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SUMMARY

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MORPHOLOGICAL STUDY OF THE THORACIC LIMB JOINTS IN DOGS

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Abstract

Dogs are one of the most morphologically diverse species, as they can range from a 1kg Chihuahua to a 100kg English Mastiff. This morphological diversity affects the appendicular skeleton, leading to a raised incidence of pathologies regarding joints (osteoarthritis, arthrosis, elbow dysplasia). This study was done on 20 dog cadavers. The joints of the forelimb were described in detail. The main purpose was obtaining photographic images which can highlight elements that were previously not mentioned in specialty literature. Following the study, some unique aspects were evidenced such as: dimensions and positioning of glenohumeral ligaments, the topography of the collateral ligaments of the elbow joint and the importance of the proper ligaments of the antebrachial-carpal-metacarpal complex in the dynamic of the movements of this joint. We have compared photographs of the dissected specimens with each other as well as with previously existent sketches from different authors who have tackled the same subject in the past, and we have conclusively pointed out the elements which we considered useful to those interested in resolving pathologies of the locomotor apparatus in carnivores.

Key words*: dogs, joints, tendon, skeleton.*

INTRODUCTION

Canids (Canidae) represent a family of semi-digitigrade, small and middle sized carnivorous mammals, spread all around the globe. The most well-known representatives of this family are: wolves, foxes, jackals, coyotes and various species of wild dogs, as well as the domestic dog.

These animals are small or middle sized, with a corporeal mass which varies from 1 to 75 kg, depending on the species. In general males are larger than females. Canids have tall, long, relatively thin legs, ending in small paws. The anterior limbs usually have five, rarely four fingers, and the posterior limbs always have four fingers with non-retractable, thick and blunt claws. (Wang & Tedford, 2007; Predoi et al., 2020).

General morphological characteristics of the limbs, presented above, make the species in the canid family good and resistant runners and swimmers (Hermanson et al., 2019; König & Liebich, 2015; Predoi et al., 2020). They catch their pray after chasing it for a long time, moving at a constant pace, in a gait or gallop.

Just as in wild species, the domestic dog kept the general conformation and structure of the limb joints. However, in time, human intervention on genetic structure led to obtaining a multitude of breeds, expressing through an extraordinary phenotypic diversity. In some cases, the dynamic performances of the animals vastly improved. Thus, greyhounds and other sighthounds are capable of speeds far superior to most wild canids. Unfortunately, genetic selection had, in other cases, a negative side. Breeds were created who were shrunken in size considerably, significantly modifying the morphology of the bones of the limbs, affecting the rapports between them, and in turn, the conformation of the joints (Serpell & Duffy, 2014; Tarafder & Lee, 2016).

The importance of this research is strongly connected to its practical application, namely orthopaedic surgery (interventions such as osteosynthesis, repairing fractures, luxations, etc.) (Stone, 1985; Zhang et al., 2015). A second reason is the wish to complete existing literature data with a series of images, taken directly from the dissected pieces which can be useful to those interested in the subject, both

from the veterinary medicine domain as well as experimental medicine.

MATERIALS AND METHODS

The study material was represented by limbs originating from eight dog bodies. After skinning, the anterior limbs were detached by sectioning the connecting muscles between the scapula and the torso, and then the basin with the posterior limbs, through a section in the caudal lumbar region, before the iliac wing.
The peri-articular musculature was carefully removed, initially maintaining the integrity of the articular capsules. Connective means were dissected through classical methods, and after the description and photographing of these structures, the capsular ligament was removed, dissecting the proper or intracapsular ligaments, when necessary.
The more suggestive images were photographed and edited on the computer in the Adobe Photosop C3 program.
The description and homologation of these structures and formations was done in accordance with Nomina Anatomica Veterinaria - 2017.

RESULTS AND DISCUSSIONS

The scapulohumeral joint is a spheroidal joint between the glenoid cavity of the scapula and the humeral head. This joint allows all types of movements, but mainly flexion and extension. A glenoidal labrum approximately 1-2 mm thick, disposed on the exterior of the edge, caudolaterally, extends the surface and the profoundness of the scapular glenoid cavity. The articular capsule forms a thin sleeve, attached proximally to the glenoidal labrum (Figures 1 and 2).
The distal insertion of the capsule is a few millimetres distally from the articular surface of the humeral head, where the continuity with the periosteum of the humeral neck can be observed. A portion of the articular capsule surrounds the origin tendon of the brachial biceps muscle, extending distally approximately 2 cm in the intertubercular groove. Its tendon and its synovial sheath are maintained in the intertubercular groove by the transverse humeral ligament.

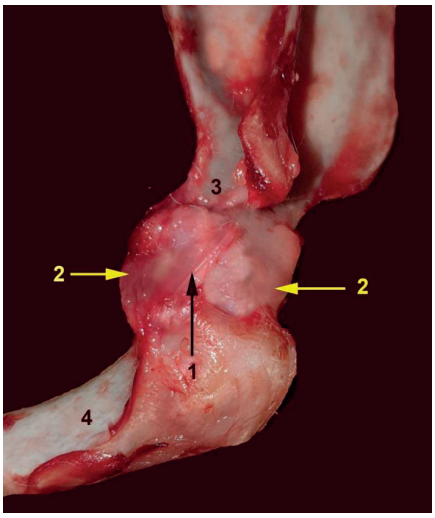

Figure 1. Right scapula-humeral joint - lateral view (original)
1 - lateral glenohumeral ligament; 2 - articular capsule; 3 - scapular neck; 4 – humerus

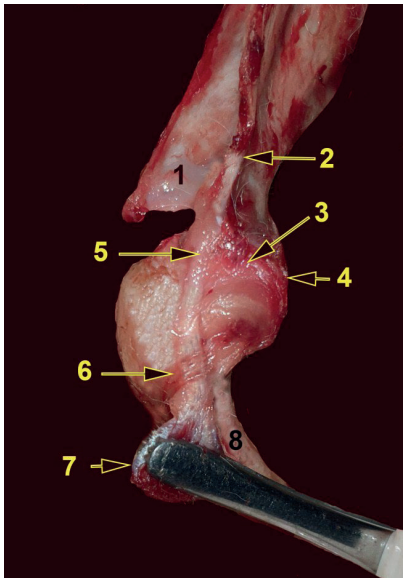

Figure 2. Right scapula-humeral joint - cranial view (original)
1- spina scapularis; 2 - supraglenoidal tuberosity; 3 - articular capsule; 4 - medial glenohumeral ligament; 5 - tendon of the biceps muscle; 6 - transverse humeral ligament; 7 - biceps muscle; 8 - humerus

The capsule unites with this ligament cranio-medially, and in the medial side with the tendon of the subscapular muscle. In the lateral side, the capsule unites with the tendons of the supraspinatus and infraspinatus muscles. In

other areas, especially caudally, the articular capsule is thin. Medially and laterally, the fibrous of the capsule is thickened, forming the lateral and medial glenohumeral ligaments. The strong tendons adjacent to the joint can be considered active ligaments. These ensure the integrity of the joint, making shoulder dislocations at this species almost impossible.

The humero-radio-ulnar (elbow) joint is a compound joint, made from the articulation of the humeral condyle with the head of the radius on one hand – the humeroradial joint – and of the latter with the semilunar notch of the ulna on the other hand. The proximal radioulnar joint has a cavity which widely communicates with the synovia of the other joints, being considered part of the elbow joint. The humeroradial joint is the joint which makes the anterior limb support the largest part of the body weight.

The humeroradial joint stabilises and restricts movements to flexion and extension of the forearm, while the proximal radioulnar joint allows the rotation movement of the forearm. Lateral movements of the articular complex are minimal, due to the strong collateral ligaments but also due to the anconeal process being profoundly inserted in the olecranon fossa of the humerus. Adding up the rotation movements allowed by the proximal radioulnar joint with the ones allowed by the carpal articular complex, permits a supination of the autopodium of approximately 90^0.

There is an articular capsule common to these three joints. In the cranial side, or the flexor side, it inserts proximally to the supratrochlear foramen, including the radial fossa almost in its entirety. In the caudal side, or the extensor side, the articular capsule forms a synovial bursa covered in adipose tissue, attaching distally to the supratrochlear foramen, so that the anterior cavity does not communicate through this foramen with the posterior one. For the most part, but especially in the cranial side, the synovial membrane attaches intimately to the articular cartilage. Medially, a synovial bursa is sent under the brachial biceps muscle and another similar one appears laterally, under the carporadial extensor muscle and the common digital extensor muscle. On the caudo-medial side there is an extension of the synovial, under the carporadial flexor muscle and the humeral portion of the profound digital flexor.

The lateral collateral ligament inserts proximally on the lateral epicondyle of the humerus. Distally it divides, and the cranial fascicle which is somewhat thicker attaches to a small eminence distally under the neck of the radius. The other fascicle, flatter, passes on the ulna (Figure 3).

At the level of the circumference of the joint, the collateral ligament is interwoven with the annular ligament (Figure 5).

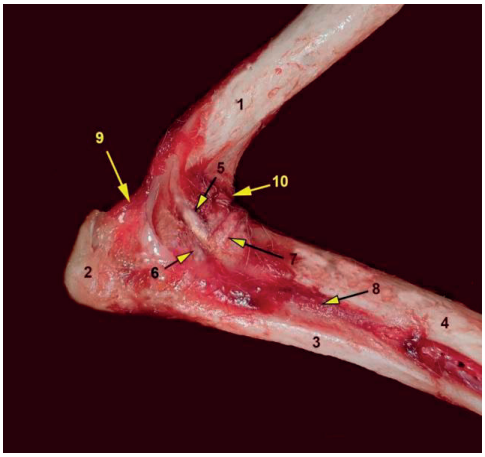

Figure 3. Right humero-radial-ulnar joint – lateral view (original):
1 - humerus; 2 - olecranum; 3 - ulna; 4 - radius; 5 - collateral lateral ligament - radial fascicle; 6 - collateral lateral ligament - ulnar fascicle; 7 - annular ligament (ring); 8 - interosseous membrane; 9 - anconeum ligament; 10 - anterior recess of the synovial membrane

The medial collateral ligament is more reduced than its ulnar counterpart, though similar. It attaches proximally to the medial epicondyle of the humerus, crossing the annular ligament distally and dividing it into two fascicles. The more weakly represented one, the cranial one, inserts proximally to the radial tuberosity. The caudal one, stronger, enters the interosseous space profoundly, where it attaches mostly on the ulna but also on the radius.

The annular ligament of the radius is a thin band which surrounds the proximal extremity of the radius (Fig. 4). It inserts on the lateral and medial extremities of the radial notch of the ulna. It is located profoundly from the collateral ligaments, interweaving with the

lateral one. Alongside the ulna, it forms a ring inside of which the radial head twists, thus rotating the forearm.

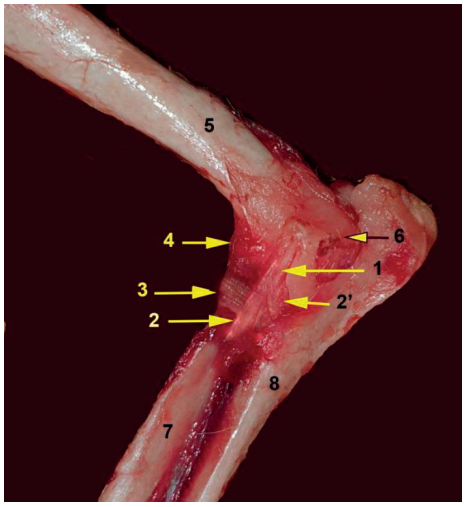

Figure 4. The humero-radial-ulnar joint - medial view (original):
1 - medial collateral ligament; 2 - radial fascicle; 2' - ulnar fascicle; 3 - annular ligament; 4 - dorsal capsular membrane; 5 - humerus; 6 - medial epicondyle; 7 - radius; 8 - ulna

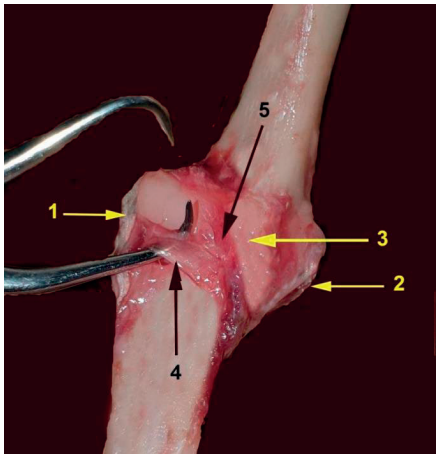

Figure 5. Humero-radio-ulnar joint - cranial view (original):
1 - collateral lateral ligament; 2 - collateral medial ligament; 3 - dorsal capsular ligament; 4 - annular ligament; 5 - oblique strengthening of the articular capsule

The oblique ligament inserts on the dorsal margin of the supratrochlear foramen. It crosses the flexor surface disto-medially, towards the tendons of the brachial biceps muscle and the brachial muscle. Once it reaches the level of the tendons it divides. The shorter portion fixes itself to the radial collateral ligament. The longer portion ends on the medial side of the radius once it forms a loop around the tendons of the aforementioned muscles.

The radius and the ulna are united through synovial joints at the extremities and through a powerful interosseous ligament completed by the interosseous membrane of the forearm.

The proximal radio-ulnar joint (already mentioned as part of the elbow joint) is a joint between the articular circumference of the radius and the radial notch of the ulna, with a depth of approximately 5 mm (Figure 6).

