

Strains	GEN	AK	TE	DOX	CIP	LEV	COT	C	A/S	CRO	TOB	AMC	CD	E	P
Staphylococcus arlettae	S	-	R	-	S	-	S	S	-	-	-	-	-	-	-
Aerococcus viridans	S	-	S	S	S	S	-	-	I	R	-	S	-	-	R
Staphylococcus epidermidis	S	-	R	S	S	S	-	-	-	-	-	-	I	I	R
Yersinia aldovae	S	S	I	S	S	S	-	-	I	R	S	S	-	-	R
Escherichia coli	S	S	S	-	S	S	-	-	S	S	S	S	-	-	R
Staphilococcus acidominimus	S	S	-	S	S	S	-	-	-	-	S	-	-	R	S
MAR index			0.4							0.66					0.8

I - intermediate resistant, R - resistant, S - sensible. MAR - multiple antibiotic resistance index
GEN-gentamicin; AK-amikacin; TE-tetracycline; DOX-doxycycline; CIP-ciprofloxacin; LEV-levofloxacin; TOC-trimethoprim; C-chloramphenicol; A/S-ampicillin/sulbactam; CRO-ceftriaxone; TOB-tobramycin; AMC-amoxicillin; CD-clindamycin; E-erythromycin; P-penicillin.

Figure 2 shows an increase in the MAR index in some of the antibiotics, which indicates a broad resistance to at least some of the tested antimicrobials and argues for their increased pathogenicity. *Pseudomonas aeruginosa, Escherichia coli, Acinetobacter lwofii, Staphylococcus eidermidis, Achromobacter xylosoxidans* and *Clostridium difficile* are considered antibiotic-resistant bacteria, according to many studies (Cheung, 2017). Some studies show that *Yersinia aldovae* is resistant to tetracycline, ciprofloxacin, ampicillin and amoxicillin and penicillin (Jamali et al., 2014).

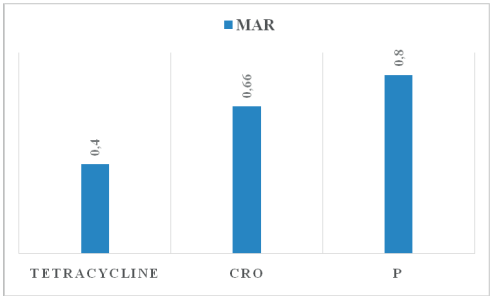

Figure 2. Multiple antibiotic resistance -MAR index in isolated strains

Dogs are part of the category of pets that live in close contact with humans. The oral cavity of clinically healthy dogs of different ages, sexes, breeds and management systems are colonized with multidrug-resistant bacteria which can act as an important source of infection for their owners and/or handlers. The most common bacterial strains isolated from the oral cavity of dogs are *Klebsiella pneumoniae* ssp. *pneumoniae, Escherichia coli, Staphylococcus aureus, Citrobacter freundii, Enterobacter cloacae, Acinetobacter calcoaceticus* and *Pasteurella* species, which can be transmitted by biting or licking. The predominant species of bacteria involved in the bite wound infection are *Staphylococcus aureus, Pasteurella multocida, E. coli, Moraxella, Pasteurella canis, Enterobacter cloacae* (Kasempimolporn et al., 2003). Multiple studies demonstrate the presence of pathogenic, zoonotic or multidrug-resistant bacteria in the oral cavity of dogs that can serve as a possible source of transmission to humans through direct contact or bite (Bata et al., 2020). Bacterial strains isolated from some of the dogs represent the normal microflora present in the skin and / or mucous membranes, digestive tract, urine, respiratory tract or are pathogenic, present in dermatitis. During the study, bacteria of the same species with different habitats, respectively patient and clinic, were isolated, supporting the interchangeability of the bacterial microflora, probably in the case of repeated visits to the same clinic of the same patient. The results of the study draw attention to the multitude of bacterial species present in the oral cavity in dogs and which, in combination with an increased resistance to antibiotics may pose a danger to staff, but also to owners or even patients.

CONCLUSIONS

According to the results obtained in this study, in order to reduce the risk of nosocomial infections, it is important to ensure a rigorous asepsis. It is important to regularly disinfect the surfaces of the clinics, including the waiting room, as this is a place where the animals stay the longest and have contact with the walls, floor and surrounding objects. Veterinarians must follow hygiene rules to prevent the spread of bacteria to patients. Given the possibility of transmitting antibiotic-resistant bacteria, a preventative measure is the correct diagnosis and choice of medication and therapeutic protocols.

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INTO THE MICROBIAL LIFE OF THE CAVES; PATHOGENS AWAITING IN THE UNDERGROUND

Sergiu Dan ZĂBLĂU, Diana Ioana OLAH, Emöke PÁLL, Florina MARIAN, Emilia UNGUREANU, Aurel VASIU, Marina SPÎNU

Department of Infectious Diseases, Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, 400372, Cluj-Napoca, Romania

Corresponding author email: sergiu-dan.zablau@usamvcluj.ro

Abstract

Caves frequently visited by animals or humans present reservoirs of pathogenic or conditioned pathogenic microorganisms. In recent years, numerous bacteria, fungi and viruses have been isolated and identified from caves as pathogens for humans and animals. Furthermore, through humans and/or animals, new types of germs can be carried into the normal microflora of caves, disturbing the microbial balance, making these locations sources of pathogenic germs with zoonotic potential. The most common diseases caused by microorganisms in caves are diseases located in the respiratory system. Visits to any underground environment should no longer be seen as a simple, risk-free tourist activity, but rather as one with potential risks to human and animal health. Numerous studies have been conducted worldwide on the diversity and abundance of pathogens in caves. In order to reduce the number of diseases associated with caves, it is necessary to raise awareness and educate about the possible dangers to people or animals that come in direct contact with such environments.

Key words: *cave, microbiome, pathogens*.

INTRODUCTION

Caves frequently visited by humans or populated by animals are reservoirs of pathogenic microorganisms. In recent years, many bacteria, fungi and viruses in caves have shown pathogenic potential for humans and animals, especially in the tropics. The zoonotic character of these pathogens is still debatable as variations of these viruses or bacteria have specific targets. For instance, historic pandemics such as MERS-CoV or SARS-CoV-2 have reached the assumption that coronaviruses from dromedary camels or bats have suffered mutations that allowed them to cause highly dangerous respiratory syndromes in humans. Although it is difficult to associate a specific infection with a single visit to an underground environment, several studies indicate that visits to caves and mining areas have resulted in fatal diseases.

Microbial life of the caves

The globe's microorganism population is extremely diverse and varied, with many specimens still an enigma to humans. These microorganisms occupy all stages of the biosphere, including the subterranean areas. Underground habitats are mainly caves, with little or no light input, with few resources of organic nutrients, relatively constant temperature and areas extracted from mineral surfaces. Specialized studies on microbial communities in karst environments are limited, most being conducted in caves in Spain, Italy, Romania or the United States. However, these studies focus on specific issues, such as taxonomy or geomicrobiology (Hernandez-Marine and Canals, 1994).

More recent studies try to highlight the biodiversity and limited distribution of bacteria in the karst environment. *Proteobacteria* represent the majority of bacteria found in caves, followed by representatives of the genus *Actinobacteria* (Barton et al., 2007). Bacteria of the genus *Actinobacteria* have been attributed mainly to soils, which represent their natural habitat, but in the last decade they have been shown to be frequently associated with the karstic environment (Schabereiter-Gurtner et al., 2002). This points out that for this group of bacteria, the microclimate conditions and the nutrient sources of the caves represent a favorable habitat.

Microclimate conditions and high salinity create certain niches and stability in microbial communities, and this leads to increased interest in in-depth studies in order to understand the natural processes that make up these microbial communities. In addition, the interaction of the composition of the karst environment, both biological and mineral, determines the biodiversity of these ecosystems, still insufficiently known.

These previously mentioned groups are very widespread in caves, and the description of new species is in a continuous flow of development due to the new molecular methods that have emerged. These methods have the advantage of taxonomically classifying the subspecies of these microorganisms. For example, after the emergence of new research methods, the genus *Mycobacterium* includes over 130 species (Mignard and Flandrois, 2008), the genus *Nocardia*, about 70 species (Rodríguez-Nava et al., 2008), the genus *Gordonia* 21 species (Blanc et al., 2007), genus *Rhodococcus* 32 species, *Brevibacterium* with 19 species and *Micrococcus*, 9 species.

Pathogens awaiting in the underground

Certain bacteria of this genus are responsible for various lung, brain or skin infections in humans.

Species such as *G. bronchialis*, *G. otitidis*, *G. aichiensis*, and *G. terrae* of the genus Gordonia are described in the literature as opportunistic pathogens responsible for bacteremia and bronchopulmonary disease (Iida et al., 2005; Blanc et al., 2007). Such species have been isolated and identified from the Grotta dei Cervi cave, located in Italy, but the possible variety of this habitat is not yet fully established. The genus *Gordonia* is of great interest in the field of biotechnology due to its ability to degrade and bioremediate sulfur from fuels (Lee et al., 2005).

The main species of the genus *Rhodococcus* are represented by *R. equi* and *R. erythropolis* potentially pathogenic bacteria, isolated from caves located in northern Spain. (Vernazza et al., 1991) (Groth et al., 1999). Regarding the genera *Brevibacterium* and *Micrococcus*, they have been little studied, and their involvement in clinical cases is rarely reported in the literature. Species such as *B. casei* or *B. epidermis* are normally part of the human skin flora, being responsible only for opportunistic infections (Reinert et al., 1995). *Micrococcus luteus* is also responsible for certain common diseases in people with immunodeficiency (Salar et al., 1997). Reverend to the *Streptomyces* family, the main pathological agent with potential pathogen is *Streptomyces somaliensis*, an accidental etiological agent of actinomycetoma in countries such as India or Sudan (Nasher et al., 1989).

The growing interest in such ecosystems is not strictly due to the diversity and taxonomic uncertainty of these bacteria, but more importantly, the role that these microorganisms could play on human and animal health. The potential pathogenic or even zoonotic risk of these bacteria coming from natural ecological niches, in this case, the underground ones, can be a real problem in human and veterinary health.

Evidence of a considerable reservoir of pathogenic bacteria present in tourist karst environments, namely species belonging to the *Proteobacteria* cluster, has also been reported. Thus, a study conducted in caves in Spain revealed the presence of a species of *Alphaproteobacteria*, called *Inquilinus limosus*, which has been implicated in the occurrence of cystic fibrosis in humans (Schmoldt et al., 2006). Studies in this direction have reported the presence of *Aurantimonas altamirensis*, a bacterium of the genus *Alphaproteobacteria*, in the Altamira Cave (Jurado et al., 2006). The bacterium was later isolated in a Canadian hospital from three patients diagnosed with cystic fibrosis, keratitis, and corneal ulcer (Luong et al., 2008).

Also, certain species of the genus *Afipia* have been associated with protozoa, and their role is suspected in the occurrence of nosocomial diseases (La Scola et al., 2002). With the help of molecular techniques, *Legionella* reservoirs have also been detected in caves in France (Bastian et al., 2009). In addition, bacteria such as *Staphylococcus aureus* have recently been isolated by researchers from karst environments of tourist value, highlighting the possible human impact. This type of bacteria is frequently associated with nosocomial infections in countries such as India or the USA (Mendes et al., 2009).

Spirochetes are also frequently associated with karst environments due to the favorable survival conditions that these habitats offer. Rodents and bats are reservoirs of leptospirosis in many tropical caves, which maximize the exposure of tourists to this disease. High heat and humidity can cause tourists to choose minimal clothing protection, and frequent contact with cave rocks can result in multiple skin microlesions. These wounds are often the main route of infection in leptospirosis (Mortimer, 2005).

Another condition commonly associated with caves in some countries is recurrent borreliosis fever. Ticks are the basic vectors of *Borrelia* spp. And they are widespread in India, Iran, Syria, Turkey, Cyprus, Egypt. In Israel, about 40-50% of caves have been found to be infested with *Ornithodoros tholozani*, the most important vector for *Borrelia persica*, the cause of recurrent tick-borne fever. *O. tholozani* parasitizes rodents and small mammals, which are the natural reservoir of the bacterium.

These bacteria, which are isolated from caves and have a significant pathogenic potential, pose a real danger to humans and animals, especially at-risk groups. However, the virulence, pathogenicity and spread of these species of microorganisms are not always constant in the strains. This suggests that the strains were isolated from niches with different characteristics, thus acquiring and developing different pathogenic factors. Also, the fact that there are few clinical observations on these microorganisms may give the false impression that only a few strains are pathogenic. This could be due to: a) relatively new detection methods, as it refers to a recently described species, b) low environmental incidence, c) the quality of an opportunistic pathogen that requires special conditions of the host's immune system. On the other hand, it would also be interesting to obtain information on the infectious capacity of strains isolated from caves through experimental research, studying their virulence capabilities with PCR methods, using virulence genes as markers and by interaction tests with other hosts (amoebae, fungi, insects, etc.).

There is a high risk, due to the presence of humans in caves, of a bacterial passage from humans to caves that can lead to the development of new lines of bacteria with different levels of pathogenicity. This phenomenon can be assessed by measuring human indicator bacteria. On the other hand, adaptation factors after a strain exchange (from human to cave and vice versa) can be recognized by transcriptome and comparative genomic studies.

Microbiological studies performed from the air of the Magurici cave, showed that the total number of germs varied between 102 and 104 cfu/m^3 of air. Gram-negative bacteria and staphylococci ranged from 0 to 102 cfu/m^3, and streptococcal and fungal values ranged from 5 × 10 to 103 cfu/m^3 air. The prevalence for these bacteria showed maximum values during the summer, close to the maternity period of the bats. The microbiological values observed during the time are comparable to those observed in the Central Park of Cluj-Napoca (NTG = 629-10479 cfu/m^3; G– = 0-786 cfu/m^3; SP = 0-1402 cfu/m^3; ST = 314- 1572 cfu/m^3; Fungi = 550-109970 cfu/m^3). In spring, autumn and winter the values were similar to the microbiology of the air in the mountain resort Păltiniș (NTG = 366 cfu/m^3; G - = 0 cfu/m^3; SP = 0 cfu/m^3; ST = 0 cfu/ m^3; Fungi = 2907 ufc/m^3) (Drăghici, 1982).

However, these values of microorganisms in the air can vary depending on the cave, the intensity of the search for food outside the habitat, the increase in maternity and most likely the number of dead youth. This bacterial contamination of the air can pose a risk to animals and humans (Hartung 1994). It is known that about 20% of infectious diseases have air as the main route of transmission, and areas with temperate climates, such as karst, are predisposing to such diseases (Manescu et al., 1993). Thermal circulation and ventilation thus play an important role in keeping the air clean, due to the fact that bats usually prefer well-ventilated areas in caves, for a cooler climate. Cave ventilation plays an important role in preventing air pollution and contributes to the health of bat populations. Bats being an inhabitant of caves but also one who often seeks nutrients in the outside world could easly carry and spread potential pathogenic agents (Borda et al., 2004).