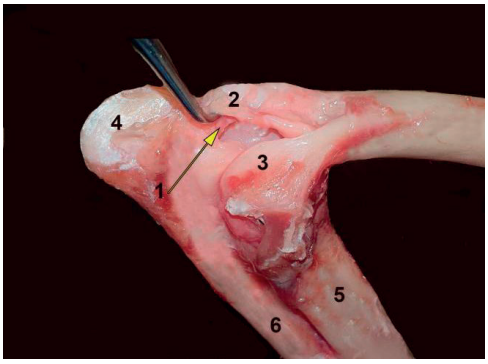

Figure 6. Right humero-radio-ulnar joint - caudo-lateral view (original):
1 - elastic ligament; 2 - medial epicondyle; 3 - lateral epicondyle; 4 - olecranon; 5 - radius; 6 - ulna

The interosseous ligament of the forearm is a short but strong ligament, which passes over the interosseous space, between two rugged lines belonging to the two bones. Its medium sizes are: 2 cm long, 0.5 cm wide and 0.2 cm thick. It is located in the middle portion of the two bones.

The interosseous membrane of the forearm is a narrow and thin septum which connects the radius and the ulna, both above and below the interosseous ligament. It interposes between the osseous margins of the radius and ulna. The membrane extends from the proximal and distal radioulnar synovial. Proximally it is perforated by the passage of the common interosseous artery and vein, as well as the interosseous nerve. Distally, a reduced perforation in the membrane allows the passage of the dorsal distal interosseous artery and vein from the palmar side to the dorsal side.

The distal radioulnar joint is between the distal extremities of the two bones, the ulna having a slight articular convexity and the radius a weak concavity. The capsular ligament in this case is represented by the distal portion of the interosseous membrane.

The **antebrachial-carpal-metacarpal** articular complex is an intricate joint. The antebrachial-carpal articulation is localised between the distal extremities of the radius and ulna and the proximal row of carpal bones. The middle carpal joint is between the two rows of carpal bones. The carpo-metacarpal joints are located between the carpus and metacarpus. The joints between the carpal bones of the same row are called intercarpal joints. The articular complex in its entirety functions as a ginglymus, allowing flexion, extension and slight lateral movements. The more ample movements are permitted by the antebrachial-carpal joint and middle carpal joint. The intercarpal joints and carpal-metacarpal joints allow more reduced movements.

There are no continuous collateral ligaments between the three major articulations of the complex (Figures 7 and 8).

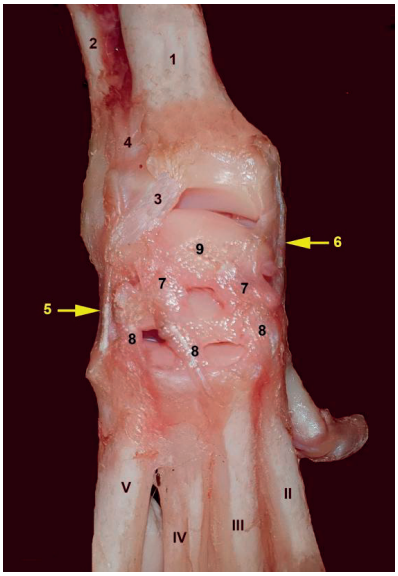

Figure 7. Antebrachial-carpal-metacarpal joint (right limb) - dorsal view (original):
1 - radius; 2 - ulna; 3 - radio-carpal dorsal joint; 4 - radio-ulnar dorsal ligament; 5 - collateral lateral ligament; 6 - collateral medial ligament; 7 - dorsal medio-carpal ligaments; 8 - dorsal carpo-metacarpal ligaments; 9 - scafo-lunar bone; II-V - metacarpals

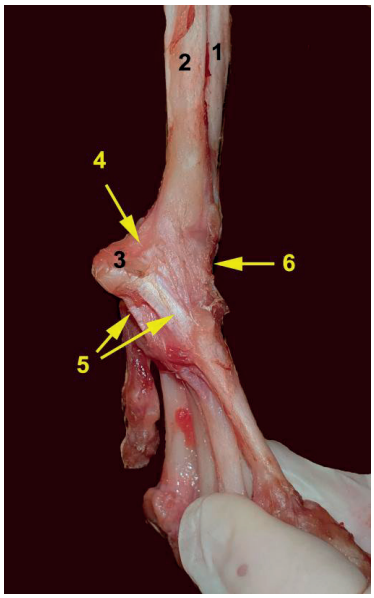

Figure 8. Autopodium joints in dog - lateral view (original):
1 - radius; 2 - ulna; 3 - pisiform; 4 - pisi-ulnar ligament; 5 - pisi-metacarpal ligaments; 6 - capsular dorsal ligament

The dorsal and palmar sides of the articular capsule are far thicker than in the case of other joints specialised in flexion and extension. The long collateral ligaments are absent. The integrity of the joint is ensured by two sleeves of connective tissue, with tendons in-between. The superficial sleeve is formed through a modification of the profound carpal fascia, while the profound sleeve is practically the fibrous component of the articular capsule (the capsular ligament).

The transverse palmar carpal ligament is a modification of the carpal fascia. It inserts laterally on the medial side of the base of the pisiform bone and widens medially, in order to insert on the styloid process of the radius and on the prominences of the most medial carpal bones. The superficial transverse palmar carpal ligament is divided in two layers. One is disposed superficially and another between the tendons of the superficial and profound flexor muscles. The carpal canal on the palmar side of the carpus is represented by the space between the superficial sheet of the transverse palmar carpal ligament, which represents the superficial wall of the canal and the palmar portion of the articular capsule, which

represents the profound wall. It includes tendons and synovial sheaths of the superficial digital flexor and profound digital flexor muscles, the radial, ulnar, palmar interosseous arteries and veins as well as the medial and ulnar nerves.

The palmar carpal fibrocartilage (carpal shield) (Figure 9) crosses the palmar surface of the carpal bones, treading on all of them except the pisiform bone. The fibrocartilage is thickened distally, this area inserting on the distal row of carpal bones and on the adjacent surfaces of the proximal portions of the III, IV and V metacarpals. The palmar carpal fibrocartilage serves as an origin to the special muscles for the II and V finger, and partially as an origin of the interosseous muscles. It levels the palmar irregularities of the carpal-metacarpal joints, smoothing the anterior wall of the carpal canal.

A series of reduced ligaments were also observed.

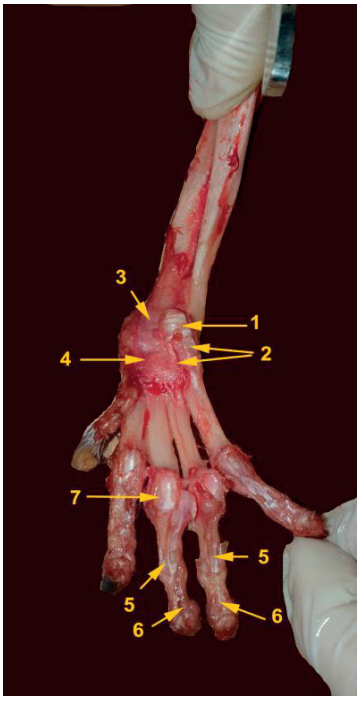

Figure 9. Autopodium joints in dog - palmar view: 1 - pisifom; 2 - pisi-metacarpal ligaments; 3 - palmar radio-carpal ligament; 4 - carpal shield; 5 - insertion of the superficial flexor muscle; 6 - insertion of the profound flexor muscle; 7 - greater sesamoids

Thus the short medial collateral ligament is represented by a straight portion and an oblique portion. The straight portion detaches from a tubercle situated above the styloid process and reaches the medial side of the carpal-radial bone (scafo-lunar bone). The oblique portion, once it detaches from the styloid process, orients towards the palmar-medial surface of the same bone. In its trajectory on the medial side of the carpus, the tendon of the *abductor pollicis longus* muscle passes between the two portions.

The intermetacarpal joints are tight connections between the proximal extremities of adjacent metacarpal bones. The synovial membrane of the adjacent carpo-metacarpal joint inserts itself for a few millimetres between the metacarpal bones. Distal to the synovial are the bones are united on variable distances through fibrous tissue, which forms the interosseous metacarpal ligaments. Distal to these ligaments are the interosseous spaces of the metacarpals.

The metacarpo-phalangeal joints are five in number. They are formed with the participation of the distal extremities of the metacarpal bones and the proximal extremities of the phalanges. Each main finger also has two palmar sesamoids. Each joint has its own capsule with two collateral ligaments. Each pair of palmar sesamoids is united through an intersesamoid ligament. This short cartilaginous ligament is represented by transverse fibres which unite the sesamoids and cover their palmar side.

The lateral and medial sesamoid ligaments are represented by short, flattened fascicles, located on each side of the joint. A first portion of the ligament inserts on the corresponding surfaces, lateral and medial, of the sesamoid bones, and then on the distal surfaces of the metacarpal bone, caudal to the proximal insertion of the collateral ligaments. A second portion orients towards the lateral and medial tubercles of the proximal phalanx. From the distal extremity of each pair of sesamoids there is a thin fascicle which attaches to the palmar side of the proximal phalanx. This is called the distal sesamoid ligament. The crossed ligaments of the sesamoid bones extend from the base of the sesamoids diagonally, to the opposite tubercle from the extremity of the proximal phalanges. In the first finger, usually, there is one sesamoid bone and thus a single ligament.

The dorsal sesamoid bones of the metacarpal-phalangeal joints are fixed through delicate

fibres to the tendon of the common digital extensor muscle and to the interosseous muscles in the proximal side, while distally, through a ligament, they are fixed to the dorsal surface of the middle phalanx.

The proximal interphalangeal joints are formed by the ends of the proximal phalanges, articulated with the fossae of the middle phalanges of the II-V fingers (Figure 9). These are "saddle" type joints. The dorsal wall of each articular capsule is thickened by a cartilaginous nucleus. The tendons of the extensors are intimately united with the capsules, which is why the cartilaginous sesamoids appear to be included in these. In the palmar area the articular capsules are intimately fused to the tendons of the flexors.

The collateral ligaments are represented by strong collagen fascicles, which are not parallel to the finger axis, but disposed vertically. They insert proximally in the fossae on the lateral sides of the distal extremities of the phalanges, and distally on the collateral tubercles of the proximal middle phalanges. At the I finger, which only has two phalanges, the collateral ligaments insert distally on the proximal extremity of the distal phalanx.

The distal interphalangeal joints, from the II to the V finger are formed by the ends of the middle phalanges articulated to the fossae of the proximal extremities of the distal phalanges. On the palmar side of the articular capsule a single cartilaginous sesamoid can be encountered; it is small and spheroidal. On the lateral sides there are thickened areas, the collateral ligaments, which insert proximally in the fossae disposed on each side of head of the middle phalanx and extend obliquely caudo-distally in order to attach on the lateral sides of the ungual crest of the distal phalanx. The dorsal ligaments are represented by two elastic chords which traverse the dorsal part of the distal interphalangeal joint, a short distance away from it. The proximal insertion is on the middle phalanx, 2 mm away from one another. It approaches distally, inserting on the dorsal side of the ungual crest. Thus the retraction of the claw is maintained passively, so that it does not reach the ground unless in the case of the contraction of the profound digital flexor muscle.

CONCLUSIONS

In the case of the scapulohumeral joint, the articular capsule though relatively thin, is consolidated both by lateral condensations (glenohumeral ligaments), as well as strong tendons, adjacent to the joint which can be considered active ligaments.

Although it allows a supination of the autopodium of approximately 90°, the elbow joint is consolidated on the lateral sides by powerful collateral ligaments. In spite of these not being able to allow "resort type" movements to the joint (like it does in equines), there is an elastic radioulnar ligament which has great importance in the passive extension of this joint.

The collateral ligaments of the antebrachial-carpal-metacarpal complex are more reduced than in herbivores. No continuous ligaments can be observed in this complex. However, there is a consolidation of the dorsal ligaments in this region, with both dorsal radiocarpal ligaments and dorsal mediocarpal ligaments encountered.

Two pisi-metacarpal ligaments can be observed: one fixed on the V metacarpal and one on the proximal extremity of the IV metacarpal. The palmar carpal shield can be confused for the majority of the proper palmar ligaments.

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MORPHOLOGICAL FEATURES OF THE SKULL IN THE DWARF KANGAROO (*Macropus rufogriseus*)

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Abstract

The study aims to analyze and describe the morphological characteristics of the skull in the dwarf kangaroo (Macropus rufogriseus). The morphological peculiarities of the skull can be useful elements for the recognition of the species. Data from the literature on the morphology of the skull in this species is limited and mostly focuses on the peculiarities of the muscles in the head. An adult dwarf kangaroo, who died of natural causes was used for this study. The study of the morphological features of the skull leadto the following conclusions: the skull is compact and elongated, the viscerocranium is more developed than the neurocranium, the external sagittal crest is absent, the zygomatic process of the frontal is replaced by a rounded crest separating the frontal surface from the orbital one, two lacrimal foramina are present, the horizontal portion of the palatine is narrow and provided with 8-10 caudo-lateral palatine foramina, there is no alar hole nor channel, the zygomatic bone has a salient spine in the rostral direction.

Key words*: dwarf kangaroo, skull, palatine, alar foramen.*

INTRODUCTION

The dwarf kangaroo *(Macropus rufogriseus)* (Desmarest, 1817) is included in the Order Marsupialia, an order that includes marsupial mammals, belonging to the Macropodidae Family, Genus Macropus.

It is a species that lives in southern and eastern Australia, in Tasmania, where their number is constantly growing, due to restrictive measures regarding the hunting of these species.

On the territory of Europe are found in zoos, and in rare cases, even pets (Bensley, 1903; Jackson, 2011)

The literature contains a small number of data on the morphological features of the skull in the dwarf kangaroo (Windle, & Parsons, 1897; Linzey, 2020; Georgescu et al., 2017; Kardong, 2009).

The study, that was performed on the skull of a dwarf kangaroo *(Macropus rufogriseus)*, aimed at presenting some features in order to be differentiated from other species within the same family, as well as with species belonging to the Order Marsupialia.

Due to the very low number of dwarf kangaroos in captivity, the morphology of the skull in this species has been very little studied in Romania.