CONCLUSIONS

Frecvent visits to caves can not only lead to the emergence of disease outsite of these envirouments but also distrupt the existing microbiom. Continuous changes in the prevalence of agents in caves may lead to the appearance of important zoonotic diseases, as proven by the 2019 coronavirus pandemic.

The bacterial microflora introduced in the underground environment can adapt to the new microclimate conditions and represent new sources of pathogenic germs or pathological conditions both for humans and for cave fauna (especially bats). Further research is needed to properly establish the possible dangers lurking in underground environments.

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ASSESSMENT OF LAMENESS SCORE AND MUSCLE ATROPHY AFTER EXTRACAPSULAR STABILIZATION OF CRANIAL CRUCIATE LIGAMENT RUPTURE IN TOY BREEDS

Alina ZĂVOI, Ciprian OBER, Liviu Ioan OANA

University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca,
3-5 Calea Mănăștur, Cluj-Napoca, Romania

Corresponding author email: andreea-alina.zavoi@usamvcluj.ro

Abstract

Ten dogs of different breeds, small size, were evaluated postoperatively, after cranial cruciate ligament rupture repair by the extracapsular method. The evolution of the lameness score and the degree of muscle atrophy were estimated at one month and three months postoperatively through discussions with the owners and/or physical evaluations. There were no pre- or postoperative complications in dogs that were involved in the study. At three months postoperatively, both the lameness score and the degree of atrophy underwent changes compared to the preoperative stage or one month postoperatively. Although the evolution of the patients was adequate, without intra and postoperative complications, the extracapsular method does not completely restore, at 3 months, the function of the affected limb.

Key words*: cranial cruciate ligament, toy breeds, extracapsular stabilization, lameness.*

INTRODUCTION

The anatomy of the knee joint in animals is complex. Over time, our understanding of the anatomy of the cranial and caudal cruciate ligament has developed, especially in the direction of microvascularization of the area. Recent work suggests the existence of a blood-ligament barrier, analogous to the blood-brain barrier, a finding that helps explain the process that lead to the progressive rupture of the fibers of the ligament matrix of both cruciate ligaments. In addition to their biomechanical role in joint stabilization, cruciate ligaments probably have key roles in joint proprioception (Muir Peter, 2017).

Cranial cruciate ligament (CrCL) rupture is one of the most common orthopaedic conditions seen in veterinary medicine. The femoral-tibial-patellar joint, because of its strategic site between the hip and hock, plays a key role in the pelvic limb. In a standing posture, as a result of the position of the menisci interposed between the femoral and tibial condyles, the stifle joint supports the bodyweight, while during the movement, it allows the transmission of the propulsive thrust towards the coxo-femoral joint and the shortening of the functional length of the pelvic limb (Kapandji, A.I., 2011; Neumann, D.A., 2017).

The role of the cranial cruciate ligament is to withstand the anterior translational and internal rotational movements of the tibia, this function having a role in preventing the anterior tibial subluxation of the knee joint (tibio-femoral and patello-femoral joint). Unlike in humans, CrCL rupture in dogs is rarely the result of a traumatic injury. The definitive etiopathogenesis of cruciate ligament rupture in dogs has not been completely clarified. It has been shown that this disorder appear during physiological daily load due to progressive degenerative changes in the stifle joint (Muir Peter, 2017).

The cranial cruciate ligament has been shown to have mechanoreceptors that detect changes in the position of the knee joint, in speed, and tension in performing movements. A key factor in limb instability after cranial cruciate ligament rupture is related to impaired neuromuscular function, secondary to reduced somatosensory information. Injuries to the cruciate ligament leads, over time, to joint degeneration (Smith et al., 2012). Most cases are not initially so easily recognized at the time of the partial rupture, and subsequently present with progressive instability. Many procedures

have been put into practice over time, but none of them have proven to be most favorable in terms of technical simplicity, associated costs, prevention of secondary pathology, rate of complications, types of complications, medium or long term results. No technique for the treatment of cranial cruciate ligament deficiency (CrCL) has been shown to be superior to the other in terms of functional outcome (Bergh MS et al., 2014; Rey et al., 2014; Kim et al., 2012; Skinner et al., 2013). In the near future, advances in understanding the effects of homeostasis of the ligament matrix on the mechanical properties of the cruciate ligaments and finding specific biomarkers, should provide insight into the mechanism of fiber rupture in the early stages of the disease and early prevention of osteoarthritis (Muir Peter, 2017).

MATERIALS AND METHODS

This study involved small dogs of different breeds who were referred to the Faculty of Veterinary Medicine, department of Anesthetics and Surgical Propaedeutics, Cluj-Napoca for an orthopedic examination to establish the diagnosis of cranial cruciate ligament rupture.

The extracapsular lateral suture stabilization technique was performed in 10 dogs.

This study included (1) Pomeranian, (1) Mixed-Breed, (3) Westie, (1) Bichon, (2) Yorkshire, (1) Chihuahua, (1) Pinscher. Their age ranged from 3 to 10 years and they weighed between 3-12 kg. The study population consisted of 5 spayed females and 5 castrated males (Table 1).

Table 1. Presentasion of cases

Nr.	Breed	Age	Sex	Affected Limb	Lamness score Preoperative	Amiotrophy Degree Preoperative
1	Pomeranian	9	M	Left	2	2
2	Mixed-Breed	5	F	Left	3	2
3	Westie	10	F	Left	3	3
4	Bichon	6	M	Left	2	2
5	Westie	7	M	Bilateral	2/3	2
6	Yorkshire	5	F	Right	2	3
7	Chihuahua	4	F	Bilateral	4/1	3
8	Yorkshire	3	M	Left	4	3
9	Pinscher	3	F	Right	3	2
10	Westie	4	M	Left	4	3

The criteria by which they were chosen for the study were based on the acute or chronic duration of the disease; bilateral or unilateral rupture; joint instability; previous surgical history; the presence or absence of meniscal damage; severity and location of OA; weight and body condition; age. Patients data were recorded on the clinical examination records. Each owner gave their written consent for the animals to be subjected to the procedures. The ruptured ligaments were repaired with a lateral extracapsular suture using an artificial nylon ligament. The degree of lameness was assessed using Fitzpatrick's lameness score (no lameness - 0; mild lameness - 1; moderate intermittent lameness - 2; severe intermittent lameness - 3; persistent lameness, no weight bearing - 4) (Fitzpatrick et al., 2010). Ligament ruptures are also classified from grade I to grade III, depending on the severity of the matrix damage. Ligaments rupture are defined biomechanically: grade I does not affect joint instability and is associated with mild damage to ligament tissue; grade II is associated with moderate fiber damage and a stretch to the point of detectable instability, and grade III ruptures are associated with severe ligament disruption and obvious joint laxity (Provenzano et al., 2002). Dogs with some joint stability have a partial cruciate ligament rupture, and those with instability present with a complete ligament rupture. Muscle atrophy was classified using a 3-point scale designed by us (normal muscles of the hip and thigh regions - 0; mild atrophy of the muscles of the hip and thigh regions - 1; severe atrophy of the muscles of the hip and thigh regions - 2) applying the thigh girth measurement in a standing position. The diagnosis in complete rupture of CrCL is usually based on the anamnesis, physical,

neurological and orthopedic examination of the patient with the demonstration of the laxity of the stifle joint by specific tests (drawer test and tibial compression test). Other tests may be needed, including mid-lateral and caudo-cranial radiography of the joint or MRI. All owners were contacted by telephone to provide information on the clinical condition of patients at one and three months postoperatively and follow up videos in order to have data on the evolution of the animal in terms of lameness score and muscle atrophy degree. The owner used the Fitzpatrick's lameness score and the 3-point scale for muscle atrophy.

RESULTS AND DISCUSSIONS

Owners have reported that their pet has experienced lameness after daily exercise, such as after jumping or playing time. All dogs that were diagnosed with a torn ligament were recorded as having a normal level of activity in daily life. The lameness that occurred due to the rupture of the cranial cruciate ligament was present for a variable period before the presentation at the clinic. CrCL rupture was complete in all dogs involved in this study, and two of them presented with bilateral rupture. The cranio-caudal instability assessed by the drawer test and the tibial compression test was present in all patients examined.

A re-examination at our hospital was recommended for owners at one month and then three months after surgery to assess the joint stability of the patients. Data collected included lameness score and degree of muscle atrophy. Four out of ten owners came with the patients for a check-up (the remaining ones were contacted by telephone with follow up videos because they never shown up for a re-examination). We reported an initial decrease in limb function, followed by a moderate increase, which usually occurs around six months after surgery, regardless of the surgical procedure used (Figure 1).

Establishing the owner's perspective on the success of the intervention remains important, as they spend most of their time with the pet. Studies documented that owners reported that their pet had a “good or excellent” result of 88.5-93% depending on the intervention (Innes John F. et al., 2000).

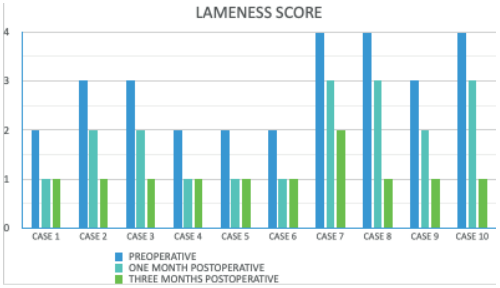

Figure 1. Evolution of the lameness score at one month and three months postoperative

However, these findings need to be interpreted with great caution due to differences in the questions asked, the method of delivering the answer and other factors that significantly influence owners reporting of the outcome such as the fact that the animal should not gain weight, because weight gaining is often cited as an explanation for the incidence of ligament rupture. This is a subjective issue and the animals in this study should have been observed and seen for a longer period of time. Consistent use of a questionnaire validated by the owner should be considered. Several questions have been created which addresses pain, limb function, quality of life, and/or patient activity. Following the physical re-examination of the four patients which presented at the clinic and telephone conversations with the other owners, we found out that at one month after the intervention, in four of the patients a slight lameness score was observed (1 out of 4) in 3 patients a lameness score of 2 out of 4 was seen and 3 of the patients showed a 3 out of 4 lameness score. At three months, nine patients presented with mild persistent lameness, and in one patient the owners reported that their pet had moderate persistent lameness. Moderate muscle atrophy of the hip and thigh regions was observed in five dogs, and in the five a severe muscle atrophy (Figure 2). After one month of the postoperative period, there was an improvement in muscle atrophy in five of the patients (being between 1-1.5), and in the others a moderate muscle atrophy due to the fact that the dog's activity during that period should be limited to the maximum. At three months, a reduced muscle atrophy of the hip and thigh regions was observed in all ten dogs involved in this study, based on the 3-point

scale for muscle atrophy. The owners noticed an improvement in the gait of their pets, with subjective observations on the lameness score and the degree of muscle atrophy. Strength deficiencies have been reported in the limb muscles up to 30% compared to the other hind limb, due to the fact that muscle atrophy contributes to weakening of the quadriceps (weakening of the quadriceps is almost ubiquitous due to injury and reconstruction of the cranial cruciate ligament) (Abbey C. Thomas et al., 2015). Early return to function is desirable to reduce muscle atrophy and maintain range of motion at the level of the stifle joint after surgery. Muscle atrophy can alter the forces, acting on the stifle joint and thus influence the progression of the degenerative processes at the joint level. Pain, edema, restriction of activity and bandaging contribute to the postoperative loss of normal movement of the affected limb, requiring a strict range of exercises as part of a rehabilitation program. The exercises are performed under controlled conditions with therapeutic aims. The aims of exercises include properly limb use, strengthening, improving joint and soft tissue mobility, promoting proprioception and eliminating weight shifts. Exercises are adapted to the stage of recovery. During the early postoperative period, exercises may include static exercises, slow walking and exercise in water where loads placed on joints are reduced (Muir Peter, 2017).

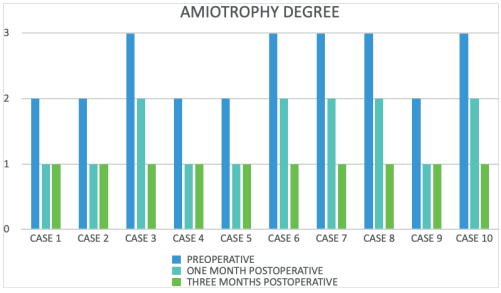

Figure 2. Evolution of muscle amyotrophy degree at one month and three months postoperative

Obesity, defined as exceeding the ideal body weight by 15-20%, puts excessive forces on the joints and articular cartilage, being exacerbated by inactivity, spreading a vicious circle of muscle atrophy and decreased desire to perform physical exercises (Impellizeri et al., 2000; Kealy et al., 2000). Although there is no clear relationship between the effect of obesity and OA, fat is considered a metabolically active tissue that promotes inflammation (Greenberg and Obin, 2006). There is growing evidence that mediators released from adipose tissue, including interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), leptin, visfatin, adiponectin, adipsin, and resistin, may play important roles in the pathogenesis of OA (Frye et al., 2016). Elevated adipsin and leptin levels are associated with OA progression in humans (Martel-Pelletier et al., 2016). It has also been suggested that leptin may play a role in the development of cruciate ligament rupture in dogs by altering the fiber ligament and collagenase activity (Comerford et al., 2005).

Since surgery is a relatively invasive procedure that presents its own risks for each patient, it is very reasonable to consider the advantages and disadvantages when discussing the choice of surgical technique. No current stabilization technique will result in a return to normal limb function, but each technique has been shown to improve lameness and patient comfort over a long period of time and result in high customer satisfaction. No current technique completely interrupts the evolution of osteoarthritis (Voss K et al., 2008; Ballagas AJ et al., 2004; Gordon-Evans WJ et al., 2013). In our study, no complications were reported after the surgery, none of the patients required another surgery.

The current recommendation for the prevention of postoperative meniscal disease is to improve the detection of pre-existing lesions at the time of the onset of ligament rupture. This is best achieved by arthroscopy (Plesman et al., 2013; Ritzo et al., 2014).