MATERIALS AND METHODS

The study material was a specimen of dwarf kangaroo *(Macropus rufogriseus)*, donated by a private individual.

The skull was thoroughly cleaned of soft tissue, then subjected to the process of controlled maceration, washing and degreasing.

The maceration was performed in vessels maintained at a constant temperature (approximately 50 days), under permanent supervision, involving a long process of putrefaction (directed, controlled, etc.).

The washing was carried out in a first step in a continuous stream of water for 24-48 hours. Rinse after maceration with the tip of a scalpel to remove all organic debris.

Degreasing was performed using common detergents diluted in wash water. Wash with slightly acidified water and check for any traces of organic matter.

The drying of the obtained bones was performed under supervision for 48-56 hours at an average temperature of 18-22°C to avoid cracking of the bone structures, in order to not compromise their integrity.

Bleaching was performed by immersing the skull in 30% perhydrol (H_2O_2) solution and then drying it.
The most interesting aspects were described and photographed. The description, identify-cation and homologation were performed according to the Nomina Anatomica Veterinaria, 6th ed. (2017).

RESULTS AND DISCUSSIONS

In the dwarf kangaroo *(Macropus rufogriseus)*, the skull has a slightly elongated appearance, with a small width at the level of the viscerocranium.
The dorsal face of the skull has the largest width at the level of detachment of the zygomatic processes of the temporal bones.
The external occipital protuberance is small and has a convex appearance in the transverse direction.
The outer sagittal crest is absent. From the level of the external occipital protuberance, two temporal lines start in the rostral direction to the level of the fronto-parietal suture.
The zygomatic process of the frontal has the appearance of a reduced, rounded crest, which separates the frontal surface from the orbital one.

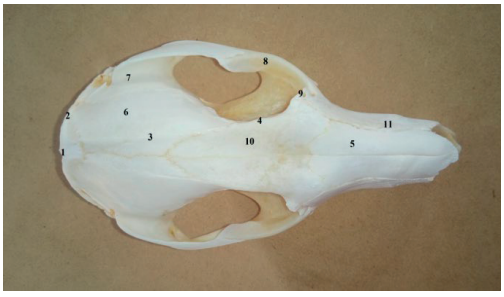

Figure 1. Dorsal face of the skull in the dwarf kangaroo (*Macropus rufogriseus*): 1. External occipital protuberance; 2. Nuchal crest; 3. Temporal line; 4. Zygomatic process of the frontal (rounded crest); 5. Nasal; 6. Parietal; 7. The zygomatic process of the temporalis; 8. Zygomatic; 9. Lacrimal foramen; 10. Frontal; 11. Nasal process of the incisor

The nasal bone in the rostral extremity ends bifidly, the medial portion being much longer than the lateral one.
There is a salient orbital process between the frontal and lacrimal bones.
The parietal bones are elongated, with a slightly convex exocranial face, being traversed by the temporal lines.
The nasal process of the incisor is widened and delimits with the oral extremity of the nasal a small nasal-incisive incision.
The lateral face of the skull has an incomplete orbit.
At the level of the zygomatic bone, a reduced spine is observed rostral, and an obvious muscular insertion spine is arranged ventrally.
The zygomatic process of the temporalis, flattened latero-medially, is wider in the caudal part, presenting ventrally a glenoid cavity elongated transversely, for articulation with the condyle of the mandible.
On the facial surface of the lacrimal bone are arranged two lacrimal foramina separated by a small tuber.
The maxillary foramen, located at the level of the pterygo-palatine fossa, communicates with the infraorbital foramen, wide, of oval aspect, through a very short infraorbital canal. A caudo-medial maxillary foramen has a salient spheno-palatine foramen.

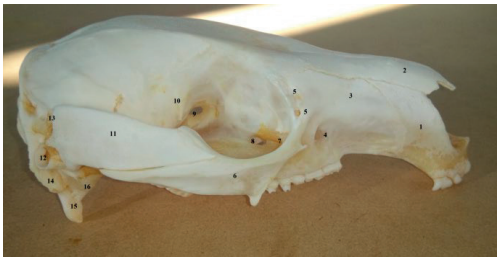

Figure 2. The lateral face of the skull in the dwarf kangaroo (*Macropus rufogriseus*): 1. Incisive; 2. Nasal; 3. Maxilla; 4. Infraorbital foramen; 5. Lacrimal foramina; 6. Zygomatic; 7. Maxillary foramen; 8. Spheno-palatine foramina; 9. Ethmoidal foramen; 10. Orbito-temporal crest; 11. The zygomatic process of the temporal;12. External auditory canal; 13. Retroglenoid foramen;14. The mastoid process; 15. Jugular process;16. Tympanic bulla

The orbital hiatus has a wide foramen, representing the common opening of the optic nerve foramen and the orbital fissure. The ethmoidal foramen is situated cranial to the optic nerve foramen. The large round foramen is separated from the optic nerve foramen by a thin bone blade. The wing foramen and the wing channel are absent.

The diastema has a concave appearance and lacks a canine socket.
The external auditory canal, of circular shape, reduced, covers in its caudal part the opening of the stylo-mastoid foramen.
The mastoid process of the petrous part is reduced.
On the ventral face of the skull is observed the very long basisphenoid, wider in the caudal extremity and narrow in its rostral extremity. The wings of the presphenoid are well highlighted, while the wings of the basisphenoid are very short.
The condyles of the occipital have an oblique arrangement, and above them, in the caudal part of the condylar fossa, the hypoglossal nerve foramen is observed. The condylar fossae are narrow and deep.
Medially the base of the jugular process is the caudal foramen lacerum and the jugular foramen. The eardrum is very small.
Near the wings of the basisphenoid there are two foramina: the rostral carotid foramen, and the caudo-lateral to this oval foramen.
The rostral carotid foramen is placed in a deep depresion at the boundary between the body and the wings of the basisphenoid.

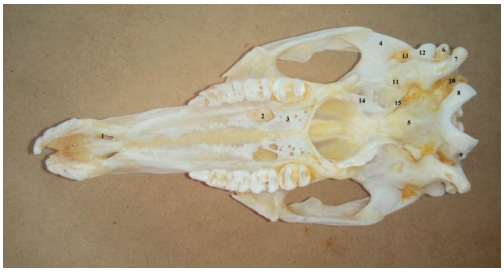

Figure 3. The ventral face of the skull in the dwarf kangaroo (*Macropus rufogriseus*): 1. Palatine fissure; 2. Palatine space; 3. Caudo-lateral palatine foramina; 4. Articular surface for the mandible condyle; 5. Basisphenoid; 6. The mastoid process; 7. Jugular process; 8. Occipital condyle; 9. Hypoglossal nerve foramen; 10. Foramen lacerum; 11. Oval foramen; 12. External auditory canal; 13. Musculotubal canal; 14. Presphenoid wings; 15. The wings of the basisphenoid

A retroglenoidal foramen is arranged caudal of the oval foramen, and the musculotubar canal is arranged medially from it.
The temporal articular surface is small, represented by an elongated transverse glenoid cavity.
The palatine arch is long, and at the junction between the horizontal portion of the palatine and the palatal portion of the maxilla, a wide maxillo-palatal space is observed that is arranged caudally to the limit between the 1st and 2nd upper molar.
The horizontal portion of the palatine bone is thin and presents 8-10 caudolateral palatine foramina with variable diameters.
In the rostral extremity of the ventral face, palatine fissures are observed, elongated and narrow.
On the nuchal surface of the skull there is a wide occipital foramen, oval in appearance, delimited by two obliquely arranged condyles.
The jugular processes are well highlighted, from their base starts in the dorsal direction a well-highlighted muscular crest, which reaches the level of the nuchal crest. Under the nuchal ridges on the sides there is a small foramen.
From the union of the nuchal ridges in the dorsal plane, a small occipital protuberance is formed, convex in a transversal direction.
The external occipital crest is evident in the upper third of the exocranial face of the occipital.

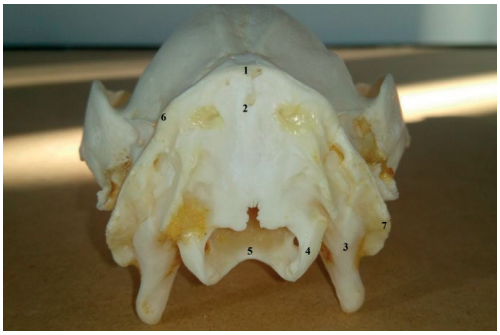

Figure 4. The nuchal face of the skull in the dwarf kangaroo (*Macropus rufogriseus*): 1. External occipital protuberance; 2. External occipital crest; 3. Jugular process; 4. Occipital condyle; 5. Occipital foramen; 6. Nuchall crest; 7. Mastoid process

The mandible of the dwarf kangaroo (*Macropus rufogriseus*) is aneven bone. On each mandible there is only one alveolus for the incisor, and the diastema is long and there is no alveolus for the canine.
The horizontal portion of the mandible is slightly convex.
The angular process, developed, is pulled much medially making a wide fossa between the shat and the branch of the mandible.

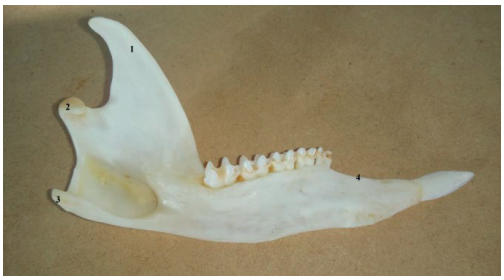

Figure 5. Mandible in the dwarf kangaroo (*Macropus rufogriseus*) - medial face: 1. Coronoid process; 2. Condilar process; 3. Angular process; 4. Diastema

The masseter fossa is deep and communicates with the pterygoid fossa through a wide foramen located in the ventral plane.
The condylar process is slightly oblique to the transverse plane, presenting the latero-medial convex-concave surface.

Figure 6. Jaw to the dwarf kangaroo (*Macropus rufogriseus*) - side face: 1. Mental hole; 2. Masseter fossa; 3. Communication port; 4. Condilar process; 5. Coronoid process

The coronoid process is abruptly curved and ends in taper.
The corono-condylar notch is wide and shallow.
On the lateral face there is a single mental foramen, and the diastema is very well highlighted.

CONCLUSIONS

On the dorsal face of the skull there is a reduced occipital protuberance, and the external sagittal crest is absent.
The zygomatic process of the frontal has the appearance of a reduced, rounded crest.
The external sagittal crest is absent, the temporal lines extend from the level of the external occipital protuberance.
On the facial surface of the lacrimal bone are situated two lacrimal foramina separated by a small tuber.
The infraorbital canal is very short.
At the level of the orbital hiatus, the ethmoidal foramen, the orbitoround foramen and common foramen, the optic nerve foramen and the orbital fissure open.
The wing hole and the wing channel are absent.
The basisphenoid is very long, with a wide caudal extremity and a narrow rostral extremity.
The tympanic bulla is not salient.
At the junction between the palatine portion of the maxilla and the horizontal portion of the palatine, a wide maxillo-palatine space is observed that is arranged caudally to the limit between the 1st and 2nd upper molar.
The horizontal portion of the palatine is thin and present 8-10 caudolateral palatin foramina.
From the base of the jugular process, an obvious muscular crest starts in the dorsal direction, reaching the nuchal crest.
The mandible has a much medial drawn angular process, with a very large masseteric fossa between the shaft and the curved branch of the mandible.
Between the two fossas, in the ventral plane, there is a communication orifice.

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INFESTATION PATTERN OF CATTLE GADFLY FLIES IN TOMPASO MINAHASA

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Abstract

The diversity and abundance of flies (FA) that infest cattle by geography in the central area of livestock rearing can be identified after the fly is caught. The purpose of the study was to find out how much diversity and abundance of some types of skin defect-causing flies were maintained at the location based on differences in temperature and humidity levels in the Tompaso Minahasa area of North Sulawesi. Catching flies is carried out in the morning in which the catch is collected to be identified and analysed its diversity and abundance. The results of identification and analysis showed that the average FA values at various humidity levels showed still in the moderate category namely: 56.1 to 60.7, with the largest abundance being 132.03 ± 7.78, on the other hand at high humidity of 60.8 to 65.4. The FA's average diversity at various temperatures shows that at low temperature levels (26.3-28.6 C) has the largest value of 127.40 ± 5.76, on the other hand at medium temperatures ranging from 28.7 to 30.8 C has an FA value of 115,83 ± 3.96.

Key words*: flies infest, cattle, minahasa, diversity, abundance.*

INTRODUCTION

Flies are naturally carriers of pathogens and play an important role in the spread of viruses, fungi, bacteria and parasites around the world (Banjo et al., 2005) in general, flies can be classified into two types, namely the licking type such as the domestic *Musca* and the type of biter and bloodsucker such as *Stomoxys, Haematopota, Tabanus* and *Hipobosca* (Ahmed et al., 2005). Tabanus spp. or horse fly is an important vector, consisting of the genus *Tabanus, Chrysops* and *Haematopot*a, with the largest size variation in the Brachycera class. The Tabanus fly is very important in Asia, as it is in China (Xu Baohai and Xu Rongman, 1992) and in Thailand (Phasuk, 2013). Haematobia irritans flies are usually found on livestock horns and are dark red in color and brown eyes with long palps of proboscis (Moon, 2002). Haematobia spp. active during the day and are obligate (Syaffitri, 2013).

The cattle system in North Sulawesi, such as in the Minahasa District, Tompaso region, is generally herded around 7.00 - 17.00, in dry fields and in moor or under trees, both in the rainy season and in the dry season. The pattern of grazing cattle as practiced by farmers does not pay attention to livestock health. Health problems in cows that are caused by flies that result in a decrease in the health condition of the cow, the flies directly suck the cattle's blood, the flies can indirectly act as vectors that cause Stephanofilariasis infection which is also called scabies disease, is a disease in cows characterized by dermatitis.