Reports on the improvement of clinical signs in surgical remediation of cruciate ligament rupture vary between 85 and 95% regardless of the surgical technique used (Moore and Read, 1995). The use of an artificial nylon ligament is the most commonly used method in small animals, and it was used in restoring the stability of the knee joint of the 10 dogs involved in this study. This can't be done completely after using the extracapsular technique, the purpose of the surgery being to reduce the clinical signs. Despite many studies showing that conservative management

remains a viable option for some patients, as long as proper care is taken in selecting cases (patients that are not limping, patiens with a partial CrCL rupture), we considered that surgery provided the best results for most patients included in this study. There will be owners who, for different reasons, will choose conservative treatment over surgical intervention. We exposed all the the pro an cons for surgery/conservative treatment and all the owners decided that surgery treatment is the best option for their animals. However, not all surgical procedures offer an equivalent level of recovery. Currently, the best available evidence provides strong support for Tibial Plateau Leveling Osteotomy (TPLO), which allows dogs to regain normal clinical limb function (Au KK et al., 2010; MacDonald et al., 2013; Berger et al., 2015). It is necessary to compare the technique chosen by us (extracapsular method - ECR) with other methods, such as TPLO, to assess the speed of recovery, which is important for both owners and animals, the two methods being the most used in toy breeds. ECR stabilizes the joint by a circumfabelar-tibial suture that mimics the function of the cranial cruciate ligament (CrCL) and tries to prevent cranial thrust of the tibia, and TPLO ensures the functional (dynamic) stability of the knee joint by eliminating cranial thrust of the tibia rather than restoring the function of the cranial cruciate ligament (Slcoum B et al., 1993). Many studies suggest a faster return to normal function of the affected hind limb after stabilization by TPLO than other surgery procedures of stabilizing the knee joint after ligament rupture. A short period of time after surgery, without strictly following an established rehabilitation program, dogs that have undergone stabilization by TPLO get a faster return to normal joint function within a year than those that have undergone stabilization by ECR. Previous studies that normally used gait to assess the long-term outcome of TPLO and ECR did not find a significant difference between them, therefore differences in the functioning of the affected limb and mild lameness can be omitted (Au KK et al. , 2010; Conzemius MG et al., 2005). Since mild to moderate lameness during more alert gait cannot be a quality factor in the life of sedentary dogs, there is a substantial impact on active and athletic dogs (Samantha Nelson et al., 2016). Usually animals that are subjected to the TPLO technique are younger than those with extracapsular stabilization. The reason for this difference may be that young dog owners may choose TPLO for financial reasons, older dog owners may be less inclined to invest in TPLO which is more expensive and time consuming in the operating room, so TPLO may be an appropriate recommendation to return to normal function as soon as possible. None of these procedures repair or reconstruct ligament rupture, and each procedure is associated with a relatively high risk of subsequent meniscal injury and OA (Slocum and Slocum, 1993; Rayward et al. 2004; Lazar et al., 2005; Morgan et al., 2010). A successfully restored function of the ligament could provide dynamic and passive stability while maintaining proprioception and avoid some of these long-term complications. However, any attempt to stimulate CrCL healing begins with the question of why the ligament fails to heal in the first place. This inability to heal is even more surprising, given that collateral ligaments heal spontaneously with minimal treatment (Frank et al., 1983a; Frank et al., 1983b; Hannafin et al., 1999). Cruciate and collateral ligament cells have similar capabilities in terms of proliferation, migration, and biosynthesis (Murray et al., 1999; Murray et al., 2000a; Murray et al., 2000b; Murray et al., 2002; Murray et al., 2007a). However, it has been found that injuries to the cruciate ligaments, unlike injuries to the collateral ligaments, do not form clots between the torn ends of the tissue; consequently, there is no reason for cell migration and tissue repair (Murray et al., 2000a). This inability to form a clot can be attributed to high intra-articular levels of plasminogen after joint injury (Brommer et al., 1992; Rosc et al., 2002). Despite the high incidence of infection and antimicrobial resistance, joint infections generally have a favorable prognosis if treated properly. The resolution of the infection was obtained in 95% of cases, and the average resolution of the lameness can be observed on day 38 (interval 15-45 days) after the initiation of treatment (Fitzpatrick et al., 2010; Savicky et al., 2013). In many cases, treatment requires prolonged

medication and leads to additional client visits, additional days of hospitalization, or other surgeries, but not all clients are willing to return with the animal (in this study six owners did not return for physical re-examination with the patients), probably not following diet or exercise restrictions and strict administration of the drugs, therefore this study is an indicative and limited one from this point of view. The frequency of injury to the knee joint requires efforts to increase our knowledge of the stifle joint. With the advent of advanced investigative methods, such as ultrasound, computed tomography, and nuclear magnetic resonance imaging, knowledge of the normal anatomy of the knee joint is essential for the correct assessment and definition of the lesion (Reed et al., 1995; Baird et al., 1998; Kramer et al., 1999). As we understand the structural and functional components of the joint, the potential of the equipment increases and procedures that will provide advanced and long-lasting treatments for complicated joint injuries. Beyond the loss of function caused by a torn cranial cruciate ligament, a major concern is the accelerated onset of osteoarthritis. An understanding of this complex issue is to define the events that take place after the initial trauma. It has been observed that immediate injection of triamcinolone after CrCL rupture reduces the amount of synovial fluid and secondary degradation of collagen (Sieker et al. 2016). Given the emerging role of joint inflammation in the pathogenesis of cruciate ligament rupture, a combination of medical and surgical treatment will likely be necessary for a result as quickly and effectively as possible. Genetic research suggests that rupture of the cruciate ligament is a complex polygenic trait (Innes JF, 2007). Dogs with such pathology are 50% more likely to have the other ligament ruptured at 12 months after the initial diagnosis (Doverspike et al., 1993; Moore and Read, 1995; de Bruin et al., 2007 a,b; Cabrera et al., 2008; Buote et al., 2009).

CONCLUSIONS

Although the lameness score decreased postoperative at both intervals studied, a slight degree of lameness persisted in the last interview with the owners.

At one month and three months postoperative, the thigh muscles developed significantly.

Although the evolution of the patients was adequate, without intra- and postoperative complications, the extracapsular method does not completely restore, at 3 months, the function of the limb.

The limits of the study are represented by the small population being observed.

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A COMPARATIVE STUDY OF CONVENTIONAL AND ARTISANAL SALAMI BASED ON THEIR PHYSICO-CHEMICAL, AND HISTOLOGICAL CHARACTERISTIC

Isabela Voichița BULAI, Raluca Aniela IRIMIA, Mara GEORGESCU, Dana TAPALOAGA

University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, 105 Spl. Independentei, District 5, Bucharest, Romania

Corresponding author email: isabelaisaconi@gmail.com

Abstract

Most conventionally produced salamis are characterized by typical characteristics of preserved meat, including colour, flavour, and significant concentrations of residual nitrates. This paper compares the physical, chemical, and histological characteristics of some salamis obtained conventionally and artisanally in Romania. Based on the results of the physicochemical evaluation, the raw-dried, boiled, and smoked products salami that was analysed fall within the requirements of Order 560/2006, but there are significant differences between samples taken from different producers. The integrity parameters of premium products correlate with significant fluctuations in quality. It is important to note that since these are commercial products, they do not have a manufacturing and storage history. Despite this, our study suggests that: there is a large variation between traditionally and artisanal processed salamis. Compared to commercially produced products that were nitrite-treated conventionally, the amount of residual nitrite in most artisanal products was lower at sampling time thus, nitrites are introduced into most of these products indirectly, through components of other ingredients. According to the results of the histological examination, abundant blood vessels, connective tissue, adipose tissue, and nerve fibres were present. It has been stated that these occasional findings are inevitable as a result of the industrial processes."

Key words*: salami, quality, integrity, microscopic structure, artisanal.*

INTRODUCTION

It has become common to use the term "artisanal food," but the concept has many aspects, and there are no definitions that precisely describe it. Almli et al., 2011. By referencing the geographical origin or traditional production methods of products, EU legislation (EC, 2006 b; EC, 2012) identifies "traditional" foods. Additionally, the Euro FIR FP6 Network of Excellence has provided a definition of traditional foods, which includes statements about traditional ingredients, traditional composition, and traditional manufacturing and/or processing methods (Almli et al., 2011; Guerrero et al., 2009).

Essentially, salami is a ground meat product made from fat and meat that is sold raw or unheated (Dong-Gyun et al., 2020). The quality and manufacturing methods of these products differ. The purpose of this paper is to compare the physico-chemical and histological characteristics of some salamis obtained conventionally and artisanal in Romania. In producing salami, there are several internal product characteristics that affect its color, taste, aroma, and texture. These indicators include water activity (Aw), pH value, protein, presence of nitrite and nitrates, and weight loss during fermentation (Feiner, 2006). In Europe, Italy produces the most traditional products and foods with a protected designation of origin (PDO) or a protected geographical indication (PGI), followed by France, Spain, Portugal, and Greece (Rocato et al., 2017). For Romanians, the most famous salami assortments from the raw-dried category are Banatean salami, Haţeg salami, and Sibiu salami (Mencinicopschi, 2006). Regarding the increasingly intensive and industrialized nature of traditional meat production and more generally food production (McEachern and Willock, 2004; Abrams et al., 2010). Consumers are concerned about the health, environmental, and social impacts of this trend, as well as the widespread use of "Without" labels (McEachern and Willock, 2004; Schleenbecker and Hamm, 2013). "Without antibiotics," "without chemical

additives," "without preservatives", and other similar claims can mislead consumers into believing that conventional products pose health and safety risks (Abrams et al., 2010). Investigation of the production process revealed that the drying-maturing process used in the production of traditionally fermented sausages varies widely among manufacturers. As a result, several authors have previously reported a wide range of pH and aw (water activity) values during the different stages of the production process of artisanal salami and traditional soppressata (Chevallier et al., 2006; Gounadaki et al., 2008; Lebert et al., 2007). There is a lack of studies in the bibliography related to the processes of product extraction in the artisanal system, and a lack of knowledge and expertise has been identified as one of the major shortcomings of the system (Zanardi E. et al., 2010). The small number of processing and transaction stages, as well as the employees involved, allow for effective communication and control (Verraes et al., 2015). A cohort study in the Veneto region of Italy investigated the production of artisanal salami and the microbiological hazards associated with these products, providing an additional useful tool for monitoring the artisanal production process and managing microbiological hazards (Rocato et al., 2017).

MATERIALS AND METHODS

Physico-chemical and histological analyses were conducted on 24 samples from the commercial chain during the validity period. These samples came from the following categories: boiled and smoked meat products (n = 12), and raw-dried meat products (n = 12). We measured the moisture, protein, lipids, nitrite content, salt (NaCl), and freshness analysis TVBN (total volatile basic nitrogen) in accordance with the standard SR ISO of the International Organization for Standardization, and the Association of Official Analytical Chemists (AOAC). The determination of moisture was carried out by drying in the drying-stove method and expressed as a percentage, according to and SR ISO 1442:2010. The nitrites content of foodstuffs is expressed in mg per 1kg product (ppm) and is regulated by - ISO 2918:1975 (last revised in 2018), using a spectrophotometer at 538 nm wavelength. The nitrate and nitrite content of meat products after the enzyme reduction of nitrates in nitrites, providing for a maximum value of 150 mg/kg for meat products. The Kjeldahl Method (AOAC, 2000 Method 928.08); and SR EN ISO 937:2007 was used to determine the raw protein content (CP=crude protein) and the extraction Soxhlet method and SR ISO 1443:2008 was used to determine the percentage of fat (ether extract) (AOAC,2000, Method 960.39). The determination of the sodium chloride content complied with the specification of SR ISO 1841-1: 2000 Part 1: Volhard method. The freshness was assessed by determining the total volatile basic nitrogen according to SR 9065-7:2007.

Statistical data analysis

The data obtained were statistically processed using MedCalc Statistical Software version 19.1.5 (MedCalc Software bv, Ostend, Belgium; https://www.medcalc.org; 2020). The statistical
tests used were: two-tailed t-test for an independent sample, one-tailed t-test for one sample, and the Wilcoxon nonparametric test for situations where the data were not normally distributed. The Shapiro-Wilk test was used to check data normality. The significance threshold is p = 0.05. The statistical indicators used in this study are the arithmetic mean, the median, the standard deviation (SD) of the mean, the standard error of the mean, the relative standard deviation, the minimum value, and the maximum value of the data set.

RESULTS AND DISCUSSIONS

a) Physico-chemical analyses

Dried raw meat products category

Statistical analysis of salamis in the category of raw dried products shows that the average value obtained for moisture content ranges from 27.62 to 31.92%, with the average moisture value of 35.02% being statistically significant (P = 0.0002; Table 1) with a probability of 95% above the value prescribed by the 2006 regulation MADR 560. The highest humidity was highlighted for sample B 24 (35.02%). These values are lower than those reported in the literature for similar meat

products (Zanardi, 2010; Van Schalkwyk et al., 2011; Demeyer et al., 2007; Ockerman et al., 2007; Conte A. et al., 2012;). We assume that the mean value of the salami sample in the category of raw dried products exceeds the maximum legal value (35%) for the parameter moisture. Determination of total fat content is performed only if limits for this indicator are included in quality standards or technical norms. If this is not the case, the free fat substances are determined. The results of the fat content gave values between 32.09 and 42.51%, with a probability of 95%. Statistical analysis showed that the average value of 37.30% is significant in comparison (P = 0.0005; Table 1) with the value prescribed by the standard. All samples are within the maximum values (50%) for this category of meat products. The obtained results indicate a higher lipid content than those obtained by other authors, such as Dobrinas S. et al. (2013), who reported values for lipid content ranging from 18.5 to 31.1% for raw dried pork and sheep meat products. The nitrite content obtained for the samples studied ranged from 0.24 to 1.12 mg/100 g, with an average value of nitrite content of 0.68 mg/100 g, significantly (P = 0.0005; Table 1) higher than the value required by Regulation MADR 560/2006. The values obtained are almost identical to those of other authors (Păduraru et al., 2010), who report values between 1-6 mg/kg, Dobrinas et al. (2013), and Isaconi et al., 2018) report 0.79-0,38 mg/kg (Isaconi et al., 2018). The value obtained for sodium chloride is reasonable, provided that the maximum value of 6% provided for in the legislation. All samples fell within the legal requirements and had an average content of 4.82%, with a confidence interval of 95% between 3.82% and 4.74%, with the average value of 4.82% statistically significant (P < 0.0001; Table 1) above the value prescribed by the standard for salami samples from the category of raw dried products. The values for sodium chloride content reported in the literature for meat preparations of the same category are similar, ranging from 3.8 to 5.5% (Zanardi, 2010; Van Schalkwyk et al., 2011; Demeyer et al., 2007) for samples of pork and sheep products.

The freshness of the samples, evaluated by the determination of TVBN, was adequate, not exceeding the maximum value of 200 mg NH3/100 g. The values obtained ranged from 46.36 to 49.90 mgNH3/100 g, with an average of 73.99%, the data being statistically significant (P < 0.0001; Table 1). A significant difference was observed in the amount of TVBN in samples B15 (34.00 mg NH3/100 g) and B18 (129.48 mg NH3/100 g). Another study (Jude et al., 2011) reported a similar variation in values for similar products, ranging between 63.59 and 176.3 mg NH3/100 g. For pork and sheep products, Dobrinas et al. (2013) reported lower than average values for the easy-to-hydrolyze nitrogen, which ranged from 17.3-32.03 mg NH3/100 g, with an average of 26.76 5.71 mg NH3/100 g.

Smoked and boiled meat products category

It was determined that the mean moisture content of boiled and smoked salami products was between 51.88% and 60.41% with a 95% probability based on the sample (n = 12). Through statistical tests, it was found that the mean value of 56.14 of the sample (P = 0.0004; Table 1) was significantly different from the value specified in the MADR 560 regulation (max. 66%). After examining the 12 samples purchased in artisanal shops, we can conclude that the boiled and smoked salami products do not exceed the maximum value (66%) provided by the legislation. In the analysis of the smoked and boiled products, we find that the average value of 15.27% obtained for the protein is statistically significant (P = 0.0005; Table 1) above the value required by legislation (min. 11%), with a confidence interval of 12.65 - 17.89%. We consider that the mean value of the salami sample in the category of boiled and smoked products is above the minimum value prescribed by the standard.

Compared with the maximum value prescribed by the regulation (30%), the determined average fat content of salami in the category of cooked and smoked products is 19.87, with a 95% confidence interval between 15.36 and 24.58%. The determined fat content value exceeds the maximum value by a significant amount (P = 0.0151; Table 1). We consider that the mean value of fat content is appropriate for the category of smoked and cooked products.

The nitrite content of the tested samples was between 1.20 and 2.52 mg/100 g with a 95%

probability. The average value was 1.86 mg/ 100 g based on statistical analysis. Table 1 shows a significant difference (P = 0.0001) from the value prescribed by MADR 560. In our view, the salami samples from the category of smoked and cooked products are significantly lower than the maximum values (max 150 mg/100 g) prescribed for this category.

The mean value obtained for TVBN (36.73 mg/ 100 g with a confidence interval between 34.81 mg/100 g and 38.64 mg/100 g) is statistically significant (P = 0.0018; Table 1). It can be said that the average value of 36.73 mg/ 100 g of salted and double smoked salami products corresponds to the conditions in which the expected value is maxed at 45 mg/100 g).

As for the sodium chloride content, a maximum of 3% NaCl is allowed for smoked and boiled products. All samples fell below the legal requirements. With an average content of 2.35% and a confidence interval between 2.00-2.59%, the average value of salt content is significantly (P = 0.0018; Table 1) higher than the value prescribed by the standard.

Table 1. Descriptive statistics for moisture, protein, fat, salt, nitrites concentrations, protein ratio and TBVN for the salami type products

Type of samples examined	Type of analysis	Order MADR 560/2006	Confidence interval 95%	Min (%)	Max (%)	Mean ± SE	Value P. (probability) *
Salami from the category of raw dried products n = 12	Moisture	**Max 35**	27,62-31,92	25,66	35,02	29,77±0,97	P = 0,0002
	Protein content	**Min 16**	20,81-25,79	14,56	25,83	22,90±0,97	P = 0,0010
	Fat	**Max 50**	32,09-42,51	12,86	46,66	37,30±2,36	P=0,0005
	Nitrite	**Max 150**	0,24-1,12	0,27	2,85	0,68±0,20	P = 0,0005
	TVBN	**Max 200**	46,36-49,90	34,00	129,48	73,99±9,58	P < 0,0001
	Salt	**Max 6**	3,82-4,74	3,02	5,35	4,82±0,20	P < 0,0001
Salami from the category of boiled and smoked products n = 12	Moisture	**Max 66**	51,88-60,41	47,00	65,21	56,14 ±1,93	P = 0,0004
	Protein content	**Min 11**	12,65-17,89	11,08	24,43	15,27 ±1,19	P = 0,0005
	Fat	**Max 30**	15,36-24,58	9,18	29,83	19,97 ±2,00	P = 0,0151
	Nitrite	**Max 150**	1,20-2,52	0,42	3,15	1,86 ±0,29	P < 0,0001
	TVBN	**Max 45**	34,81-38,64	33,00	42,30	36,73 ±0,86	P < 0,0001
	Salt	**Max 3**	2,00-2,59	1,5	3,5	2,35 ±0,49	P = 0,0018

* The mean of the population from which the sample with value imposed by order 560/2006 was compared. The bilateral t-test was performed for each of the two types of products separately

b) Histological analyses

Microscopically, in some sections of the raw dried products (salamis), various tissues were highlighted: striated muscle tissue, various types of connective tissue in abundance, fatty tissue, vascular structures, and nerve filaments (Figure 1). In the sections of the raw dried products stained with the conventional technique hematoxylin-eosin (reds), the homogeneous appearance of muscle fibers and their non-uniform distance from the endomysium can also be observed, the appearance is related to the process of dehydration after treatment with salt. The cell morphology is moderately preserved in the center, while the periphery of the product is completely distorted (Figure 2).

In sections of boiled and smoked products (Figure 3), most muscle fibers are homogeneous, have a broken sarcolemma, sometimes with lysed nuclei. Occasionally, streaks are seen in the sarcoplasm. Adipocytes retain their cellular outlines, with most of them lacking nuclei. Plant fragments of spicules and evenly distributed basophilic amorphous masses are observed throughout the thickness of the product. Fiber integrity is moderately preserved. Microscopically, no parasitic, fungal, or bacterial elements were detected in any product category. The products of the meat processing industry do not consist exclusively of materials of animal origin. Simple microscopic observation using conventional H&E (hematoxylin - eosin) staining easily identifies components of plant origin in their traditional form (Pospiech M. et al., 2011). Vanha and co-workers argue that the identification of constituents in meat products combined with an estimate of their actual quantity allows monitoring the quality of meat

products using the same staining (Vanha et al., 2011).

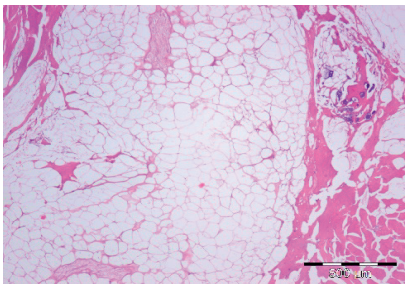

Figure 1. Dry summer salami, HE stains (Ob. 4x), striated muscle tissue, various types of connective tissue in abundance, fatty tissue, vascular structures, and nerve filaments

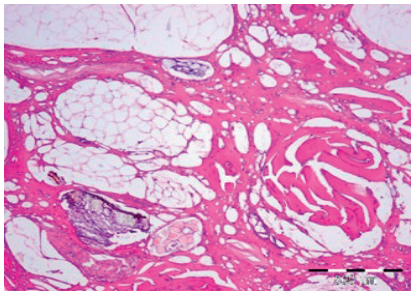

Figure 2. Banat salami - dry raw product HE stains (Ob.4x). The cell morphology is moderately preserved in the center, while the periphery of the product is completely distorted

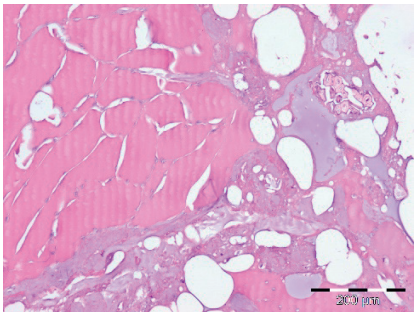

Figure 3. Rustic Salami - boiled and smoked product, HE stains (Ob. 10x) - muscle fibers are homogeneous, have a broken sarcolemma, sometimes with lysed nuclei

CONCLUSIONS

According to the physic-chemical analysis, raw, dried, boiled, and smoked products complied with Regulation 560/2006, but there were significant differences between samples taken from different producers.

Quality differences between high-quality products correlate with changes in integrity parameters.

Our study suggests the following: Artisanal and conventionally processed salamis differ significantly.

We found that residual nitrite levels were lower in artisanal products than in products made with conventional nitrite additions. Therefore, nitrites are introduced indirectly into most of these products through other ingredients. The results of the histological examination showed the presence of blood vessels, various types of connective tissue, adipose tissue, and nerve fibers in abundance. These occasional findings were described as "unavoidable in view of the technological process."

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STUDIES FOR OPTIMISING THE COST OF ANIMAL HEALTH PROGRAM FOR AVIAN INFLUENZA IN ROMANIA

Ana-Maria ZORLESCU, Teodor Stefan IONESCU, Marius CAMPEANU, Doina DANEȘ

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

Corresponding author email: zorlescu_ana@yahoo.com

Abstract

In the first half of the last century, avian influenza (AI) caused great losses in the poultry farming sector, practically all over the world, especially in Europe and it is still causes. Risk analysis, cost-benefit analysis and cost effectivennes analysis of eradication, control and monitoring program for avian influenza. Biosecurity measures in the poultry industry - applying the "all-in, all-out" policy - are able to protect for avian influenza. The first line of defense against avian influenza is the early detection of outbreaks, followed by a rapid response. This is closely linked to the communication between veterinarians, farmers and the performance of veterinary services. People who come into close contact with infected poultry, such as the families of poultry owners and workers in this sector, remain at risk. In conclusion, the risk analysis for AI identified as a major risk the location of the poultry farm in areas with a high density of migratory birds. Sensitivity analysis shows that, if more than 15% of the poultry population,are exposed to the risk of disease, the cost of the surveillance program is justified.

Key words: *Avian Influenza, risk analysis, cost-benefit analysis, cost-effectiveness analysis.*

INTRODUCTION

Through this study, we aimed an overview of avian influenza control program and his efficacy, characterise the present landscape , and predict possible scenarios, taking into account the Romanian and international status.
The main objective was to draw some suggestions and/or opinions to optimise existing AI program in Romania. Furthermore, to take a picture of all factors supporting decision-makers to improve animal welfare and, last but not least, an improvement in human well-being and health.
When the goal is to control a zoonosis, it is desirable to eradicate it as quickly as possible, in order to restore the freedom of movement of humans and/or animals.
The fact that seasonal or abnormal weather changes strongly influence many infectious diseases suggests that they will continue to be increasingly influenced by more extended climate changes (Patz et al., 2000). Climate can affect the transmission of the disease through influence on the replication and movement (and probably on the evolution) of microorganisms and their vectors.

MATERIALS AND METHODS

The materials used were the national, international and European Commission legislation in force. In addition, there are also guidelines, promoted by the awarness campains, on how the human population that may be affected by specific zoonoses should behave.
We used the data, guidelines, recommendations and/or acts, norms, regulations issued by the OIE, WHO, EFSA, EC, etc.
The European Commission has published updated rules on biosecurity and risk mitigation measures, unanimously supported by Member States (MS).
These biosecurity measures shall comply with the EFSAs` recommendations and shall, basicaly, aim to prevent the contact with carrier animals and to implement the early detection systems. Furthermore, the implementation of these measures by the poultry industry and the traditional backyard farming is critical to prevent future outbreaks of zoonotic diseases.
The Directive 2005/94/E.C. on avian influenza (AI) is based on the experiences of MS in controlling major disease outbreaks and considers the latest scientific knowledge on

avian influenza to be able to meet the challenges facing Europe today. Its primary objectivs are a better prevention and a better control of outbreaks. Food, contaminated equipment and manure must be destroyed or treated to inactivate the virus.
According to the EU legal provisions, all Member States implemented contingency plans for AI (approved by Commission Decision 2007/24/E.C.), the most appropriate measures being implemented immediately.
Preventive hygiene measures, such as cleaning and disinfection, are essential at the farm level. Disease awareness among farmers and the cooperation of all people involved in the poultry sector must ensure that the strictest biosecurity measures are applied to prevent the spread of the disease.
Each MS may decide whether it is necessary to introduce avian vaccination against avian influenza as emergency or preventive measure. However, before resorting to vaccination against avian influenza, the State Veterinary Authority must submit a detailed vaccination plan, including the appropiate surveillance measures.
Since 2003, EU Member States have had to carry out avian influenza surveillance programs to detect avian influenza virus infections belonging to the H5 and H7 subtypes in poultry, due to their potential to generate into the highly pathogenic or zoonotic form of the virus (Crawford et al, 2005).

Risk analysis of the prevention/ surveillance/monitoring program for avian influenza in Romania

As they evolve, the risk analysis of outbreaks is identifying the critical points for the early assessment and detection of fatalities, the risk factors for the introduction and spread of avian influenza virus, and is providing data for the outcome assessement of the applied biosecurity policy.
The risk of the introduction and spread of avian influenza virus remains high in breeding activites, mainly when the movement of animals, the restriction of access throughout the production cycle and/or contact with wild birds is not controled/eliminated. If poultry cannot be fenced during periods of high risk, it is recommended to prevent direct contact between wild birds and poultry by reducing the size of the outdoor area and/or using the net. In addition, food and water must be provided under a roof or horizontal fabric.
All subtypes of influenza A virus, regardless of hemagglutinin and neuraminidase, can cause infection in birds.
The risk level has been scored with 1 for the maximum probability, with 0 for uncertainty and for the highest magnitude of consequences with 5. Criteria and risk factors considered as associated with the introduction of the virus into poultry holdings include:
- direct or indirect exposure to wild birds, in particular those of the identified target species, or the existence of poultry farms near wetlands, ponds, swamps, lakes, rivers or on the shores of the sea where migratory birds may congregate on water;
- the location of the poultry holding in areas with a high density of migratory wild birds, in particular those birds which are identified as "target species" for the detection of the highly pathogenic strain H5N1 as listed in Decision 2010/367;
- the location of the poultry holding close to the resting and breeding grounds of migratory waterfowl, in particular where these areas are related to the movements of migratory birds in areas where highly pathogenic strains such as H5N1 are known to occur in wild birds;
- poultry farms reared outdoors or poultry farms where they are raised outdoors, wherever contact with wild birds cannot be effectively prevented;
- low level of biosecurity in poultry farming, including improper storage of feed and use of surface water.
At the end of 2011, according to the reports of the county sanitary veterinary and food safety directorates, in Romania, there were:
a) 269 commercial broiler farms; according to Decision 2010/367 of EC (chickens from commercial farms should not be tested);
b) 173 commercial laying hen farms;
c) 44 commercial holdings of chicken farmer;
d) 6 commercial turkey farms for fattening;
e) 2 commercial ratite farms;
f) 6 commercial pheasant farms;
g) 8 commercial quail holdings;
h) 717 risk areas ("target" localities);
i) 2 commercial palmipedes holdings.