This disease is caused by worms from the genus Stephanofilaria and is transmitted by fly vectors. This condition easily occurs because the breeding of flies in decomposing dung and garbage habitats full of bacteria and pathogenic organisms, coupled with high fly populations can disturb the peace of livestock.

MATERIALS AND METHODS

In order to characterize the abundance of flies, the following indicators were used: number of flies, of which area of cattle body and type of flies density per square centimetre.

The period analysed in this study was 2019-2020.

The data, collected from Pinabetengan Village, have been statistically processed and interpreted, building the trend line and recommendation.

RESULTS AND DISCUSSIONS

The average abundance of cow fly at various humidity is presented in Figure 1. From this figure shows that at moderate humidity levels (56.1-60.7) the largest abundance of nuisance flies causing defects is 132.03 ± 7.78, on the other hand at high humidity (60.8-65.4) has an abundance of flies that cause the smallest defect, namely 114.17 ± 5.81.

The difference in fly abundance according to humidity is largely due to the conditions of agricultural land and plantations where cattle are grazing.

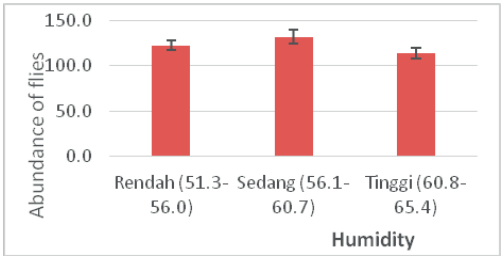

Figure 1. Abundance of flies and humidity, by area

The average and diversity of flies that cause cow defects at various temperatures are presented in Figure 2.

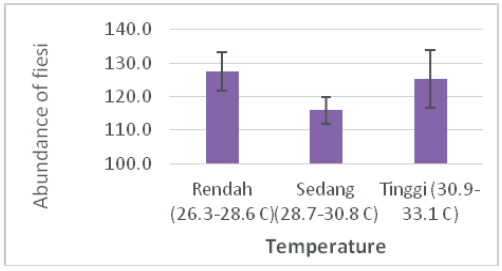

Figure 2. Abundance of flies and temperature, by area

From this figure shows that at low temperature levels (26.3-28.6°C) has the largest insect abundance that causes defects, namely 127.40 ± 5.76, on the other hand at moderate temperatures (28.7-30.8°C) has an abundance of insects causing the smallest defects, namely 115.83 ± 3.96.

The abundance of flies that causes defects is greatest at low temperatures, namely in areas of low humidity which have the largest cattle population, with agricultural land conditions that are dominated by secondary crops such as maize, beans, and plantation land such as coconut. The condition of the settlements of the adjacent settlements also contributed to the abundance of insects, this condition was followed by a larger population

Identification and analysis showed the average abundance of flies at various humidity showed that at moderate humidity levels (56.1-60.7) had the largest fly abundance, namely 132.03 ± 7.78, on the other hand at high humidity (60.8-65.4).

The average diversity of fly abundance at various temperatures shows that at low temperature levels (26.3-28.6°C) has the largest fly abundance, namely 127.40 ± 5.76, on the other hand at moderate temperatures (28.7-30.8°C) has an abundance of flies, namely 115.83 ± 3.96.

CONCLUSIONS

The results of identification and analysis showed that the average FA values at various humidity levels showed still in the moderate. The FA's average diversity at various temperatures shows that at low temperature levels has the largest value of flies.

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PARASITOLOGICAL INVESTIGATIONS IN AN ARABIAN HORSE BREEDING FARM IN ROMANIA

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Abstract

Endoparasite control is one of the major challenges of equine health management, and requires constant surveillance. The present study aimed to investigate the prevalence of intestinal parasites in an Arabian horse breeding farm. Of the 115 samples examined by flotation and sedimentation methods, 69 (60%) were positive for parasitic infestation. The prevalence of Strongylidae was 35.65%, Parascaris equorum 19.13% and Eimeria leuckarti 23.47%. An initial treatment with ivermectin paste (20 mg/ 100 kg) was administered to all the animals positive for helminth infestation. Two weeks after the treatment, samples were collected and examined again, using the same methods. The prevalence of Strongylidae decreased to 6.95%, while the prevalence of Parascaris equorum remained close to the initial value, at 18.26%. Horses tested positive for Parascaris were treated with fenbendazole paste (7.5 mg/kg), leading to a decrease in prevalence to 2.60%. The remaining horses received a second dose of ivermectin, which had no effect on the prevalence of Strongylidae infestation. As a final result, we changed the control program for endoparasites from scheduled prophylactic treatment "in blind" every three months, to a control program based on diagnosis and monitoring of therapy.

Key words*: anthelmintics, equine parasites, Parascaris equorum, Strongylide.*

INTRODUCTION

Arabian horse breeding has a long history and tradition, being one of the first equine breeding practices to ever be documented (Lewis, 2016). The breeding of top-quality show horses depends largely on the proper management of several factors, such as nutrition, hygiene, environmental factors and health status, particularly endoparasite control (Matthee, 2003).

Horses are prone to infestation with multiple parasite species that can impair the animal's health and well-being. In contemporary establishments, subclinically infested horses are one of the hidden sources that can generate and maintain contamination of the environment. Subclinical parasitism is often difficult to detect, since the animals appear normal, however, it can be associated with poor performance, such as diminished growth rates in young animals, reduced reproductive rates in mares and can even impair the balance of the immune system, all of which can translate into high management costs for the breeder (ESCCAP, 2019).

Studies have demonstrated that treating horses "in blind" for parasite infestation can be more costly and detrimental to the animals' health then implementing a customized parasite control program, based on diagnosis and fecal egg count reduction tests (Kaplan, 2009). Also, rotational deworming has been proved to promote anthelmintic resistance, by the selection pressure under which sensitive parasites are eliminated and the resistant ones survive and reproduce (Nielsen et al., 2014).

The aim of this study was to determine the prevalence of intestinal parasites in an Arabian horse breeding ranch and to implement an evidence-based approach for the treatment program.

MATERIALS AND METHODS

The investigations were carried out from November 2020 to January 2021 in an Arabian Horse breeding farm, in Giurgiu County, Romania. At the time, the horse population consisted of 95 Pure Breed Arabian horses - 23 foals, ages 3 to 18 months and 72 adult horses.

Also on the farm lived 20 Haflinger mares which were used as surrogates in the reproduction process. The Arabian horses included prized animals, used for breeding purposes, and occasionally for participating in beauty competitions around the world. The horses were subject to a prophylactic deworming schedule every three months, using commercially available products containing ivermectin, praziquantel and pyrantel pamoat. Prior to anthelmintic administration, the horses' weight was only estimated, not determined precisely. Also, parasitological examinations had never been performed on the farm, the prophylactic treatments were done blindly, therefore, there was no data available concerning the efficacy of the deworming program.

In order to determine the existing parasitological burden in the horse population, fresh fecal samples were collected from each horse. The samples were labeled and examined immediately in the establishment's parasitological laboratory. Examinations were carried out by flotation method using supersaturated saline solution to reveal the presence of light parasite eggs and larvae and by sedimentation method for heavy eggs (such as tapeworm eggs and *Eimeria* spp. oocysts). A McMaster counting chamber was used to perform fecal egg counts (FEC) in order to determine the degree of infestation (number of eggs per gram of feces - EPG) of the positive samples. Based on the FEC, the horses were categorized into low (<200), moderate (200-500) and high (>500) worm egg shedders (Kaplan, 2009). The horses were also tested for *Oxyuris equi* infestation using the scotch test method, by applying a transparent adhesive tape to the skin of the perianal region, and them removing and examining it microscopically. The horses with positive samples were administered an oral paste containing ivermectin, according to the producer's specifications, after each horse was weighed individually, on a livestock scale. All the positive horses were dewormed during the first phase of the treatment schedule, regardless of their FEC results. Two weeks after the initial treatment, the parasitological examinations were repeated and the horses still positive for *Strongylidae* infestation received a second dose of ivermectin paste. The horses that tested positive for *Parascaris* infestation after the first treatment (mainly the young horses), received a fenbendazole based product for the second treatment. Two weeks after the second treatment, fecal samples were collected again and FECs were performed to determine the efficacy of the treatment schedule.

RESULTS AND DISCUSSIONS

The initial parasitological exams revealed that 69 of the 115 horses were positive for parasitological infestation. The highest prevalence was noticed for parasites belonging to the *Strongylidae* family (35.65% - Figure 1); *Parascaris equorum* (Figure 2) registered 19.13% and *Eimeria leuckarti* 23.47%.

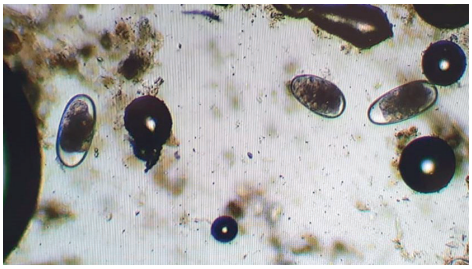

Figure 1. Strongyle eggs identified by flotation method (100x)

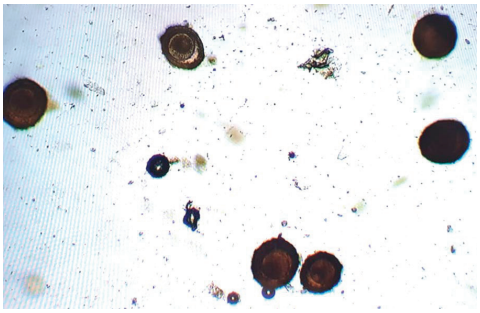

Figure 2. *Parascaris equorum* eggs identified by flotation method (100x)

Although 20 of the adult horses presented characteristic symptoms of pinworm infestation, such as pruritus and tail-rubbing, the diagnosis of *Oxyuris equi* infestation could be confirmed by the scotch test method only for one of the horses (0.87%). Of the 22 samples positive for *Parascaris equorum*, 12 were collected from foals and the remaining 10 were from adult mares.

Of the 69 positive samples, 18 presented poly-parasitic infestation (2 or 3 genera of parasites identified in the same sample). The results of the initial examinations are summarized in Table 1.

Table 1. Prevalence of intestinal parasites at initial examinations

Specification	Number	Percentage
Number of samples	115	
Positive samples	69	60%
Samples positive for *Strongylidae*	41	35.65%
Samples positive for *Parascaris equorum*	22	19.13%
Samples positive for *Eimeria leuckarti*	27	23.47%
Samples positive for *Oxyuris equi*	1	0.87%
Horses infested with a single genre of parasites	41	35.65%
Horses infested with 2 genera of parasites	14	12.17%
Horses infested with 3 genera of parasites	4	3.47%

The fecal egg counts revealed that the majority of the strongyle infested horses were low (70.80 mean EPG) or moderate eggs shedders (355 mean EPG), and only a small percentage had severe infestations (950 mean EPG). Most of the horses infested with *P. equorum* had an EPG of over 200 with a mean of 335, and a few were high egg shedders, with an mean EPG of 687.5 (Table 2).

Table 2. Results of the initial fecal egg count: number of horses categorized into low, moderate or high egg shedders

Parasite genus/ species/ family	Low egg shedders (<200 EPG)	Moderate egg shedders (200-500 EPG)	High egg shedders (>500 EPG)
Strongylidae family	27	11	3
Parascaris equorum	5	15	2

Contrary to information provided by literature (Dubey and Bauer, 2018), *E. leuckarti* was identified mostly in adult horses, both male and female, and despite the high percentage of positive samples, the number of oocysts shed in feces was very low. No *Anoplocephala* spp. eggs were found during the examinations, despite the fact that all the horses have access to pastures occasionally.

Since there were no data available on the potential anthelmintic resistance on the farm, an ivermectine based product was chosen for the treatment of all the horses who tested positive for helminth infestation. The ivermectine was chosen considering its efficacy against both adult and larval stages of large and small strongyles (Cernea, 2008).

The second parasitological examination revealed that 8 horses (6.96%) were still positive for *Strongylidae* infestation. All of the positive samples had an EPG of under 100. During the 14 days following the first treatment, some of the horses that tested positive for *P. equorum,* shed adult roundworms in their feces (Figure 3).

However, the prevalence of roundworm infestation remained close to the initial value, at 18.26% (21 positive horses), showing only a decrease in EPG values. The prevalence of *E. leuckarti* was 12.17% (Table 3).

Figure 3. Adult *Parascaris equorum* worms shed after initial deworming

Table 3. Prevalence of intestinal parasites after the first treatment

Specification	Number	Percentage
Number of samples	115	
Positive samples	36	31.3%
Samples positive for *Strongylidae*	8	6.95%
Samples positive for *Parascaris equorum*	21	18.26%
Samples positive for *Eimeria leuckarti*	14	12.17%
Samples positive for *Oxyuris equi*	0	0%
Horses infested with a single genre of parasites	28	24.34%
Horses infested with 2 genera of parasites	8	6.95%
Horses infested with 3 genera of parasites	0	0%

After the second dose of ivermectin was administered to the strongyle positive horses, there was no major decrease in the prevalence of *Strongylidae* (6.08%), nor the EPG values. After treating the *P. equorum* positive horses with fenbendazole paste the prevalence of infestation decreased to 2.60% (Table 4). It was decided not to treat the *Eimeria* positive horses considering several factors: the oocysts were identifies only in samples taken from adult horses (none of the foals tested positive for *E. leuckarti*), that showed no symptoms of eimeriosis, and the shedding of oocysts was inconsistent. Also, none of the adult horses that tested positive for *E. leuckarti* were in direct contact with the foals at the time, therefore, the risk of contamination for the young horses was low. Of the 115 samples examined, 5 (4.35%) were positive for *E. leuckarti* infestation even though no treatment was administered for coccidiosis.

Table 4. Prevalence of intestinal parasites after the second treatment

Specification	Number	Percentage
Number of samples	115	
Positive samples	11	9.56%
Samples positive for *Strongylidae*	7	6.08%
Samples positive for *Parascaris equorum*	3	2.60%
Samples positive for *Eimeria leuckarti*	5	4.34%
Samples positive for *Oxyuris equi*	0	0%
Horses infested with a single genre of parasites	7	6.08%
Horses infested with 2 genera of parasites	4	3.47%
Horses infested with 3 genera of parasites	0	0%

The prevalence of intestinal parasites found in the initial study, before and for the purpose of designing the therapeutic protocol was high (69%), compared to a similar study carried out in Bucharest and Ilfov county in 2015, that revealed a prevalence of 28.57% for helminth infestation in pure breed horses kept in similar conditions (Bulgaru and Tudor, 2015). However, surveys carried out in other regions of the country revealed similar results to the current study. For example, in 2013, Ionita et al. found a prevalence of 87.97% for parasitic infestation in two stud farms from center and northeastern Romania.

A study on working horses from villages in the northeastern and southeastern parts of the country, showed a prevalence of intestinal parasites of 70.3% (Buzatu et al., 2013). Different results were obtained by a study on horse population located in Western Romania, Arad County, where the prevalence of parasite infestations was 100%, with a 73.21% prevalence for digestive strongyles, 28.57% for *P. equorum*, 12.50% for *O. equi* (Morariu et al., 2012). Also, both studies cited above reported positive results for *Strongyloides westeri* and *Anoplocephala* spp., parasites that were not found in the horses included in the present study.

Parasite control is a global concern among scientists and horse breeders. In Italy, a study on strogyle infestations in horses found a 39.5% prevalence of strongyle egg shedding, with 86.4% of the stables investigated having at least one positive animal (Scala et al., 2020).

A literature review on parasites of equines in Iran revealed a pooled prevalence of parasitic infestations of 28.8%, with helminths having the highest prevalence (47.6%) (Khamesipour et al., 2021). Parasitological investigations carried out on horses living in a tropical climate, in Cuba, determined a prevalence of 97% for strongyles and 10% for *Parascaris* spp. (Salas-Romero et al., 2017).

Despite the fact that the horses subjected to the current study were being dewormed regularly and frequently before the start of the study, the percentage of infested horses found in the initial examinations was very high. A similar deworming schedule (in blind treatments, every three months, with the rotation of anthelimintics) applied in a stable from Bucharest yielded very different results, with a prevalence of helmith parasites of 6.67% (Bulgaru and Tudor, 2015).

The high prevalence of intestinal parasites in the investigated farm appears to be the result of the related action of some factors, such as: the subjective approximation of weight could lead to under-dosing of anthelmintics, which represents a risk factor for the development of drug resistance (Matthee, 2003); even though the overall health of the horses was closely monitored at all times, no coproparasitological

exams were performed to assess the parasitelogical infestation status of the animals. It has been proved that appropriate monitoring of the parasitic infestation, considering the biology of the parasites and parasite-host interactions is essential for the efficacy of the control schedule (Kaplan, 2009; Nielsen et al., 2014; Buzatu et al., 2015).Parasitological surveillance of the herd is imperative because animals from other countries are regularly introduced into the herd, each one of them harboring its own parasitological fauna. A study carried out on 40 horse farms in Brazil showed that more than 50% of the farmers introduce newly acquired horses on their properties without performing any prior examinations, and only 38.5% of the farmers used quarantine as a precautionary measure (Rosa et al., 2018).

CONCLUSIONS

Of the 115 samples examined, 60% were positive for parasitic infestation. The prevalence of digestive strongyles was 35.65% before treatment, and dropped to 6.08% after two anthelmintics administrations.

P. equorum infestation was identified in 19.13% of the tested animals. The first treatment with ivermectin paste was unsuccessful, however, after the second treatment with fenbendazole paste, the prevalence decreased to 2.60%.

The prevalence of *E. leuckarti* varied between the 3 exminations performed, even though no treatment was administered showing a inconsistency in oocyte shedding.

At the end of the investigations described in the current study, the prevalence of intestinal parasites on the farm had decreased to 9.56%

The conclusions of this study demonstrate the effectiveness of considering antiparasitic therapy as an action to be undertaken into a surveillance and monitoring program.

The study represents the first step in establishing an effective program for controlling internal parasites on the farm.

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THERAPEUTIC APPROACH IN VETERINARIAN ONCOLOGICAL EMERGENCIES

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Abstract

Oncological emergencies can occur at any time during an neoplastic pathology or during specialized treatment, and must be identified and properly addressed as early as possible, so as to determine a maximum favorable response. Emergencies in cancer can be represented by iatropathic disease (Cringanu, 2013), paraneoplastic syndromes or the cancer itself. The earlier we diagnose, the better we can establish a treatment with a successful result. This article aims to present treatment protocols in the most common cases of veterinary oncological emergencies. The main objective of our research was to establish a unitary conduct for a fast and correct diagnosis and an efficient treatment of oncological emergencies, before their effects became irreversible.

Key words*: oncology emergency, paraneoplastic, chemotherapy, disease.*

INTRODUCTION

The emergency events in oncology can be defined as acute pathological conditions determined by the clinical evolution of paraneoplastic syndromes, a consequence of the chemotherapy disease or the evolution of the neoplastic disease.

Assessing the diagnosis and performing specific therapy should be rapid and address the syndromes in order of their severity (Cervantes & Chirivella, 2004). The diagnosis of the oncology patient's emergency must be determined in order of priority and type of emergency.

The choice of therapeutic strategy and the combination of techniques and methods is made personalized according to the clinical condition of the patient at the time of diagnosis by anamnesis, general physical examination evaluation, TNM clinical staging, monitoring of vital functions, histopathological type and appreciation of the response to the therapies already performed (Cringanu & Crivineanu, 2009)

Oncological emergencies order of frequency from most to least frequent, are:

- Paraneoplastic metabolic syndromes: hypoglycemia, cachexia, hyponatremia, hypercalcemia, hypoalbuminemia, hyperkaelaemia and hypocalcemia (Krug & Michl, 2018).
- Hematologic syndromes: lymphocytosis, neutrophilia, anemia, thrombocytopenia (Cringanu, 2013).
- Cancer related: TLS[1], thoracic hemorrhagic effusions, peritoneal effusions, pathological fractures from bone neoplasia (Ehrhart et al., 2013), etc.
- Obstructive or structural emergencies, due to space-replacing tumors: pain (Nolen, 2004).
- Iatrogenic emergencies include: chemotherapy induced toxicity, local subcutaneous extravasation or anaphylactic reactions. (Shmuel & Cortes, 2013).

Less than 25% of animals have adverse effects to chemotherapy (Chun et al., 2001).

[1] Tumor lysis syndrome

Although not all of these emergencies are documented in this paper, it is important that we check for early signs, because oncology emergency may also be aggravated by pre-existing liver disease, kidney disease, hematologic disease, neurological disease, disease evolution or therapy associated toxicity and if we recognize the possibility of an emergency we can prevent it.
A clear goal is to establish the diagnosis and specific therapy based on standardized protocols, to be used by all clinicians. Another major objective of this paper is the description and screening of toxic effects of chemotherapy previously administered to some of the cancer patients, and how to prevent the iatropathic syndromes that occur when treatment is combined with metabolic paraneoplastic syndromes.
The 14 cases studied have been selected for the variety of symptoms and their evolution has been documented for teaching purposes.
The goal of the oncological therapy is to control the pain, to restore metabolic balance, to alleviate the paraneoplastic symptoms and eventually restore the morpho-structural integrity of affected tissues.

MATERIALS AND METHODS

We used the medical observations resulted from first time examination and follow-ups of specific oncologic cases over a period of 12 months, on 14 dogs of different breeds, genders, weight and ages with malignant tumors in advanced clinical stages, III and IV. Five of the cases that presented with oncological emergencies benefited from chemotherapy, previously administered at different clinics. The 14 cases are divided into 3 lots, depending on the oncological emergency determined by a certain type of cancer.
Lot no.1 is comprised of 2 cases of Non-Hodgkin malignant lymphoma exhibiting compressive or obstructive syndromes and 3 cases with inflammatory carcinoma (mastitis carcinomatosa[2]) exhibiting lymphedema and stasis edema. Also, we included one case of osteosarcoma with pain syndrome due to a pathologic fracture.
The second lot is comprised of cases that display metabolic syndromes such as histamine discharge, liver and kidney failure in 3 cases of cutaneous mastocytoma and one case of osteosarcoma that was included as an example of severe hypercalcemia due to tumor and bone lysis.
The 3rd lot includes patients that suffer from iatropathic diseases and different types of chemotherapy toxicity (renal, hepatic). We selected 4 cases, 2 mammary gland adeno-carcinomas and 2 lymphoma cases, that exhibited important symptoms.
The examined patients were part of case studies of the Internal Medicine Department and Oncology Department. We gathered complete hematology and biochemical panels for each of the patients. Result from adenograms, fine-needle aspiration biopsies from both cancer tissue and healthy tissue were collected and biopsies by excision were also attached to the patient files. Samples have been stained by May-Grunwald-Giemsa or Trichromic Masson methods. The imaging departments provided us with ultrasounds, X-rays, CT scan results completing the diagnosis protocol in order to identify soft tissue and bone modifications in each case, as needed.
In these emergencies, the investigations used were chosen strictly in the context of each case to establish the optimal therapy and only relevant images have been included in the article.
The cytopathology and/or histopathology diagnosis was already established for the cases, upon arrival at the Oncology Clinic. The present paper highlights the treatment of the oncological emergency for each case.
Out of the 14 patients, 2 have died (one lymphoma and one mastocytoma patient), necropsies were performed and histopa-thology samples from both dogs were taken (lymph node samples, liver and kidney samples). The most representative images are illustrated in this paper.

[2] MC

The treatment protocol for each emergency was established taking into consideration acute/life-threatening symptoms and TNM of each patient.
The canine patient's clinical record also include basic data: weight, age, medical history and co-morbidities in order to establish an exact protocol for the chemotherapy and an oncological emergencies protocol.

Table 1. Drugs used in the treatments in this article

Drug	Recommended dose
Dexamethasone	0.2-0.5 mg/kg, up to twice a day
Hydrocortisone Hemisuccinat	10-15 mg/kg, i.v.[3], shock dose
Prednisolone 5 mg	0.5-1.0 mg/kg
Tramadol 50 mg/ml	3.0-4.0 mg/kg i.m., every 8 to 12 h
Sedam 35 mg/ml	1.0-3.0 mg/kg, p.o., as needed
Ketamine	0.2-0.3 ml/kg i.m.[4]
Metamizol	25-35 mg/kg, as needed
Piroxicam	0.3 mg/kg, p.o.[5], every other day
Meloxicam 5mg/mL	0.2 mg/kg, s.c.
Meloxicam ointment	as needed
Mibazon ointment	as needed
Cefort	50 mg/kg/zi i.v.
Cefa-cure 50 mg	20 mg/kg, p.o., every 24h
Granisetron	0.6 mg/kg, s.c., i.v.
Emeset	0.5-1.0 mg/kg, i.v., every 12-24 h
Ondansetron	0.5-1.0 mg/kg, i.v., every 12-24 h
Osetron	0.5-1.0 mg/kg, i.v., every 12-24 h
Ranitidine	1.0-2.0 mg/kg, p.o., every 12 h
Zantac	1.0-2.0 mg/kg, p.o., every 12 h
Arnetin	1.0-2.0 mg/kg, p.o., every 12 h
Cimetidine	5.0-10.0 mg/kg, p.o. every 6-8 h.
Leukeran	2 mg/10 kg, p.o. once a week
Holoxan	200 mg/m2, i.v. every 14 days
Carboplatine	50 mg/m2, i.v. every 21 days
Cyclophosphamide	50 mg/day, p.o. every 14 days
Furosemide 20 mg/ 2 ml	10-20 mg, i.v.
Mannitol 20%	200 mg/kg, i.v.
Aspatofort	10 ml i.v., 2 times a day in Glucose solution
Etamsylate	10-15 mg/kg, every 6 h, i.v.
Vitamin K	2.2 mg/kg s.c.
Adrenostazin 1.5 mg/5 ml	1.5-2.0 mg/20 kg. s.c.
No-Spa 40 mg/2 ml	40 mg., s.c.[6] every 24 hours
Metoclopramide syrup	0.2-0.5 mg/kg., every 6-8 h
Enteroguard	1 tb/3 kg, p.o. for 7 days
Entero-Chronic	4 g/day/30 days p.o.
NaCl 0,9 g/100 ml, Glucose 5%, Ringer Lactate, Aminoplasmal Solution	as need/body mass/ parameters i.v.

[3] intravenous administration
[4] intramuscular administration
[5] oral administration

RESULTS AND DISCUSSIONS

Lot 1 - Compressive/obstructive syndromes and pain syndrome in 2 non-Hodkin´s Lymphoma (NHL) patients (*Patient A*, common breed, 12 years, M;
Patient B, Corsican Dog, 8 years old, F) an osteosarcoma patient (*Patient C*, Setter, 11 years, F) and 3 inflammatory carcinoma MC patients (*Patient D*, Bichon Frise, 9 years, F; *Patient E*, common breed, 11 years, F; *Patient F*, French Bulldog, 8 years old, F).
Image procedures used in our diagnosis are: x-ray for patient C with osteosarcoma (Figure 5) and CT scans for patients A and B.
The cranial vena cava syndrome (CVCS) occurs once the tumor compresses the vein in its path. Venous obstruction may be due to compression, invasion, deep vein thrombosis or vein fibrosis. In the 2 NHL patients it was secondary to the malignancy. As a result of the cancer invasion/compression, an increase in central venous pressure occurs determining edema and collateral circulation (Figure 1).
Diagnosis is clinically established relatively easily, in the initial stages of CVCS and can be confused with heart failure (a feature of differentiation being the absence of jugular pulses and absence of tachycardia).
Symptoms suggestive of CVCS consist of progressive edema of the neck, followed by

[6] subcutaneous administration

facial and forelimb edema, dyspnea associated with tracheal compression, head, neck and chest edema, superficial venous ectasis over the chest.