Wild birds have a proven role in the global epidemiology of avian influenza viruses, playing a significant role in their evolution, maintenance and spread. The main wild species involved were waterfowl, seagulls and seabirds; however, the virus seems to pass easily between different species of birds.
The incidence of the infection seems to be seasonal, reaching the highest isolation rate in young birds, during the autumn. Thus, several routes of exposure of poultry to wild bird viruses have been documented or suspected to be the source of the outbreaks.
Direct exposure of poultry to wild birds is the most likely transmission event.
The identification of an avian influenza strain shall be performed utilising laboratory tests. Subsequently, a risk assessment shall be performed using the available data. At the same time, a risk analysis can be achieved with existing data on the evolution of avian influenza strains over time and the prediction of a future situation. Therefore, the risk assessment was based on existing information and its extrapolation.
The changes that may occur from a previous preliminary risk assessment for an on-site evaluation (in the outbreak) are quite diverse, with an increased likelihood of spreading a strain of avian influenza in the affected areas: from one infected farm to another. This is increasing the likelihood of an avian influenza strain spreading from an area known to be affected to an extent known as "moderate to high risk" through animal fairs, movements oflive poultry and through informal/illegal bird movements for this period.
There is always a delay in reporting human cases compared to the period between infection and their presentation for treatment.

What is the likelihood that AIV will spread from an infected farm to an uninfected farm in the affected areas?
The increase in poultry marketing activities has been associated with an increased risk of infection with the H5N1 avian influenza virus (AI) in humans and poultry (Soares Magalhaes et al., 2012). However, the presence of the avian influenza strain in poultry with subclinical evolution remains unnoticed during the period in which they are present along the market chain will not interrupt commercial activities unless effective surveillance of healthy birds and mitigation measures are taken whenever the virus is detected (Therestrial code - OIE).
Many farms/households are involved in integrated production systems for broilers, where the movement of chickens, humans and feed is the most likely source of infection between farms. Chickens for sale at animal fairs for human consumption are a significant source of infection for humans but are less likely to spread between farms. At the same time, however, there is a threaten of the virus spreading on farms through transport vehicles whenever biosecurity measures are not applied.
There is an increased likelihood of the bird flu strain spreading during the winter season on an infected farm.

What is the probability that AIV will spread from a known affected area to a "moderate to high risk" area?
As the highest probability of spreading AIV to poultry is associated with their non controled movement, the areas of moderate to high risk are those where there is an increase in poultry consumption and occasional poultry trade movements. More reliable surveillance data are needed to proper assess the potential increase in the emergence of avian influenza during the holiday season when meat consumption is increased.
For some "moderate to high risk" areas, live birds subject to movement must be submited to serological tests at the farm of origin and border crossings if the situation so requires. In this way, it must be further guaranteed that poultry farms were not infected with the avian influenza strain at least a few weeks before their dispatch. This should reduce the likelihood of the virus spreading. In addition, the closure of markets may result in the emergence of traders looking for alternative destinations for poultry, which would lead to increased poultry traffic and thus to the uncontrolled spread of the avian influenza virus.

What is the probability that AIV will spread from an area known to be affected to a low-risk area?
Given that, by definition, an uninfected low-risk country or area does not trade directly with affected countries or regions, the likelihood of

the avian influenza strain spreading from a known affected area to a "low-risk" area through trade it is considered unchanged. The possibility of spreading the bird flu virus, implicitly through trade, depends on the regulatory frameworks applied in low-risk counties and the level of illegal trade.

What is the likelihood of a human being infected with AIV from a potentially infected bird in the affected areas?

As poultry marketing activities may be associated with an increased risk of AIV infection in humans and poultry. This could be mitigated be mitigated by the awareness campains about. The risk is also associated to the traditional slaughter practices, leading to closer contact with live birds. In addition, poultry infection may go unnoticed without clinical signs.

Increased attention can be paid to improve biosecurity in animal fairs, especially in areas that have been previously affected. However, biosecurity and prevention measures appear to be variable and, in some cases, very limited. In addition, the effects of the temporary closure of markets in areas where the virus has been confirmed, and in particular the measures to be taken in the markets, may reduce the number of new cases.

Measures to reduce the risk of spreading the avian influenza virus from an infected farm/unit to an uninfected farm/unit in the affected areas and in an area known as a "moderate to high risk" area, are biosecurity measures for animal fairs, from the poultry sources to the market and that there are adequate surveillance systems in place.

The devastating economic consequences of influenza outbreaks appear both for the poultry industry and the national economy and for the society. Job losses can be significant; to control outbreaks, healthy birds often need to be slaughtered; the presence of highly pathogenic strains restricts international trade in live birds and poultry; public opinion can be affected, reducing both travel and tourism in the affected areas, reducing the consumption of poultry meat.

People in close contact with infected birds are at risk of acquiring bird flu as there is a potential nature of bird flu to infect the human population. Although many human cases are limited to conjunctivitis or a mild respiratory illness, some strains tend to cause serious illness. However, there is no evidence that the consumption of poultry meat or eggs could transmit the bird flu virus to humans. Therefore, as a precautionary and regulatory measure, birds that have been slaughtered as a measure to control an outbreak of avian influenza are excluded from the human and animal food chain.

Critical public health messages for the public in the affected areas are aimed at disrupting the epidemiological chain through warnings such as:

- "avoid contact with chickens, ducks or other birds, if not necessary";
- "prevent children from coming in contact with poultry and their waste or feathers";
- "poultry are not pets";
- "wash your hands with soap and water after coming in contact with poultry or their droppings in the affected areas";
- "clean the equipment - overalls, gown, shoes - outside the house";
- "seek for medical care if you are unwell."

Important public health messages for professionals and people handling sick birds or farm decontamination must be endorsed, as:

- Get vaccinated with the flu vaccine to avoid the simultaneous infection of the human flu virus and bird flu and to minimise the possibility of reassignment of the virus genes to people at specific risk of inhalation of possible infected materials.
- Do not allow people at high risk for severe complications from the flu (for example, immunocompromised people over the age of 60 or with known chronic heart or lung disease) to work in high-risk areas.
- Carry out serological surveillance of workers exposed to animals and veterinarians.

Cost-benefit analysis of the prevention/ surveillance/monitoring program for avian influenza in Romania

The literature reveals some empirical and theoretical contributions to analysing the costs and benefits of controlling and preventing animal diseases in developed and developing countries. Therefore, the different ways that have been used to quantify the costs and

benefits of various disease-related control and prevention measures are critically analysed to identify an appropriate methodology for analysing the mitigation measures used to control/prevent the occurrence of highly pathogenic avian influenza.
In addition to the financial losses caused by the euthanasia/neutralization and mortality of birds, there are significant costs for measures to monitor, prevent and control AI given by zoonotic strains, such as H5N1, and for production losses, such as banning business for some time.
Indirect losses include exacerbated effects (such as price shocks and demand), trade impact, spillover effects (such as effects on tourism and the services sector), and effects in the broader society, such as job losses due to restriction of activity and staff illness. Many of these effects are related to society's reaction to the presence and risk of zoonotic AI strains (H5N1).
The cost-benefit analysis requires economic, epidemiological and demographic investigations, investigations that the Romanian veterinary services have already carried out in the implementation of the serological and virological surveillance program in the population of domestic and wild birds for the detection of avian influenza virus and at the same time complete reports as requested by the EC and the OIE regarding the financing or evolution of avian influenza in Romania.
The assessment of the political feasibility of the serological and virological surveillance program among the populations of domestic and wild birds for the identification of the IA virus was also carried out by the Romanian veterinary services and endorsed by the European institutions at the start of its co-financing project.
The assessment of the physical feasibility of the serological and virological surveillance program among the populations of domestic and wild birds for the identification of avian influenza virus was carried out at the time of submission to the EC of the application for co-financing of the program; as such, Romania, through the state institutions, has the necessary physical resources for the implementation of the program, a fact physically proven by its implementation since 2011.
Expected benefits are the elimination of zoonotic AIV from the territory of Romania - immeasurable, prevention of new cases (costs necessary to limit the spread of an outbreak and its liquidation, costs for protective equipment, etc.) - difficult to quantify due to the wild birds natural reservoir, reduction of financial impact due to the transmission of viral infection to humans (avoidance of a pandemic) - difficult to quantify;
EC is co-financing: 50% of the total annual cost of the program: € 359,275 X 50% = € 179,637.5 reimbursed annually by the EC.
We listed and quantified costs for one year generated by restrictions on the movement of animals , losses by limiting the commercial circulation of birds, by stamping-out in case of outbreaks, the necessary expenses for the repopulation, acquisition and performance of tests to determine inhibition of H5 and H7 haemagglutination (€ 42,000 X € 12 / test = € 504,000), collecting and transporting samples to the laboratory (21,000 X 0.5 € / sample = 10,500 €) , costs for virological surveillance of wild birds (€ 38,000); information materials for public awareness (€ 318,100), disinfectant materials, protective equipment, administrative costs, training, etc .(€ 200,000)
We proposed the minimum period for obtaining relevant results at five years considering the seasonal evolution of avian influenza, depending on the migration path of wild birds, the pathogenicity peculiarities of strains circulating at a given time in Romania, the early reaction of veterinary services for liquidation of affected outbreaks; as such, the results of a cost-benefit analysis over a longer or shorter period of time could alter the fidelity of the results.

Choosing and applying a discount rate:
VV = VP (1 + r) n VP = VV / (1 + r) n
r = interest or discount rate = 0%, with the benefits listed in the whole population of wild animals, domestic animals and among the human population by eliminating the costs of treatment;
n = time period (in years) = 5 years
***Selecting acceptance criteria*:**
NAV - discounted net value;
B / C - benefit-cost ratio;
IRR - internal rate of return (average yield);

VPB (Present Value Benefits); VPC (present value costs).
In year 1: VPB = € 1,709,016.88 if there were outbreaks in 10% of the entire bird population.
VPC = € 1,885,041.88
VNA = VPB-VPC = 1,709,016.88 - 1,885,041.88 = - € 176,025
VNA <0, the project is not economically feasible
B / C = VPB / VPC
B / C = 1,709,016.88 / 1,885,041.88 = 0.91
B / C <1, the project is not economically efficient
RIR = discount rate = 0%
In years 2, 3, 4 and 5:
VPB = 1,709,016.88
VPC = 1,885,041.88
VNA = VPB-VPC = 1,709,016.88 - 1,885,041.88 = - € 176,025
VNA <0, the project is not economically feasible
B / C = 1,709,016.88 / 1,885,041.88 = 0.91
B / C <1, the project is not economically efficient

Sensitivity analysis if the disease occurs only in +/- 5% of the herd

Variant: - 5%
In year 1. VPB = € 1,034,145.94
VPC = € 1,210,170.94
VNA = VPB-VPC = 1,034,145.94 - 1,210,170.94 = - € 176,025
VNA <1, the project is not economically feasible
B / C = 1,034,145.94 / 1,210,170.94 = 0.85
B / C <1 the project is not economically efficient
Variant + 5%
In year 1. VPB = € 3,396,194.23
VPC = € 2,559,912.82
VNA = VPB-VPC = 3,396,194.23 - 2,559,912.82 = € 836,281.41
VNA> 0, the project is economically feasible
B / C = 3,396,194.23 / 2,559,912.82 = 1.33
B / C> 1, the project is economically efficient

Writing and presenting the report

We assumed in performing the cost-benefit analysis:
- because some benefits are challenging to quantify, their values were considered 0;
- the 'costs' (losses) that arise due to restrictions on the movement of animals during the evolution of outbreaks of avian influenza being challenging to quantify were also considered 0;
- when there are no cases of avian influenza in Romania, they recover, and the effects cancel each other out.

In variant 1, the cost reduction benefits were applied to the risk of disease estimated at 10% of the poultry population, concluding - thus, the fact that the project is not efficient and is not economically feasible.
In variant 2, when applying the sensitivity analysis, one of the variables used was to consider that, the cost reduction benefits were applied to the risk of disease estimated at 5%. from the flock of birds, concluding that the project is not efficient and not economically feasible.
In variant 3, when applying the sensitivity analysis, one of the variables used was to consider that, the cost reduction benefits were applied to the risk of disease estimated at 15%—from the flock of birds, concluding that the project is efficient and economically feasible.
Considering the three variants presented and taking into account the fact that the evolution has explosive and zoonotic potential, as well as the seasonal manifestation , the project is feasible and efficient, significantly since the evolution of avian influenza exceeds 15% of the total bird population; even if the costs of monitoring the program are constant, they are much lower than the costs of controlling outbreaks affecting a large number of birds, such as B / C> 1 and NAV> 0.

Cost-effectiveness analysis of the avian influenza eradication, control and monitoring program

Direct costs are including activation and ongoing administrative, human and logistical resources, such as the use of enhanced personal, protective equipment, as part of alert response measures (Yock et all 2009).

Stages of cost-effectiveness analysis and its links to the decision-making process and the animal health environment (there should be a feedback loop):

a) the seasonal presence of avian influenza viruses with zoonotic potential, on the Romanian territory in wild/migratory birds and in domestic birds;

b) implementation of the program for surveillance and control of avian influenza coordinated at the central level;
c) serological and virological surveillance in domestic birds and active and passive surveillance among the population of wild birds. From the perspective of the program, only the results and costs faced by this program are taken into account, while from a social perspective, all significant results and costs are taken into account, regardless of who pays or who benefits;
d) acquisition of laboratory tests , costs of collecting, transporting and performing samples, administrative costs, costs of consumables, etc.;
f) the analysis of the estimated costs of the program and its effectiveness, in relation to the number of birds and the number of people to be protected from avian influenza as a result of early virus detection programs and biosecurity measures;
g) establishing the accuracy of the data and correcting them with fundamental values where necessary;
h) budget sizing (tailoring) for the control program of the avian influenza by obtaining all the necessary financement from the decision-makers.

Cost-effectiveness checklist:
1. Identify the problem and establish the conceptual model. What is the issue addressed? What is the purpose? Defining the expected result.
The problem addressed is the seasonal emergence of avian influenza in Romania among the population of wild/migratory birds and domestic birds.
The aim is to early identify the emergence of avian influenza in the wild birds through the active and/or passive surveillance and limit it spread toward the domestic poultry population. All this for the prevalence of zero cases of avian influenza among the human population in Romania.
2. *Establishing the analytical perspective.* What approach is used? The method to be used is established, whether only in terms of social impact or only in the actual program for the surveillance and control of avian influenza among the population of wild/migratory and domestic birds.
The average cost of the serological and virological surveillance program of the poultry population and active and/or passive surveillance of the wild bird population was about 720 thousand euros.
The costs of the program for the serological and virological surveillance of the poultry population, as well as for the active and/or passive surveillance of the wild bird population, include costs related to:
- acquisition and performing of laboratory tests (inhibition of haemagglutination, RT-PCR and virus isolation) ;
- sampling and samples transport ;
- warning campaign to the public and/or the biosecurity measures required for the population of domestic birds and even for the human population, etc.
During 2011-2017, on the Romanian territory, the avian influence evolved seasonally. For example, in 2015, between March and April, 118 dead pelicans were registered in the Danube Delta .
In 2016, in November, in the area of the of Constanța harbor, 4 dead wild birds were registered, one of which confirmed as being infected with the H5 subtype; in December 2016, in Tulcea County, the presence of an outbreak of avian influenza was confirmed, 191 birds being slaughtered from households; in January 2017, in Prahova County, 52 domestic birds were killed. In the wild bird population during 2016-2017, there were 21 cases identified in 7 counties (Constanța, Teleorman, Tulcea, Iași, Bacău, Giurgiu and Ialomița).
The target was to limit the impact of AIv present in the wild by the avian surveillance and control program in the poultry population, to avoid/reduce the number of outbreaks with the lowest burden of costs. The effectiveness of a such program is seen in the limited number of cases of avian influenza reported during 2011-2017 in Romania and the sporadic evolution of avian influenza in all MS during this period.
3. *Identify and estimate costs.* What elements need to be included and given real value for costs in conjunction with the avian influenza surveillance and control program for the poultry and wild/migratory bird population?

What are the alternate options? What is the source of the costs, and how robust are they?
The elements to be included are:
- laboratory investigations for HPAI subtypes (H5 and H7), as RT-PCR testing, virus isolation,
- sampling and samples transport
- administrative costs,
- travel and on-the-spot inspections in the event of outbreaks of avian influenza,
- training,
- neutralising materials,
- emergency slaughter equipment,
- consumables and protective equipment, etc.

The avian influenza surveillance and monitoring program in domestic and wild/migratory bird populations can be enlarged, including cases of human influenza and human-induced AI deaths.

4. *Identifying and estimating results.* What are the estimated results? How were they derived? In what time frame do they appear? How safe are the results?

This type of results can be quantified in the periods when there are no avian influenza outbreaks in the populations of domestic birds in Romania. However, the results are not 100% reliable as they depend heavily on active and passive surveillance of wild bird populations, the existence and continued funding for the implementation of the avian influenza surveillance and control program.

5. *Cost-effectiveness estimation and sensitivity analysis.* The cost-effectiveness calculation is made by relating the costs of the intervention to its efficiency. Performing a sensitivity analysis: how robust are the results? what are the key assumptions?

The cost-effectiveness ratio for the period 2016-2017 (Cost of intervention / Efficiency of intervention) is approximately 1 million euros / 21 cases of avian influenza identified in the wild birds population and 243 domestic birds slaughtered and neutralised in the Romanian outbreaks. Therefore, the cost for eradication the avian influenza outbreacks was, on average, 1 million euros.

Although at first glance, it may seem that from the mathematical approach, this ratio is zero, in fact, "21 cases of avian influenza identified among the population of wild birds and 243 domestic birds slaughtered and neutralised in the outbreak" tends to the desired goal (“a few cases of avian influenza to zero cases”) as such the cost-effectiveness ratio is maximum.

The efficiency of the intervention is represented by the decrease of the number of avian influenza outbreaks, these being reduced by more than 70%. Thus, in 2005-2006 on the Romanian territory, were notified 53 outbreacks in 5 commercial poultry farms and 122 households. Therefore, when reporting the program to the social impact, its effectiveness is significantly reduced because no avian virus infection in humans have been reported in Romania.

They calculate the program's cost-effectiveness for the eradication, control and monitoring of avian influenza for the period 2016-2017 to the social impact. As a result, it was found that approximately 1 million euros were spent to control disease in birds, plus 7.5 million euros, the cost of the human influenza vaccine .

The obtained results contain figures extracted from the official reports (Romanian Ec etc.) ; as such, they are robust data and present the current status on the Romanian territory.

Adapting sampling criteria to the circulation of HPAI strains would help retailoring the biosecurity measures and public awareness campaigns. The higher the number of samples analyzed - negative for avian influenza, the more it can be argued that the prevalence of avian influenza in Romania is lower.

The critical points identified are the monitoring of avian influenza in Romania, the improvement of the communication between veterinary services and public health services, the improvement of the the attitude of humans in order to ask doctors for the proper conduct, , through awareness of the risks related to "self-diagnosis" and "self-treatment".

6. *Feedback.* What are follow-up measures needed? How will the results be used/shared to help improve the program and further inform decision-makers?

It is necessary to continue the surveillance and control of avian influenza in Romania, with the appropriate funding by the institutions in charged, out/without of the EC co-financement. Communication through veterinary and public health channels is needed to make the human population aware of the risks related to outbreaks and on the measures that poultry

keepers can and may take to limit the emmergence of avian influenza. (applying minimum biosecurity measures, including in households).
Cost-effectiveness analysis requires multidisciplinary teams - veterinarians, disease control experts, epidemiologists, economists, etc. to measure the contributions of prevention and control interventions to the overall results of strategies and policies for eradicating, controlling and monitoring avian influenza.
By applying cost-effectiveness concepts and models, the allocation of limited resources can be improved during animal health programs and projects.

RESULTS AND DISCUSSIONS

Biosafety measures in the poultry industry - by applying the "all-in, all-out" principle - can protect this sector from AIV.
The first line of defense against bird flu is the early detection of disease outbreaks, followed by a rapid response. This is closely linked to the high degree of communication between veterinarians, animal owners and the performance of veterinary services.
Implementing warning systems and preventive measures is essential as part of an effective avian influenza prevention and control strategy. However, this approach must be combined with the preparation for eradicating a potential outbreak.
When AIV is detected in poultry flocks, the stamping-out is the requested policy to control and rapidly eradicate the disease .
Controlled disposal of infected birds, movement restrictions, improved hygiene, biosecurity, and proper surveillance lead to a significant reduction in the potential for viral contamination of the environment.
A more robust understanding of virus diversity and the trends of viral evolution could inforce biosecurity efforts in the bird and/or animal population where the virus could spread (Machalaba et al., 2015).
Early warning systems do not question whether the highly pathogenic strain of avian influenza exists in a population at a specific location and time. The limited frequency of the HPAI in wild birds and the apparent grouping of these cases present additional challenges in addressing this goal.
Although finding an infection with a highly pathogenic strain of avian influenza (H5N1) is statistically more likely in birds found dead, the absence of dead birds does not indicate the absence of the disease.
AIV risk is a real threat for traditional farms where the contact of domestic birds with the wild ones is open. The owners of subsistence farms live in direct contact with their poultry, a way of life that offers epidemiological oportunities for the transfer of AIV strains.
Traditional breeding must reconsider the growth of poultry in the open air, in order to limit the contact with wild birds and poultry in households must be kept away (isolated) from ducks, geese and wild birds, which are the natural host/reservoirs of the virus outside farms.
Many wild birds - shore and laguna birds - can become infected without developing any clinical signs. Therefore, poultry should not be in contact with these.
People who come into close contact with infected poultry, such as the families of poultry owners and workers in this sector, remain at risk.
The actions that can be taken to limit the spread of AIV are:
- sheltering poultry indoors, mainly in areas with a high density of wild birds;
- avoid keeping in the households the elements that can attract the wild birds;
- maintaining strict control over access to poultry and limiting it, to as few people as possible;
- avoiding the introduction of flocks of birds with unknown disease status;
- close monitoring and reporting of existing diseases and deaths in bird population to the veterinary services.
Globally, there has been evidence that there is no risk of human infection through the consumption of heat-processed poultry products, as this treatment inactivates the virus. Therefore, all measures to prevent and control avian influenza, followed by the supply chain, may be cancelled if improper handling of food by the producer/consumer.
The most common errors, with the potential to increase the risk of transmitting AIV, are:

- the slaughter of poultry in the household - due to preferences for "warm" or "fresh" meat or religious preferences and social/cultural practices ;
- the use of the same tools (knives, utensils and chopping boards) to process raw meat, without cleaning, and sanitation, to process raw products (vegetables);
- freshly cooked - even if there is no concrete evidence of the transmission of the avian influenza virus to humans through food consumption, this possibility cannot be ignored (AIV is inactivated above 70°C);
- improper waste management and improper disposal of hazardous waste, such as meat, skin, feathers, blood, bones, etc., outside homes and in open areas, poses potential risks not only to the uninformed consumer but also to people in the neighbourhood. Moreover, such a practice attracts other pets, such as the domestic pig, which acts as a host for the viral recombination of AIV.

CONCLUSIONS

Risk analysis is a technically sound and socially responsible way to assist industry, government and the general public.
The risk analysis carried for AI identified as significant risk factors the presence in the geographical area of Romania of susceptible wild species (wild birds); the direct or indirect exposure to wild birds and the presence of the poultry holding near wetlands, where migratory waterfowl may congregate.
The cost-benefit analysis for AI identified as non-quantifiable or difficult to quantify characteristics, the goals as: the „0" human AIV cases, the prevention of emmergency and reemergency, the the costs of losses imposed by restrictions on the movement of animals; the losses due to limiting the commercial movement of birds.
The cost of liquidating the outbreaks is challenging to quantify out of 13 elements included in the calculation.
Considering these variables, the sensitivity analysis shows that, compared to the risk of illness of more than 15% of the population, the cost of the surveillance program is justified, the B/C ratio being higher than 1.
By applying cost-effectiveness concepts and models, the allocation of limited resources can be improved during animal health programs and projects.

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HISTOLOGICAL EVALUATION OF TWO EUTHANASIA METHODS IN A TOXICITY STUDY IN LABORATORY RATS

Cristin COMAN[1, 2], Diana ANCUȚA[1, 3], Teodoru SOARE[3]

[1]"Cantacuzino" National Medico-Military Institute for Research and Development, Preclinical Testing Unit, 103 Splaiul Independentei, Bucharest, Romania
[2]"Spiru Haret" University, Faculty of Veterinary Medicine, 253 Basarabiei Road, Bucharest, Romania
[3]University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, 105 Splaiul Independentei, Bucharest, Romania

Corresponding author email: comancristin@yahoo.com

Abstract

Toxicity tests are mandatory preclinical regulatory studies for the authorization of a medicinal product. The testing protocol include a complete analysis of the possible toxic action of the tested product. The most complex and defining analysis is post-mortem histological analysis. Depending on the place of action of the tested product and to avoid the interference of euthanasia methods with the results of analyzes, different euthanasia methods may be chosen. In a study of toxicity in rats for a substance with action on the nervous system, two methods of euthanasia were chosen, namely anesthetic overdose and euthanasia by decapitation with deep sedation of animals. Histological evaluation of the main organs revealed congestion in the analyzed organs regardless of the euthanasia method used in most animals. Diffuse hemorrhage, perivascular edema and pulmonary edema have also been observed. Lesions were identified in both test and control groups, male and female. Statistical analysis showed significant differences between the two methods, euthanasia by overdose of anesthetic producing more lesions than decapitation, the latter being considered more appropriate for this type of study.

Key words*: euthanasia, anesthetic overdose, decapitation, histological evaluation.*

INTRODUCTION

For the market authorization of medicinal products and medical devices, preclinical and clinical studies are mandatory (Steinmetz & Spack, 2009). Preclinical studies refer to *in vitro* and *in vivo* on laboratory animal studies (Henderson et al., 2013). Laboratory animal studies can target biocompatibility and toxicity regardless of its type (acute, chronic toxicity, genotoxicity, cytotoxicity, etc.). Animal toxicity studies are usually performed on 2 species (rodents and non-rodents), but it is also approved to perform them on a single species, in the case of target drugs (Prior et al., 2018). In order to avoid erroneous results in toxicity studies, the elimination of all factors that may interfere with the obtained values is essential. In addition to microclimate factors (temperature, relative humidity, noise, light, etc.) and those related to the animal (gender, age, body weight, type of barrier in which they were breeding) an important influence on the results may be the study methods used, methods related to the administration of test substances and the collection of samples for analysis. In a toxicity study, all the parameters that can determine the possible toxic actions of the test substances (weight gain, food consumption, immunological, ocular, hematological and biochemical parameters) are analyzed (Vandivort & Eaton, 2014).

The most important analysis remains the histological analysis which can detect in detail the possible toxic effects of the test substance (Crismann et al., 2004).

However, histological analysis can also be affected by certain factors that need to be considered, the most important of which is how euthanasia is performed.

Euthanasia, which is the act of ending life with good methods, is strictly regulated by national legislation, there are methods on species, ages and weights, and the person performing the euthanasia must be trained for this purpose.

The Guide for the Care and Use of Laboratory Animals indicates the appropriate euthanasia method depends on many criteria, including compatibility with research objectives. It further states, "The selection of specific agents and methods for euthanasia will depend on the species involved and the objectives of the protocol. Euthanasia, as a process, separates the presentation of new variables, treatments or environmental changes to the living system from the terminal collection of tissues and blood for additional study or analysis" (National Research Council, 2011). In itself, the euthanasia method can alter physiologic parameters and responses (Close et al., 1997).

There are numerous studies that have analyzed the effects of different euthanasia methods on the value of the different parameters studied (hematological, hormonal, and histological) (Brooks et al., 1999, Schoell et al., 2009, Artwohl et al., 2006, Shomer et al., 2020). Regardless of the study, there were no euthanasia methods that did not interfere with some of the test results (Pierozan et al., 2017).

In a chronic toxicity study for a substance with an effect on the central nervous system, a study performed on rats, two methods of euthanasia, anesthetic overdose and decapitation under deep sedation were used.

The option for two euthanasia methods was justified by the exclusion in the interpretation of the results of the changes generated by the euthanasia method. The results obtained at the necropsy and histopathological examination were analyzed.

MATERIALS AND METHODS

All procedures during the study were performed in "Cantacuzino" National Medico-Military Institute for Research and Development (CI), Department of Research and Development, Preclinical Testing Unit and in the pathological anatomy laboratory of the Faculty of Veterinary Medicine. Animal studies have been approved by the CI Ethics Committee and authorized by the competent authority, in accordance with the provisions of national and European regulation on protection of animals used for scientific purposes, respectively Law 43/2004. CI is an authorized unit under current legislation as a user of animals for scientific purposes.

Wistar rats, male and female, were used for the toxicity study, with an average body weight of 280-320 g at the beginning of the study. The animals were kept in identical conditions in open cages, with wood chips as bedding. Food and water were provided *ad libitum*. The microclimate conditions were temperature 20-24^{0}C, relative humidity 45-65%.

Two groups were created, each of 40 animals, of which 20 females and 20 males, a batch inoculated with the test substance and a control batch inoculated with saline solution. The test substance which is still under clinical study, effective on disorders of the nervous system has been inoculated intramuscularly, as well as saline solution (Hemofarm, Romania) that was use as control substance. Weekly administrations were given, and the study lasted 90 days.

Rats were daily inspected and food consumption was recorded for each group once a week. Weight measurements were performed for each rat every 14 days during the entire study period. Blood collection from the retro-orbital sinus was performed on days 0, 30, and 60, under general anaesthesia, using a cocktail of Acepromazine 1% (5 mg/kg; Sedam, Farmavet, Romania) and Ketamine 10% (100 mg/kg; Vetased, Farmavet, Romania). At the end of the study, the animals were euthanized; blood and samples were taken for histological examination.