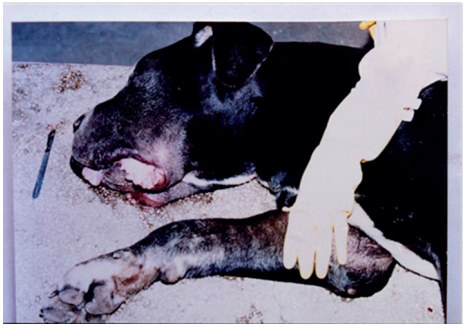

Figure 1. Severe edema of the head (Patient A)

The treatment is symptomatic with oxygen therapy for dyspnea, Furosemide to reduce edema and Dexamethasone or Hydrocortisone in high doses, according to the prospectus, in order to reduce inflammation. The prime cause established to be neoplastic in origin, is treated with chemotherapy. Leukeran 2 mg/10 kg once a week, or Holoxan 200 mg/m^2 once every 14 days recommend after the acute episode.
Unfortunately Patient A died. During the necropsy, we collected lymph nodes that presented specific NHL lymphadenopathy (Figure 2).

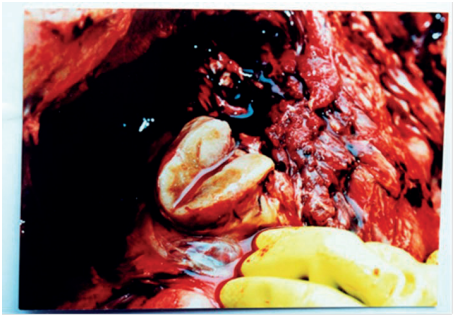

Figure 2. Necropsy lymphadenopathy (Patient A)

Obstructive syndromes due to the thromboembolic disease (TED), also known as venous thromboembolism (VTE), includes a broad spectrum of manifestations, from the most common - acute venous thrombosis (AVT)- to the most severe - deep venous thrombosis (DVT) and pulmonary embolism (PE). In this study, we encountered AVT in all of the 3 cases of inflammatory carcinoma (MC[7]) (Figure 3).

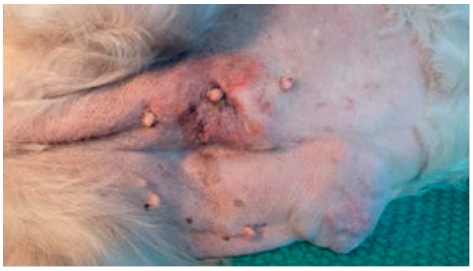

Figure 3. Thromboembolic disease (AVT) in MC (Patient D)

The increased risk of thrombosis is due to tumor release of a procoagulant TF[8] responsible for triggering the extrinsic coagulation cascade.
For the treatment of this type of inflammatory carcinoma, we used for the symptomatic treatment of the emergency: locally: Mibazon ointment alternating with Meloxicam ointment, intravenous: Cefort and Mannitol, subcutaneous: Furosemide at 24 hours, intramuscular: Tramadol at 12 hours. This is a palliative solution that relieves paraneoplastic syndromes. It is recommended to start oncological treatment with intravenous Carboplatine at 21 days for a long term result.
The pain syndrome is a specific and frequent syndrome in cancer patients. The definition proposed by the International Association for the Study of Pain considers pain to be an unpleasant sensory and emotional experience, associated with actual or potential tissue destruction, or described in terms of such destruction. A quick and reliable method we use to clinically asses the dogs in acute pain (emergency evaluation) in this lot, is the short form of the Glasgow Composite Measure Pain Scale

[7] mastitis carcinomatosa
[8] tissue factor

(GCMPS). Analgesic intervention was made at a 6/24 (or 5/20) level (Figure 4).

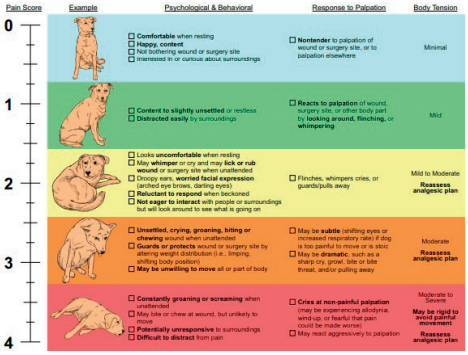

Figure 4. Veterinary Anesthesia & Analgesia Support Group www.vasg.org/pdfs/ CSU_Acute_Pain_ Scale_Canine.pdf

In our cases, the pain syndrome presented as an emergency for the patient with osteosarcoma and two of the dogs with MC. All of the 14 patients experienced some form of pain, but only these three were considered an emergency because of the high level of pain on the GCMPS and acute presentation (spontaneous fracture - Figure 5 - due to the osteolytic process in the radius with osteosarcoma and intense inflammatory reaction overlapping severe infection in the patients with MC).

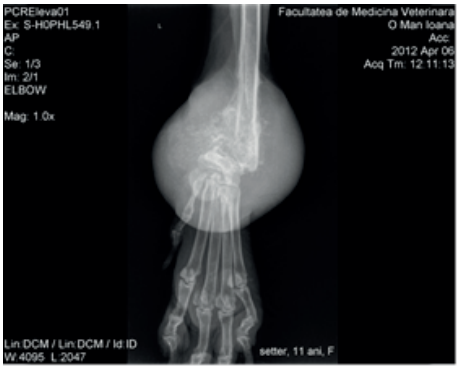

Figure 5. Spontaneous fracture due to the osteolytic process in a radius with osteosarcoma (Patient C)

Pharmacological agents used were non-steroidal anti-inflammatory drugs, opioid substitutes and pyrazolones (Table 1). In addition to analgesics, substances with sedative or tranquilizing properties (a combination of Ketamine and Sedan) can be administered to reduce anxiety, pain and improve the effectiveness of analgesics (opioid substitutes: Tramadol and nonsteroidal anti-inflammatory drugs: Piroxicam and Meloxicam).

Lot 2 - Metabolic syndromes or paraneoplastic syndromes are clinical or morphological manifestations defined as "distant effects" produced by the tumor-induced release of peptide hormones that initiate an immune reaction. This leads to clinical and lab parameter modifications, without a dependency to the primary tumor's size or spread (Krug & Michl, 2018). These syndromes occur in addition to the local, systemic or non-metastatic manifestations that may occur. It is expressed in the form of a complex functional and pathological manifestations associated with the tumor-specific symptoms, with an initial insidious evolution but with increasing gravity as the tumoral cells disseminate in the body.

The following were the most common syndromes accompanying the cancer disease of our 3 patients with MCT[9] (*Patient G,* Shar-Pei, 8 years, F; *Patient H*, Shar-Pei, 11 years, M; *Patient I*, Boxer, 7 years, M) and one patient with osteosarcoma (*Patient J*, Great Dane, 7 years, M).

Patient H and patient I presented with cachexia. This is a specific metabolic imbalance, characterized by severe decrease of body weight, through loss of muscle mass. Cancer induced alterations in host energy metabolism or cachexia are probably the most common paraneoplastic syndromes in human and veterinary medicine.

Hypocalcemia caused clinically significant symptoms in our two patients: muscle spasms and convulsions.

Hipoalbuminemia is the primary plasma change caused by the "hunger" of malignant tumor cells.

[9] mast cell tumors

Hypoglycemia is a frequent metabolic manifestation in oncology by the exaggerated consumption of carbohydrates by tumor cells.

Hypercalcemia defined as elevated calcium levels over normal limits is the most common metabolic disorder associated with bone metastases and/or bone lysis and was a problem in patient J.

Hyperkalaemia is the major potentially fatal electrolyte disorder defined as a potassium increase of more than 5.0 mEq/l, due to the release of cellular debris through cell destruction. The most severe manifestation in our patients G and I has been ventricular arrhythmia.

Hyponatremia by volumetric overload is caused by the accumulation of fluids in the extracellular compartment by reducing the circulating volume

The treatment of this type of metabolic abnormalities is symptomatic and includes Aminoplasmal and Ringer's lactate rehydration solutions to compensate for electrolytes and metabolic acidosis.

Table 2. Paraneoplastic syndromes and patient parameters

Paraneoplastic syndrome	Case	Value	Normal Range
Hypocalcemia	Patients H, I	> 9 mg/dL	9-12 mg/dL
Hypoalbuminemia	Patient G, I	> 2.0-2.5 g/dL	3.5-5 g/dL
Hypoglycemia	Patients G, H, I	60-65 mg/dL	75-125 mg/dL
Hypercalcemia	Patient J	18.8 mg/dL	9-12 mg/dL
Hyperkaelaemia	Patients G, H, I	< 5 mEq/l	3.5-5 mEq/l
Hyponatremia	Patient G	< 120 mEq/l	136-145 mEq/l

The clinical signs of mastocytoma may be complicated by signs attributed to the release of histamine, heparin, and other vasoactive amines. Occasionally, mechanical manipulation during examination may lead to degranulation and erythema in the areas adjacent to the formation. This phenomenon was given a name: Darier's sign (Figure 6). Healing after surgical excision is also difficult due to the enzyme gelatinase secreted by malignant mast cells. This is also the reason for the appearance of specific edema. When mast cell degranulation occurs ulcers form in the stomach or intestines, in different stages of evolution and cause vomiting and loss of appetite (patients G and I), lethargy and melena (patient H).

H2 receptor antagonists counteract the effects of degranulation and prevent the aggravation of the disease (Table 3). Less commonly, these chemicals and compounds can cause anaphylaxis (Pinard, 2020). Although none of our patients exhibited this type of emergency, we recommend antihistaminic treatment preventively (Table 3).

The treatment must also include antibiotics (Cefort) and anti hemorrhagic therapy (Etamsylate and vitamin K)

Table 3. Selected treatment for MCT patients

Drug action	Product	Case
Corticosteroids	Hydrocortisone	Patients G, H, I
	Dexamethasone	
	Prednison	
H2 receptor antagonists	Ranitidine	Patients G, H, I
	Zantac	
	Arnetin	
	Cimetidine	

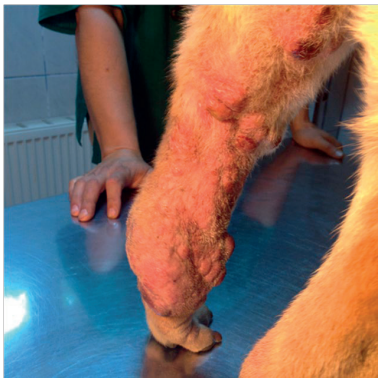

Figure 6. Severe edema and infiltrative cutaneous mastocytosis. Darier's sign (Pacient G)

Lot 3 - The iatrogenic disease is the complete palette of pathological syndromes induced by chemotherapy toxicity, the whole variety of symptoms that occur following the non-selective action on the entire

organism due to the chemotherapy overlapping the neoplastic disease and paraneoplastic syndromes. Four of the 14 patients have exhibited at least one of these symptoms as emergencies.

The emergencies we encountered in the 4 cases included in this lot, 2 mammary gland adenocarcinomas (*Patient K*, common breed, 13 years, F; *Patient L*, Maltese, 12 years, F) and 2 lymphoma cases (*Patient M*, Boxer, 8 years, M; *Patient N*, Dalmatian, 6 years, M)

Emesis is the reflux of gastric contents caused by digestive tract damage.

In our cases, the emesis was precocious - determined by the administration of an oral chemotherapy drug with digestive absorption (Cyclophosphamide) for patient M and N and ultra precocious - based on the Pavlovian reflex created by an experience before chemotherapy for patient K and L.

Table 4. Selected treatment for iatrogenic emesis

Symptome	Drug action	Product	Case
Emesis	5-HT_3 antagonist antiemetic	Granisetron	Patients M, K
		Emeset	
		Ondansetron	
		Osetron	
		Zophren	
	H2 receptor antagonists	Ranitidine	Patients K, M, N
		Zantac	
		Arnetin	
		Cimetidine	

Enteric syndrome - Diarrhea occurs as a consequence of the local irritation by some chemotherapy agents or oral treatments at high doses and for prolonged periods, which accentuates peristalsis, information obtained through ultrasound examination (patient M) and has been treated symptomatic with No-Spa 40 mg/2 ml s.c. (40 mg every 24 hours), Entero-Chronic (4 g/day/30 days) and Enteroguard M (1 tb/3 kg, for 7 days) for patient M

Chemotherapy immunosuppression is a syndrome that initially affects cell mediated and then humoral mediated immunity, due to mechanisms such as: inhibition of stem cell proliferation, destruction of circulating or fixed immunocompetent cells, decreased circulating antibody concentration.

Thrombocytopenia in patient L, a value of 60 thousand/microL (normal value 117-460 thousand/microL) was determined by mega-karyocyte cytolysis at the central medial level by the metabolic inhibitor effect. Clinically, it was manifested by a purpura syndrome following administration of the chemotherapy agent Cyclophosphamid. We decided to give the pacient a blood transfusion and recommend a long term treatment with immunomodulatory drugs after the patient overcomes the acute episode.

Acute interstitial nephritis and hemorhagic cystitis diagnosed in patient M are caused by the excretion of the chemotherapy he had received with Cyclophosfamide at renal level. The toxic effect of the chemotherapy determined an acute reaction in the kidneys and severe irritation of the mucosa in the urinary bladder, visible in the ultrasound. The selected therapy was a cocktail of Etamsilate every 4h and Adrenostazin to stop the bleeding, vitamine K p.o., and Manitol i.v. for 3 days after the patient overcomes the acute episode.

Induced jaundice syndrome is the excess accumulation of conjugated bilirubin in the tissues or blood stream. Clinically, jaundice has been noted in pigmented skin, mucous membranes and sclerotic of patient M and we classified it as: hepatic - caused by large and repeated administration of Cyclophosphamide as an agent of alkylation (Table 5). This type of symptoms are determined by the oxidative degradation that causes the occurrence of toxic liver metabolites in the form of acrolein and chloroacetaldehyde. The emergency choleretic therapy used Metoclopramid syrup p.o every 4 h, No-Spa s.c. every 8 h foar 24 h and Colebil p.o. for 3 days.

Table 5. Blood parameters for patient M

	UM	Results	Normal value
GPT/ALT	U/L 37°C	385	5-60
GOT/ASAT	U/L 37°C	72.4	>35
GGT	U/L 37°C	12.4	>9
Bilirubin	mg/dL	2.12	>0,90
Urea	mg/dL	68	>50
Creatinine	mg/dL	2.9	>1.80
Alkaline phosphatase	U/L 37°C	386	>200
Acid phospatase	U/L 37°C	132	<125
Prostatic phosphatase	U/L 37°C	189	Sub 190

Unfortunately patient M (Boxer, 8 years, M) has died during the case study. Kidney and liver samples were taken in order to confirm organ reaction to the chemotherapy (Figures 7 and 8).

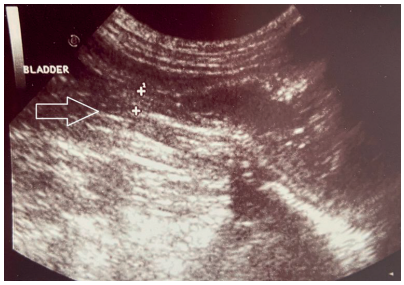

Figure 7. Bladder with visibly thickened wall due to the action of the chemotherapy (Patient M)

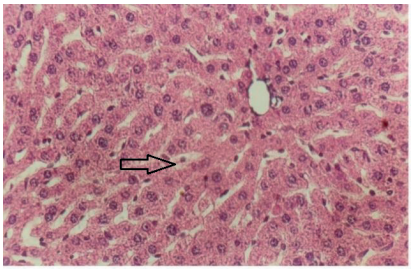

Figure 8. Liver cell necrobiosis, with caryolysis and cytolysis caused by Cyclophosphamide. Necropsy sample from patient M

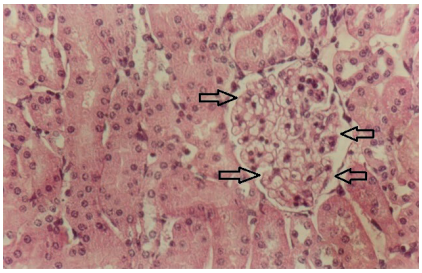

Figure 9. Glomerular edema after Cyclophosphamide therapy. Necropsy sample from patient M

CONCLUSIONS

Emergency oncology has the same principles as any other kind of medical emergency. Triage is of up-most importance. It saves time and it saves lives.

In order to have a correct and fast triage, we recommend a complete medical chart of the patient, after the initial threat is treated (hemorrhage stopped, edema treated, pain alleviated, etc.). The more we know, the better we can treat.

Speed. The information must be gathered fast and must be selected regarding the prime emergency (example: an x-ray for severe pain to confirm a fracture, in a patient with osteosarcoma, after pain management therapy has begun)

Paraneoplastic syndromes are frequent and agressive, but respond well to re-balancing therapy.

A printed Glasgow Composite Measure Pain Scale (GCMPS) is of great help in emergency situations. Also printed protocols and doses for the emergencies presented in this article are very useful. The combination of sedatives and tranquilizing drugs will improve the effectiveness of analgesics. Also edema and mastocytoma erythema respond well to high shock doses of Hydrocortisone combined with Dexamethasone

The majority of oncology emergencies have reversible effects if caught in time.

Once the emergency has passed, the correct chemotherapy and regular follow-ups will help keep things under control.

ACKNOWLEDGEMENTS

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BENIGN PROSTATIC HYPERPLASIA - PREVALENCE AND CLINICAL-SONOGRAPHIC FEATURES IN DOGS

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Abstract

Benign prostatic hyperplasia (BPH) is the most common canine prostatic disorder being diagnosed in almost 100 per cent of sexually intact male dogs over the age of seven years, as well as in animals treated with androgenic hormones. The present study reports the incidence of benign prostatic hyperplasia, regarding age and breed among dogs, commonly clinical signs, and incorporating in the clinical picture the paraclinical examinations especially ultrasonography in order to obtain an diagnosis. The research was made between 2018-2019, within the Clinic of the Faculty of Veterinary Medicine Bucharest and within private veterinary practices. Benign prostatic hyperplasia was diagnosed in 30 dogs with insidious and asymptomatic evolution recording frequently hematuria, polakisuria and prostatomegaly. The paraclinical investigation with ultrasonography aims to establish the definite diagnosis, detecting ultrasound changes suggestive -prostatomegaly with dimensions between 20-90 mm with regular homogeneous appearance or lacunar microcystic aspect. All the research that is included in the study, it is appreciated that it is of considerable importance in fundamental research on the incidence of primary factors, responsible for pathogenetic vulnerabilities useful in clinical activity in structured screening.

Key words*: benign prostatic hyperplasia, prevalence, ultrasonography, dogs.*

INTRODUCTION

Benign prostatic hyperplasia is an increase in the volume and number of non-inflammatory types of prostate epithelial cells, in simple or complex form (cystic hyperplasia) (Nizanski, 2014). It arises spontaneously in the gland as a consequence of ageing and endocrine influence and may begin as early as 2-3 years of age, becoming cystic over 4 yrs of age (Sun, 2017).
Benign prostatic hyperplasia mainly affects intact male dogs, almost 100%, as well as animals treated with androgen hormones, being rare in cats (Dhivya, 2012).
The age is recorded as a predisposing factor, and it is estimated that approximately 50% of intact male dogs will develop BPH by the age of 5, 60% by the age of 6 and 95% by the age of 9 (Juodziukyniene, 2016).
Estrogens act by increasing the number of prostate receptors for androgens, respectively by forming free-radical metabolites that affect the prostate by altering its response to the action of androgens (Golchin, 2019). Androgens act through dihydrotestosterone (resulting from the transformation of testosterone by the 5th reductase) and increase the number and size of epithelial cells and prostate stroma (Jubb, 2015).
Clinical signs associated with the condition are constipation, hemospermia, tenesmus and hematuric syndrome being triggered by dysuria and urethral stenosis due to enlargement of the prostate (Renggli, 2010; Pinheiro, 2017) or as a result of hyperplastic tissue with increased vascularity (Golchin, 2019).

MATERIALS AND METHODS

The research took place between 2018-2019, within the Clinic of the Faculty of Veterinary Medicine Bucharest and within the private veterinary practices.
The studies and investigations initiated and carried out started from the suspicion and confirmation of the diagnosis of benign prostatic hyperplasia which involved the development of clinical examination and paraclinical methods translated by performing

urine analysis, biochemical and hematological profile and imaging methods.
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.
Determination of hematological parameters was performed with IDEXX VetAutoread Hematology Analyzer and Scil Vet Abc Plus and Genrui - 5-Part Auto Hematology Analyzer KT-6610 and biochemical parameters with Spotchem EZ SP 4430 (Figure 6) and SkylaTM VB1 Veterinary Clinical Chemistry Analyzer.
Imaging was performed using the Esaote Veterinary MyLab 30 ultrasonography.

RESULTS AND DISCUSSIONS

Benign prostatic hyperplasia was diagnosed in 30 dogs between age 3 and 18 years, of different breeds (Figure 1).

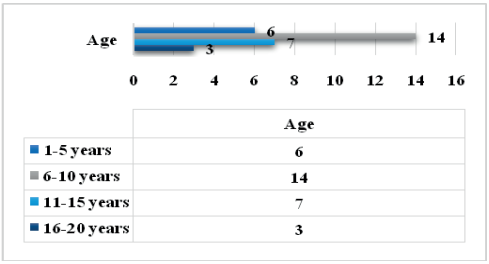

Figure 1. Distribution by age in dogs with benign prostatic hyperplasia

The medical history of the patiens was followed in order to identify possible pre-existing conditions and possible repetitive clinical signs relevant to a diagnosis, so the data collected consisted of the manifestation of blood urination in 8 individuals, urination with a high frequency (n = 7), urination in impermissible places (n = 1), pain on palpation in the pelvic area (n = 1), a urination with an increased amount (n = 1) or lack of urination (n = 1) and the presence of inhomogeneities at the testicular level (n = 1).
The evaluation of patients involved recorded body temperature (°C) with the following values 38.3 (n = 7), 39.2 (n = 2), 38.2 (n = 2), 38.1 (n = 3), 38.7 (n = 3).), 39.3 (n = 2), 38.4 (n = 2), 38.5 (n = 3), 38.9 (n = 3), 38.6 (n = 2) and 37.9 (n = 2), data on the general condition of the animal, assessed as good (n = 30), with absent uremia (n = 30) and clinical information on the frequency of urination being of a pollakisuric nature in 5 patients, normal in 24 individuals and with strangulation in one animal.
The urine was examined visually from a colorimetric point of view, being noticed by the owner a urine of red color (n = 7), yellow - orange (n = 5) and yellow in a percentage of 60.00% (n = 18).
Urethral discharges independent of the act of urination with initial/incipient hematuria suggest the existence of a prostate condition. The nature of the prostate condition was confirmed by subsequent evaluations.
The clinical evaluation was completed by the physical examination of the urinary and genital tract, the following aspects being reported:

- absence of physical changes detectable through inspection and palpation (n = 30);
- absence of renal pain sensitivity (n = 30);
- absence of pain sensitivity in the bladder (n = 30);
- by the method of clinical exploration with the help of the index called transrectal digital palpation, a prostatomegaly was found in a percentage of 100% (n = 30) with painful manifestation in 15 patients (30.00%) or without manifestation of painful sensitivity in 15 individuals.

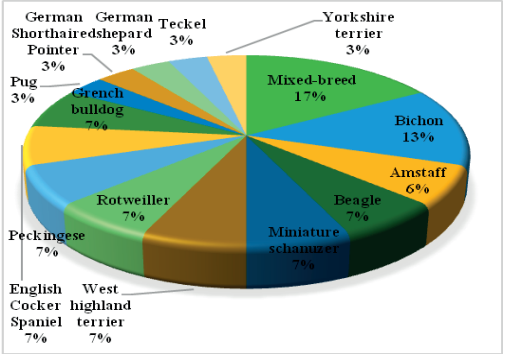

Figure 2. Distribution by breeds in dogs with benign prostatic hyperplasia

The correlative character of the registered clinical-anamnetic data indicates an insidious and asymptomatic or poorly expressed

evolution in benign prostatic hyperplasia, with reduced and uncharacteristic systemic changes, and as a dominant clinical for the algorithm od diagnosis were - hematuria (43.55%), polakisuria (19.35%) and prostatomegaly (100%).

Urinary parameters were evaluated by diagnostic strips or with the help of the automatic analyzer obtaining the following values - leukocyturia of different intensities, urobilinogen, a urine sample with positive nitrates, proteinuria, urinary pH with 6.5 in 10 patients, pH 7 in 7 individuals, pH 6 (n = 7), 9 (n = 2), 5, 5.5, 7.5 and 8, the presence of blood suspicion with + (n = 11), ++ (n = 5), +++ (n = 12), ++++ (n = 2) and urinary density was 1030 in 11 animals, 1020 in 7 individuals, 1025 (n = 2), 1035 (n = 2), 1045 (n = 2), 1040 (n = 2), 1050 (n = 2), 1000 and 1009.

Examination of the urine sediment by light microscopy confirmed the presence of red blood cells in the urine, the presence of leukocytes, epithelial cells, ammonia-magnesium phosphate crystals, reduced microbial flora and bilirubin crystals.

The paraclinical investigation through the ultrasound imaging method aims to establish the definite diagnosis, detecting ultrasound changes suggestive of benign prostatic hyperplasia:

- prostatomegaly correlated with age and species with dimensions between 20-30 mm at a percentage of 60.00% (n = 18), 30-40 mm (n = 4), 40-90 mm (n = 10) (Figures 3, 4, 5, 6, 7, 8);
- regular homogeneous appearance in 19 individuals in a percentage of 63.33%;
- lacunar microcystic appearance in 11 patients.

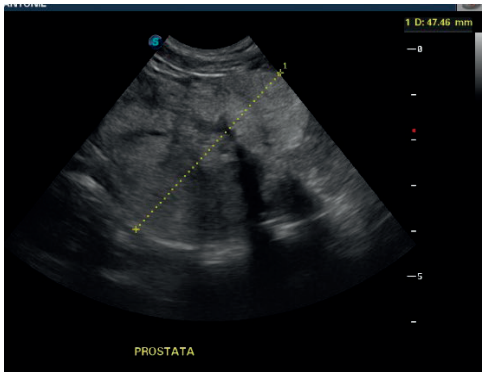

Figure 3. Benign prostatic hyperplasia - prostatomegaly (47.46 mm)

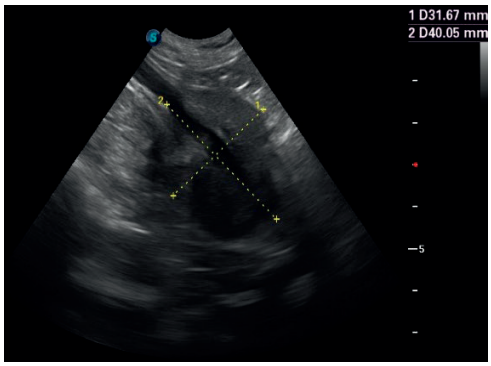

Figure 4. Benign prostatic hyperplasia - prostatomegaly (31.67 mm)

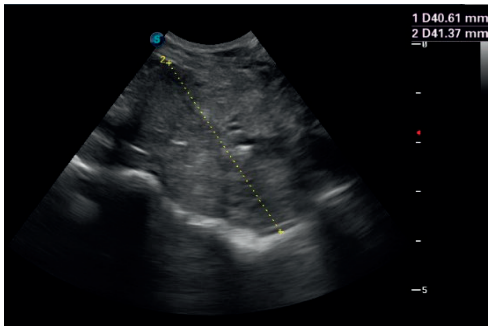

Figure 5. Benign prostatic hyperplasia - prostatomegaly (41.37 mm)

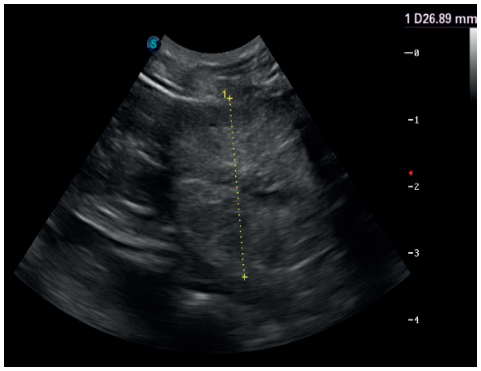

Figure 6. Benign prostatic hyperplasia - prostatomegaly (26.89 mm)

In addition to the initial hematuria, dysuria, and tendency to constipation, which suggests prostate damage, ultrasound examination allows the most accurate assessment of changes in volume, contour, echogenicity, and echostructure in benign prostatic hyperplasia, prostatitis, and intra / paraprostatic cysts.