As an anesthetic we chose a combination of the injectable dissociative agent, α2-adrenergic receptor agonists and phenothiazine tranquilizer as sedative to potentiate the effects of anesthetic.

Ten animals in each groups and for each gender were euthanized with an overdose of Ketamine 10% (Vetased, Farmavet, Romania)/Xylazine 2% (Xylazin Bio, Bioveta, Czech Republic)/Acepromazine 1% (Sedam, Farmavet, Romania) "cocktail" - mix 300 mg/ml Ketamine, 50 mg/ml Xylazine, and 30 mg/ml Acepromazine in a 3:3:1 ratio and injected 1.5 - 2.1 ml/kg intraperitoneally.

Ten animals were euthanized by guillotine decapitation after sedation with 3% isoflurane (Anesteran, Rompharm, Romania). The

procedure was undertaken skillfully and rapidly by a trained operator.

After euthanasia, necropsy was performed and organs with vital functions were collected: brain, heart, lungs, kidney, and liver. The organs were paraffin embedded and stained with hematoxylin-eosin. Histological examinations were performed on 5 µm sections.

All data are shown as positive samples at the number of samples analyzed. 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 < 0.001 were considered statistically significant, and < 0.05 less significant.

RESULTS AND DISCUSSIONS

No mortality was recorded in any of the groups during the study. All animals gained weight and the clinical and blood parameters were normal. The results obtained at necropsy and histopathological examinations are highlighted in Tables 1 to 4. As there were no differences between the groups of test substance versus saline, the results were combined to better highlight the differences between the 2 euthanasia methods.

Table 1. The result of necropsy in females depending on the method of euthanasia

Euthanasia methods	Organ	Female	
		Congestion	Hemorrhage
Anesthetic overdose	Heart	9/20	0/20
	Lung	12/20	2/20
	Liver	12/20	4/20
	Kidney	7/20	4/20
	Brain	11/20	1/20
Decapitation	Heart	1/20	0/20
	Lung	4/20	0/20
	Liver	2/20	0/20
	Kidney	3/20	0/20
	Brain	1/20	0/20

Table 2. The result of necropsy in males depending on the method of euthanasia

Euthanasia methods	Organ	Males	
		Congestion	Hemorrhage
Anesthetic overdose	Heart	11/20	3/20
	Lung	13/20	2/20
	Liver	10/20	3/20
	Kidney	9/20	4/20
	Brain	12/20	1/20
Decapitation	Heart	3/20	0/20
	Lung	2/20	0/20
	Liver	2/20	0/20
	Kidney	2/20	0/20
	Brain	2/20	0/20

Table 3. The result of histological analyses in females depending on the method of euthanasia

Euthanasia methods	Organ	Females			
		Congestion	Diffuse hemorrhage	Petechiae	Edema
Anesthetic overdose	Heart	11/20	3/20	11/20	3/20
	Lung	13/20	2/20	13/20	2/20
	Liver	10/20	3/20	10/20	3/20
	Kidney	9/20	4/20	9/20	4/20
	Brain	12/20	1/20	12/20	1/20
Decapitation	Heart	3/20	0/20	3/20	0/20
	Lung	2/20	0/20	2/20	0/20
	Liver	2/20	0/20	2/20	0/20
	Kidney	2/20	0/20	2/20	0/20
	Brain	2/20	0/20	2/20	0/20

Table 4. The result of histological analyses in females depending on the method of euthanasia

Euthanasia methods	Organ	Males			
		Congestion	Diffuse hemorrhage	pointed hemorrhage	Edema
Anesthetic overdose	Heart	15/20	4/20	3/20	0/20
	Lung	16/20	4/20	2/20	3/20
	Liver	13/20	5/20	1/20	0/20
	Kidney	11/20	6/20	1/20	1/20
	Brain	15/20	2/20	3/20	1/20
Decapitation	Heart	4/20	0/20	0/20	0/20
	Lung	6/20	1/20	0/20	0/20
	Liver	6/20	1/20	0/20	1/20
	Kidney	5/20	2/20	1/20	0/20
	Brain	4/20	0/20	0/20	0/20

The analysis of the results highlights differences between the two euthanasia methods. Congestion and hemorrhages were observed on the necropsy examination. The differences between the two methods of euthanasia were significant regardless of organ in congestion ($t < 0.001$), and less significant ($t < 0.05$) in the case of hemorrhagic lesions, being more obvious and more numerous in groups euthanized with anesthetic overdose. Regarding the results from the histopathological examinations, there were congestive lesions, diffuse hemorrhages, edema and pointed hemorrhages lesions being the consequence of the action of euthanasia methods. The differences between the method of euthanasia by an overdose of anesthetic were more numerous and more obvious on histopathological examination than by the method by decapitation. Regardless of the organ, the differences were significant ($t < 0.001$) in congestive lesions and less significant in diffuse hemorrhage lesions, even insignificant in the case of edema and pointed hemorrhage. The analysis of the results between the genders did not show significant differences.

The definition of euthanasia is a good death, and if we are trying to provide a good death for an animal, we should do that at all costs (Person et al, 2020). The two methods of euthanasia have different actions, one being of chemical origin (anesthetic overdose) and one physical (decapitation).

Ketamine is a short acting anesthetic agent being widely used, but has emerged as an abused drug in recent years. Ketamine is a dissociative anesthetic developed in 1963 to replace phencyclidine and is being currently used for human anesthesia and veterinary medicine (Dinis-Oliveira, 2017). Ketamine is not acceptable for euthanasia when used alone but can be humane when used in conjunction with sedatives and tranquilizers. However, it is not very efficient as it requires very high doses. Xylazine hydrochloride is a thiazine derivative that acts by activation of central presynaptic α2 receptors, producing sedation and muscle relaxation. As an anesthetic, it is typically used in conjunction with ketamine.

Acepromazine is a phenothiazine tranquilizer that blocks dopamine receptors in the CNS and depresses the reticular-activating system, resulting in sedation. Acepromazine also blocks alpha-adrenergic receptors. Acepromazine is a sedative that potentiates the effects of other anesthetic agents. The combination of the 3 substances works very well for anesthesia, and in overdose, as we used it, it quickly induces euthanasia in animals.

Physical methods of euthanasia have a high potential for being inhumane and are only acceptable when scientifically necessary and must be performed by carefully trained personnel. Physical methods are acceptable for fully sedated animals (Kongara et al., 2014). It is very important that we make sure that the animal is dead as a result of the euthanasia action. Very deeply anesthetized animals may appear dead; yet, they may recover from the anesthesia at a later time. Rapid euthanasia of laboratory rodents without the use of anesthesia is a necessary research technique whenever there is the likelihood of anesthesia or stress

interfering with the chemistry of the tissues under investigation. Decapitation has long been the procedure of choice under such circumstances (Holson, 1992). Recently, the American Veterinary Medical Association (AVMA) panel on euthanasia recommended that decapitation to be avoided on the grounds that the decapitated head may be conscious and suffering for as much as 15 seconds (AVMA, 2020).

The histopathologic changes caused by various methods of euthanasia were studied in rats, mice, guinea pigs, and rabbits. Lesions resulting from particular methods of euthanasia were consistent from species to species. Each method studied affected the organs to some degree, ranging from mild congestion to edema and alteration of vascular permeability. Euthanasia of experimental animals by overexposure to CO_2, or intraperitoneal injection of concentrated sodium pentobarbital seemed most suitable for pulmonary studies. Decapitation (mice, rats, guinea pigs), cervical dislocation (mice), CO_2, and intracardial injection of sodium pentobarbital were more suitable for examination of abdominal viscera (Feldman & Gupta, 1976).

However, it is clear that the applicability of these euthanasia methods may change with the model of study, experimental treatments and other factors.

Consequently, euthanasia should be assigned cautiously and preferably after preliminary studies to prevent aberrant research results. Equipped with the basic principles of euthanasia, investigators can make informed decisions that meet current standards of animal care while still achieving the scientific goals of their research studies.

CONCLUSIONS

The choice of euthanasia methods to generate as few side effects as possible is important in preclinical toxicity studies. The methods chosen in the presented study, respectively overdose of anesthetic and decapitation with deep sedation generated gross and histopathological changes such as congestion and hemorrhage in most organs examined. They were significantly more common with an anesthetic overdose. The method of decapitation with prior sedation can be considered an acceptable method of euthanasia from this point of view.

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THE RAT AS AN ANIMAL MODEL FOR THE EVALUATION OF THE CUTANEOUS WOUND HEALING

Fabiola IONIȚĂ[1, 2], Cristin COMAN[1, 3, 4], Mario CODREANU[2]

[1]Cantacuzino National Medico-Military Institute for Research and Development, Bucharest Romania
[2]University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, 105 Splaiul Independenței, Bucharest, Romania
[3]Fundeni Clinical Institute, Translational Medicine Centre of Excellence, 258 Fundeni Road, Bucharest, Romania
[4]Spiru Haret University, Faculty of Veterinary Medicine, 256 Basarabia Blvd, Bucharest, Romania

Corresponding author email: comancristin@yahoo.com

Abstract

The healing of cutaneous wounds is a dynamic process including overlapping phases of inflammation, proliferation, re-epithelialization and remodeling. Proper wound healing is essential for the reestablishment of structural and functional integrity of the damaged tissue. Rodents are valuable biological tools for understanding tissue repair process and for developing effective treatment strategies, despite anatomical and physiological differences between human and animal skin. The purpose of the study is to evaluate the cutaneous wound healing assessment for an excisional wound model in rats, for further testing with innovative medical devices loaded with biological active compounds. CD-SD female rats were surgically operated to excise one full-thickness circular skin patch, 20 mm diameter, in the dorsal region. Patches applied were changed every other day and samples of wounds/scars were collected on the 7th and 14th postoperative days. Macroscopic monitoring and histopathological examination assessed the wound healing process over time. Results showed that rats provide an optimal animal model for cutaneous wound healing, as data obtained can provide valuable translational information and can contribute in optimizing treatment protocols.

Key words: *wound healing, excision model, animal model, rat.*

INTRODUCTION

Skin, the largest organ of the human body, plays a crucial role in the protection against microorganisms, vitamin D synthesis initialization, excretion, thermal regulation and detection of sensory information about the external environment (Abdallah et al., 2017; Tottoli et al., 2020; Reinke & Sorg, 2012).

A wound is a breakdown in the protective function of the skin, a disruption of cellular, anatomical, and functional continuity of the cutaneous tissue. A wound may be described by its aetiology, anatomical location, whether it is acute or chronic, by the method of closure and by the appearance of the predominant tissue types in the wound bed. It may be produced by physical, chemical, thermal, microbial, or immunological insult to the tissue (Negut et al., 2018; Thakur et al., 2011; Wilhelm et al., 2016).

Wound healing is the interaction of a complex cascade of cellular and biochemical actions leading to the restoration of structural and functional integrity with regain of strength of injured tissues (Gurtner et al., 2008). Wound healing involves multiple cell populations, the extracellular matrix and the action of soluble mediators such as growth factors and cytokines (Gonzales et al., 2016; Velnar et al., 2009). The healing process consists of a sequence of overlapping events including inflammatory responses, regeneration of the epidermis, shrinkage of the wound and finally connective tissue formation and remodeling (Alizadeh et al., 2007; Hasamnis et al., 2010).

Wound healing models have been developed over many decades in attempt to understand the tissue repair process and test new treatment protocols (Masson-Meyers et al., 2020). Although *in vitro* models have been important in underlying the mechanisms of this wound

repair process, *in vivo* models remain the most predictive models, allowing to obtain information on the multifactorial nature of the wound healing process, which may be influenced by external factors (Dorset-Martin 2004; Gottrup et al., 2000; Wong et al., 2011).
The advantage of using animal models is that the wound healing process is accelerated in animals and it is possible to study the process over days rather than longer periods of time needed in humans (Chang et al., 2019; Mogford, 2001). Currently used animal models for wound healing research are: rodents (mouse, rat), rabbit and pig.
Rats have been widely used in the study of skin wound healing by allowing the standardization of the type, size, shape, and depth of the wound injury (Dorset-Martin 2004). This particular animal species is often selected for its wide availability, tractable nature and cheapest cost in terms of housing, maintenance, and reproduction. Also, a wide variety of specific reagents are available for research purposes. Despite of their small size, rats are large enough to provide a suitable skin area for wound healing studies. (Grada et al., 2018; Masson-Meyers et al., 2020).
The purpose of the study was to evaluate the wound healing assessment for an excisional wound model in CD-SD female rats. This experiment is part of a study that will evaluate the *in vivo* healing potential of an innovative medical device loaded with biological active compounds. The following aspects were assessed during the experiment: clinical examination and general appearance, macroscopic wound monitoring and histopathological examination of samples collected at the end of the study. Blood samples were collected for hematological analysis.
Results showed that rats provide an optimal animal model for wound healing, as data obtained can provide information for a better understanding of the benefits and limitations of this model in translational applications.

MATERIALS AND METHODS

This study was carried out in compliance with the principles of ethics and 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.
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 food and the water were administered *ad libitum* during the entire experiment period The animals were kept under standard conditions, temperature 18-24°C, humidity 35-75% and in light controlled conditions (12 h/12 h light and dark cycles). During the study, the animals were housed into individual cages.
For this study, 20 CD-SD female rats, weighing 200-300 g, 12 weeks age were surgically operated to excise one full‑thickness skin patch, in the dorsal region. The animals were anesthetized by intraperitoneal injection of a cocktail of medetomidine (0.5 mg/kg; Biotur) and ketamine (75 mg/kg; Farmavet). The back of the animals was shaved and the selected area was disinfected using 70% ethanol and 3% betadine solution. The animal was placed on the lateral side and one circular full-thickness wound (20 mm in diameter) was made on the dorsum cervical region, using a sterile straight surgical scissors, a tissue forceps and a scalpel blade (no. 24). Bleeding was controlled with gauze compresses until hemostasis. Each wound was covered with an untreated textile patch (25 x 25 mm) and then covered with sterile gauze and flexible, self-adhesive bandage (Petflex). Patches were changed every other day after hydration with 0.9% saline solution (Figures 1 and 2).
Animals were randomly divided in 2 groups (n = 10 in each group), according to the moment of tissue sample collection. Half of the animals were euthanized using an anesthetic overdose on the 7th post-operative day and the other half of the animals on the 14th post-operative day.
Clinical examination of the animals was performed daily and wounds were measured using a vernier caliper (length and width). Wounds measurements, macroscopic descrip-

tion of the lesions and evaluation of the healing process were performed every 48 h. For reproducibility, the measurement of the wound area was performed by a single observer throughout the experimental time.

Figure 1- Surgical instruments used for skin patch excision and wound measurement

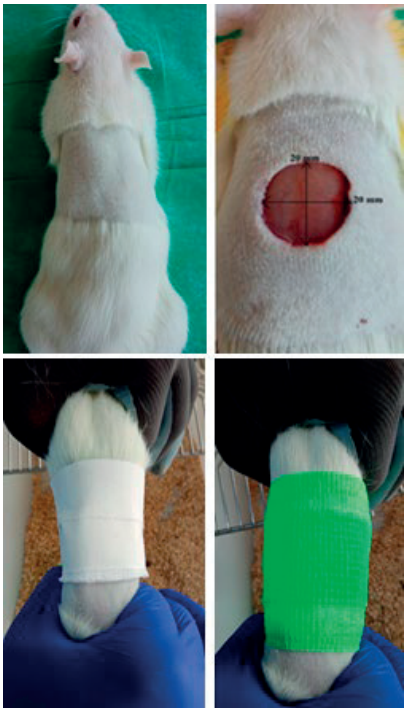

Figure 2- Surgical excision and wound coverage

At the end of the study, blood samples for hematological tests were collected from the retro-orbital sinus. For hematological tests, blood was sampled in EDTA pre-conditioned tubes and IDEXX ProCyte Dx 5 Diff analyzer was used.

Tissue specimens were obtained from the wound site by sharp dissection using the same instruments (surgical scissors and scalpel blade), on the 7th and 14 th post-operative days and histopathological examination was performed.

The full thickness wound tissues, including the adjacent skin, were fixed immediately in formalin and paraffin embedded for routine histological processing. A 4 μm section obtained from each paraffin block was stained with hematoxylin and eosin (H&E) and evaluated using a light microscope with specific image analysis software (Olympus SC 50).

RESULTS AND DISCUSSIONS

All the animals showed good general health condition throughout the study, as assessed by their weight gain, food consumption and mobility. Temporarily, some of the animals presented pruritus on the dorsal region, but without interfering with the wound healing process. No signs of intercurrences in wound healing, such as edema, erythema or suppuration in the wound area was observed during the experiment.

The average wounds area (mm^2) was calculated every 48/72 h by measuring the two dimensions (length x width). By day 7, the average wound area for group 1 had been reduced from 400.58 mm^2 to 170 mm^2, representing 42.43% from the initial excisional wound. By day 14, the average wound area for group 2 had been reduced from 400.82 mm^2 to 69.36 mm^2, representing 17.30 % from the initial wound. The measurement results are presented in Tables 1 and 2 and graphically represented in Figure 3. Macroscopic images of the wound-healing process over time are presented in Figure 4 (group1) and Figure 5 (group 2).

Table 1- Wounds area during the study for group 1 (mm^2)

Animal ID	Day 0	Day 2	Day 4	Day 7
1	400	360,99	239,94	164,64
2	403,6	384	285,65	171,2
3	400	364,7	255,76	166,44
4	400	362	269,01	195,3
5	400,8	354,9	277,24	149,03
6	400,2	355,12	251,12	219,45
7	400,6	361	264	163,8
8	400	299,28	159,6	129,36
9	400	329,42	225	142,74
10	400,6	370,54	250,56	198,12
Average	400,58	354,195	247,788	170,008
STDEV	1,04861	22,4723	33,844	26,0612

Table 2- Wounds area during the study for group 2 (mm^2)

Animal ID	Day 0	Day 2	Day 4
1	403,8	399,96	361,6
2	401,2	380,79	305,04
3	400,8	362,34	267
4	400	387,93	327,69
5	400,6	356,57	294,84
6	400,4	382	306,55
7	400,4	341,9	241,08
8	400	362,18	363,3
9	400,4	372,37	304
10	400,6	384,07	342,09
Average	400,82	373,01	311,31
STDEV	1,0486	16,377	37,054
Animal ID	**Day 7**	**Day 11**	**Day 14**
1	283,24	248,89	106,02
2	236,88	147,6	69
3	191,54	98,4	27,36
4	149,16	100	50,3
5	202,94	105,3	78,1
6	306,44	121,18	57,53
7	237,8	167,99	95,16
8	354,65	175,44	69,93
9	348,52	163,52	78,96
10	303,4	136,8	61,32
Average	231,1	146,512	69,368
STDEV	65,914	43,568	21,237

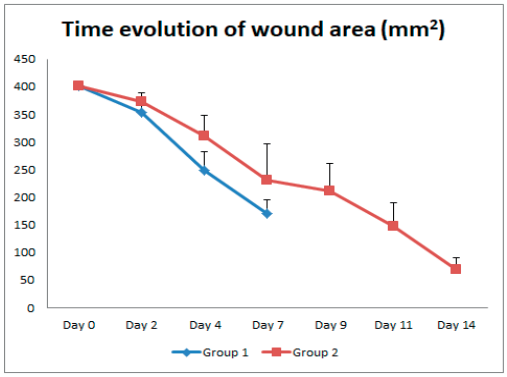

Figure 3 - Time evolution of wounds area for each group

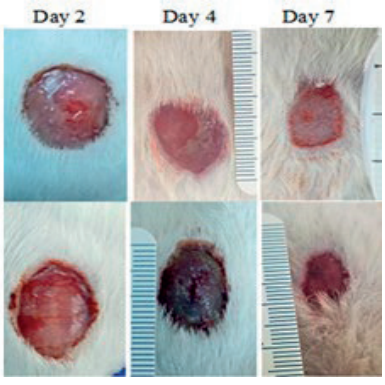

Figure 4 - Macroscopic images of wound-healing process on 2nd, 4th and 7th post-operative days (group 1)

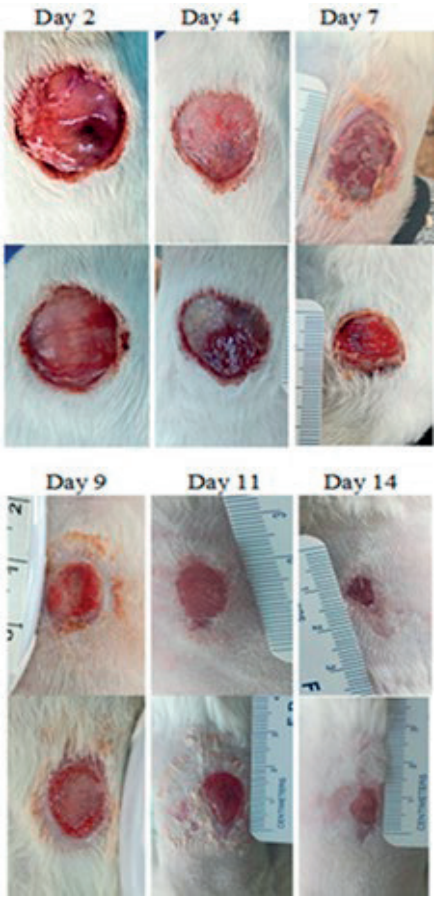

Figure 5- Macroscopic images of the wound-healing process on 2nd, 4th, 7th, 9th, 11th and 14th post-operatives days (group 2)

Hematological analyzes performed at the final day did not reveal significant differences between animals euthanized on 7th post-operative day and the ones on 14th day, for none of the determined cell lines (erythrocyte/leukocyte/platelet). Hematological inflammation markers (total WBC count, lymphocyte/granulocyte count) registered low degree variations, with values within the normal reference range and were not influenced by the wounds area.

Histopathological evaluation of tissues samples of wounds/scars collected at the end of the experiment included: bridging of cells and keratinization, inflammatory cells, neoangiogenesis, proliferation of fibroblasts and neocollagenesis. Histopathology aspects are presented in Figures 6 and 7.

Day 7

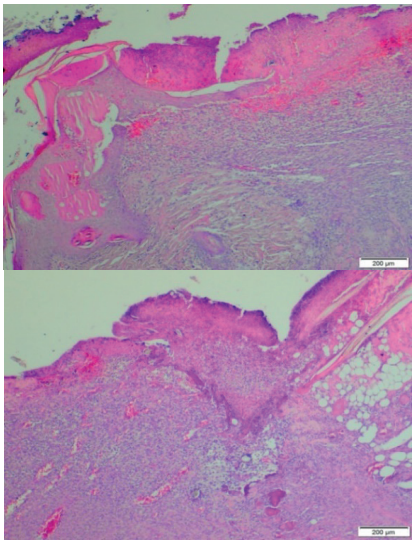

Figure 6 - Histopathology of tissue samples at 7th post-operative day, stained with H&E, 4X (Olympus SC 50) Early epithelialization with granulation tissue extending from the surface of the defect to the hypodermis and skeletal muscle layer; Abundance of polymorphonuclear and mononuclear inflammatory cells; Perivascular inflammatory cells and mast cells; Frequent fibroblasts and thin collagen fibers; Activation of local microvascular endothelial cells lining the inner surface of blood vessels (neoangiogenesis)

Day 14

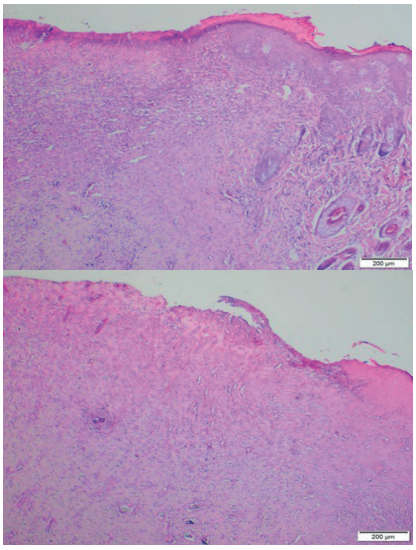

Figure 7- Histopathology of tissue samples at 14th post-operative day, stained with H&E, 4X (Olympus SC 50) Epidermal hyperplasia with parakeratosis, low granulation tissue, the defect being largely covered by epithelialization; moderate to low number of mononuclear inflammatory cells; dense collagen fibers, firmly orientated; Vertical orientation of the blood vessels in the granulation tissue with discreet angiogenesis

The purpose of this study was to establish a reproducible, predictable and quantifiable rat model of excisional wound healing, for further wound healing medical device testing.

Wound healing process was assessed by the measurements made during the experiment and histopathologic analysis in both time points (days 7th and 14th).

The gradual maturation of the granulation tissue and subsequent transformation into the primary fibrous scar is considered one of the important morphological features of wound healing progression. Neovascularization is critical for efficient wound healing, since it is required for the delivery of nutrients and maintenance of oxygen homeostasis, to allow cellular proliferation and tissue regeneration to occur (Pastar et al., 2021).

Murthy et al., (2013) created an excisional wound model by surgically removing a full-thickness skin area of 500 mm2 and assessed the wound healing process. The rate of wound healing in control rats was 21.6% to 68.3% from day 4 to day 12 and 80.6% to 98.1% from day 14 to day 20, while complete epithelialization and healing were observed on day 24.

Caetano et al. (2014) tested the efficacy of wound healing materials by performing two circular full-thickness wounds on rats dorsal region by using 15mm diameter punch and collected tissue samples on days 2,7,14 and 21. Macroscopic and histological analysis showed similar results to this study, most wounds being completely healed from the 14th day on.

Santos et al. (2021) performed 8 mm wounds on the back of Wistar rats for the wound healing assay. On day 7, granulation involved the full-thickness of dermal tissue and re-epithelialization was limited to the marginal area of the wound. On day 14, a remarkable increase in collagenesis, as well as reduction of the interfibrillary spaces was observed, with most of the defect being fully re-epithelialized.

Rat cutaneous wound healing does not perfectly mimic human skin wound healing because the skin morphology is different (Abdullahi et al., 2014, Petersen et al., 2016).) Rat skin is unique in having a subcutaneous panniculus carnosus layer (a thin muscle layer between the subcutaneous fat and dermal layer), that facilitates skin healing by both wound contraction and collagen formation

(Davidson & Opalenik, 2013). Consequently, wound contraction, which is usually more rapid than epithelialization, causes a decrease in the overall healing time of rat wounds (Chang et al., 2019; Masson-Meyers et al., 2020; Wong et al., 2011). In contrast, human wounds heal by re-epithelialization and granulation tissue formation, important differences to consider when assessing the translational relevance of rodent studies (Rouselle et al. 2018). The inherent differences between human and rat skin should be considered in determining whether rats are appropriate in wound-healing models. Rats have been classified as "loose-skinned animals", primarily because of their skin's elasticity and its lack of a strong adherence to the underlying structures compared to humans (Abdullahi et al., 2014). Efforts have been made to create modified models, where contraction is retarded to more closely mimic the physiology of human wound healing (Sharpe & Martin, 2013). Grada et al. (2018) discusses the limitations in using rats as a model due to contraction wound healing mechanism and mentions the use of splinting technique in order to avoid healing primarily via contraction. Son DO & Hinz, (2021) also describes a procedure to splint the edges of full-thickness rodent skin with a sutured plastic frame to prevent wound closure by granulation tissue contraction. Therefore, the wound will heal through granulation tissue formation and re-epithelialization, similar to the process in humans.

The diferences between human and rat skin are also present internally, as rats possess the enzyme l-gluconolactone that converts l-gluconogammalactone to vitamin C, therefore rats do not require diets with added vitamin C. This is particularly relevant in wound healing as vitamin C plays a vital role in collagen synthesis (DePhillipo et al., 2018). Main characteristics of human and rat skin are presented in Table 3.

Developing an animal model that can mimic the complexity of human healing process may seem an unattainable goal, because non-healing and delayed healing wounds in humans are often the result of a combination of external factors (Davidson & Opalenik, 2013; Dorset-Martin, 2004). Despite their limitations, rats are often selected for their availability, easy manipulation, low cost, and small size, as well as defined genetic backgrounds. Rats are large enough to provide a suitable area of skin for studies which require larger or more numerous wounds per animal (Grada et al., 2018).)

Table 3- Characteristics of human and rat skin

Trait	Human	Rat
Epidermis	Thick	Thin
Dermis Thick	Thick	Thin
Skin adherence	Tight	Loose
Panniculus carnosus	Absent	Present
Hait coat	Sparse	Dense
Hair growth	Mosaic	Patches
Vitamin C source	Exogenous	Endogenous
Keloid/ hypertrophic scar	Possible	No
Wound Healing Mechanism	Re-epithelialization	Contraction

Excisional wounds are the most commonly used wound healing models, generated by the surgical removal of all skin layers. Excisional models commonly use the rat's dorsum as the wound location as dorsal sites tend to be more effective in keeping the animal from reaching and manipulating the wound. This model allows the investigation of inflammation, granulation tissue formation, re-epithelialization, angiogenesis and remodeling tissue. (Masson-Meyers et al., 2020; Dorset-Martin, 2004).

CONCLUSIONS

Rats provide a valuable animal model for cutaneous wound healing and further research will improve wound assessment methods to provide a better understanding of the benefits and limitations of this model in translational applications.

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