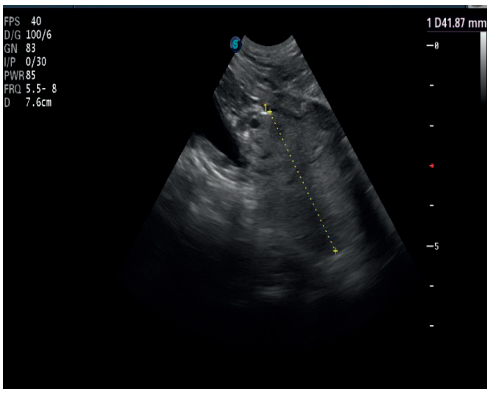

Figure 7. Benign prostatic hyperplasia - prostatomegaly (41.87mm)

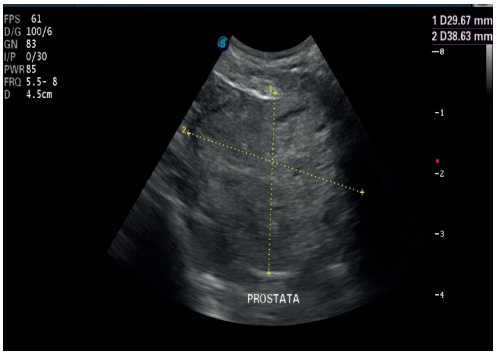

Figure 8. Benign prostatic hyperplasia - prostatomegaly (29.67 mm)

Investigations of the blood biochemical and hematological profile performed in 10 patients allowed to obtain the paraclinical picture of the animal presented in the clinic and to discover the existence of risk factors for the development of certain diseases, referring to the normal values of the device and the existing context (Tables 1 and 2).

Table 1. Results of analysis in patients with benign prostatic hyperplasia

Parameters	10 years	18 years	11 years	6 years	8 years	7 years
AST *(U/l)*	**18**	**48**	**18**	**20**	**24**	**-**
ALT *(U/l)*	**187**	**18**	**91**	**40**	**39**	**30**
GGT	**23**	**0**	**-**	**0**	**10**	**0**
Crea *(mg/dL)*	**1**	**1.4**	**1.3**	**1.7**	**0.9**	**0.8**
Uree *(mg/dL)*	**28**	**34**	**20**	**91**	**21**	**7**
TP *(g/dL)*	**6.9**	**6.1**	**5.7**	**-**	**6.2**	**6.2**
Glucosis *(mg/dL)*	**116**	**99**	**91**	**86**	**103**	**97**
ALKP *(U/l)*	**127**	**66**	**-**	**1004**	**114**	**99**

Table 2. Results of analysis in patients with benign prostatic hyperplasia

Parameters	10 years	13 years	18 years	10 years	8 years
AST *(U/l)*	**9**	**-**	**-**	**-**	**10**
ALT *(U/l)*	**63**	**54**	**43**	**-**	**134**
GGT	**20**	**3**	**1**	**-**	**-**
Crea *(mg/dL)*	**0.7**	**0.7**	**1.6**	**-**	**0.7**
Uree *(mg/dL)*	**30**	**16**	**32**	**-**	**20**
TP *(g/dL)*	**6.7**	**6**	**7.3**	**-**	**-**
Glucosis *(mg/dL*	**76**	**98**	**106**	**-**	**98**
ALKP *(U/l)*	**461**	**434**	**76**	**-**	**-**
HGB *(g/dL)*	**18**	**14.3**	**11**	**14.7**	**-**
HCT *(%)*	**51.9**	**42**	**6.7**	**44.3**	**-**
Grans *(%)*	**82**	**75.9**	**85**	**73**	**-**
Grans *(K/µL)*	**6.2**	**10.4**	**13.7**	**6.8**	**-**
WBC *(mii/mm³)*	**7.5**	**7.17**	**16.1**	**9.3**	**-**
PLT *(K/µL)*	**68**	**532**	**936**	**667**	**-**

CONCLUSIONS

Benign prostatic hyperplasia can vary in severity, with clinical signs that are often non-specific leading to common diagnoses of prostatic syndrome.

Our study indicates that a correct diagnosis will be obtain by peforming an complete physical examination including transrectal digital palpation of the prostate that should always be included in a male health check and by ultrasonography that has a great value for diagnosis.

Ultrasound imaging was the examination of choice by visualizing the prostatic gland, its urethra, the bladder and the locoregional lymph nodes.

All the research included in the study, it is appreciated that it is of considerable importance in fundamental research on the incidence of primary factors, responsible for pathogenetic vulnerabilities useful in clinical activity in structured clinical and paraclinical screening .

The complex form of prostatic hyperplasia, which usually occurs in geriatric dogs, is an ideal research model due to its unique pathological feature, including not only glandular hyperplasia, but also an increase in the stromal components of the prostate**.**

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ANTIGEN EXPRESSION ENHANCEMENT OF *Mycobacterium bovis* AN5

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Abstract

The revealing value of the purified protein antigens designed for the bovine tuberculosis screening registers considerable variations between the different batches of product, even in the case of the same obtaining protocol and of the same operator.
In order to limit variations in the potency of the purified protein extract and to improve the expression of immunodominant proteins, different culture protocols have been designed, which, under reproducibility conditions, could reduce the variation limits between batches.
The study of the genetic profile identified the AN5 strain used as a virulent Mycobacterium bovis strain belonging to the bovis-caprae lineage. The potency of the purified protein extract was quantified by testing dilutions on guinea pigs. The metabolism modulation of the studied strain, by alternative culture conditions, improved its antigens profile, these being the key compounds for the potency of the tuberculin.

Key words*: AN5 strain, bovine tuberculin, guinea pigs, Mycobacterium bovis, potency.*

INTRODUCTION

Bovine tuberculosis is an infectious disease caused by M*ycobacterium bovis* that affects cattle, other domesticated animals and certain free or captive wildlife species. It is usually characterised by formation of nodular granulomas known as tubercles. Although commonly defined as a chronic debilitating disease, bovine tuberculosis can occasionally assume a more progressive course. Any body tissue can be affected, but lesions are most frequently observed in the lymph nodes (particularly of the head and thorax), lungs, intestines, liver, spleen, pleura, and peritoneum. It should be noted that other members of the *M. tuberculosis* complex, previously considered to be *M. bovis*, have been accepted as new species despite identical 16s RNA sequences and over 99.9% identity of their genome sequences. These include *M. caprae* (Aranaz et al., 2003) (in some countries considered to be a primary pathogen of goats) and *M. pinnipedii* (Cousins et al., 2003), a pathogen of fur seals and sea lions. These two new species are known to be zoonotic. In Central Europe, *M. caprae* has been identified as a common cause of bovine tuberculosis (Prodinger et al., 2005). Disease caused by *M. caprae* is not considered to be substantially different from that caused by *M. bovis* and the same tests can be used for its diagnosis.

The rigorous application of tuberculin testing and culling of reactive cattle has eliminated *M. bovis* infection from farmed bovine populations in some countries, but this strategy has not been universally successful. Extensive investigations of sporadic *M. bovis* reoccurrence have shown that wildlife reservoirs exist in some countries and can act as a source of infection for cattle, deer and other livestock. The risk that these reservoirs of infection constitute for domestic animals and humans is quite variable depending on the specific epidemiological situation for the species and the environment (Corner, 2006; Morris et al., 1994). The detection of infection in a wildlife population requires bacteriological investigation or the use of a valid testing method for the species involved (the tuberculin test is not effective in

all species) together with epidemiological analysis of information. The badger (*Meles meles*) in the United Kingdom (Wilesmith, 1991) and the Republic of Ireland (O'Reilly & Daborn, 1995), wild boar (*Sus scrofa*) in Spain (Naranjo et al., 2008), the brush-tail possum (*Trichosurus vulpecula*) in New Zealand (Animal Health Division, 1986), and several wild living species in Africa have been shown to be capable of maintaining *M. bovis* infection. Other species of *Mycobacteria*, potentially harmful to humans, such as *M. avium*, *M ulcerans*, *M. marinum* and *M. chelonae*, have been isolated from reptiles (Lupescu & Baraitareanu, 2015). Control of transmission from the wildlife population to farmed species is complex and, to date has relied on the reduction or eradication of the infected wildlife population. The use of vaccination to control the disease in some species continues to be investigated.

Although cattle are considered to be the true hosts of *M. bovis*, the disease has been reported in many domesticated and nondomesticated animals. Isolations have been made from buffaloes, bison, sheep, goats, equines, camels, pigs, wild boars, deer, antelopes, dogs, cats, foxes, mink, badgers, ferrets, rats, primates, South American camelids, kudus, elands, tapirs, elks, elephants, sitatungas, oryxes, addaxes, rhinoceroses, possums, ground squirrels, otters, seals, hares, moles, raccoons, coyotes and several predatory felines including lions, tigers, leopards and lynx (De Lisle et al., 2001; O'Reilly & Daborn, 1995).

Mycobacterium bovis has been identified in humans in most countries where isolates of mycobacteria from human patients have been fully characterised.

The official ante-mortem diagnosis relay on the tuberculin's intradermal administration in order to develop cell mediated type IV hypersensitivity, tuberculin most frequently industrially obtained from the AN5 or Vallee *M. bovis* strains.

These products' potency is currently established by statistical analysis using the parallel lines method, based on results of in vivo hypersensitivity tests carried out on guinea pigs and, to the possible extent, on the target species, as compared to the WHO (World Health Organization) reference standard (WHO International Laboratory for Biological Standards). Due to an ongoing concern for the health and welfare of laboratoy animals (Coman et al., 2019), currently, there are attempts to replace the animal tests with in vitro experiments (Spohr et al., 2015).

Tuberculin represents a complex mixture (over 800 proteins) whose production and application did not essentially change since conception (Robert Koch, 1980; Landi, 1982). The concentrate's characterization is challenging, because thermal and chemical treatments applied during production affect the proteins structure and antigenicity (Nagai et al., 1981).

The batch of bovine PPDs should be controlled for its diagnostic value, more specifically for the ability of secreted proteins and somatic components - released from autolysis, replication, mechanical and chemical interventions during production - to induce a delayed type IV cell mediated hypersensitivity reaction (Mattow et al., 2003; Sonnenberg et al., 1997). It is a well-known fact that the proteins profile of *in vitro M. bovis* cultures depends on a serie of factors, such as type of media, temperature, time, stirring, etc, which explains the differences between commercial tuberculins obtainned by different producers, or different batches of the same producer. (Sonnenberg et al., 1997; Andersen et al., 1991; Downs et al., 2012).

Also, in field studies in certain epidemiological circumstanses, differences of senzitivity and specificity have been demonstrated between PPDs produced with the same strain by different manufacturers (Rennie et al., 2010). The worldwide use for PPD production of the glycerol adapted AN5 strain, unlike field *M. bovis* strains, has led to the emergence of local variants depending on the cultivation and storage conditions of the producer. Comparative proteomics studies of different AN5 variants (UK, Brazil, Korea, Spain, Italy, Holland), but also of MTC strains (MTC, *Mycobacterium tuberculosis complex*) have proven the existence of a variable number of common proteins to *bovis* (AN5) or *tuberculosis* PPD products, but also particularities regarding the presence or expression level of *M. bovis* specific major antigens, involved in T cells activation, such as MPB70, MPB83 and ESAT-6 like (Roperto et al., 2017; Cho et al., 2015; Borsuk et al., 2009; Prasad et al., 2013; Pelayo

et al., 2009). Comparative genome analysis of the virulent and attenuated, or virulent and AN5 *M. bovis* strains, has demonstrated, on one hand, the lack of deletions or major restructuring, highlighted in the case of the BCG strain, and on the other hand, an acummulation of SNPs (single nucleotide polymorphisms), responsible for the alteration of the global gene expression profile (Pelayo et al., 2009; Canevari et al., 2014; Smith et al., 2003; Smith et al., 2006; Inwald et al., 2003).

MATERIALS AND METHODS

Bacterial strain and cultivation methods. The *M. bovis* AN5 strain was cultivated on modified Dorset-Henley (mDH) medium, with occasional returns to the glycerinated potato. A *M. avium* strain was used as a negative control for the taxonomic classification per genus/species, gene expression and, implicitly, membership of MTC.

For the purpose of the current study, the *Mycobacterium* strains were inoculated on Lowenstein-Jensen agar (LJ, Becton-Dickinson) and glycerinated potato/mDH medium at 37°C for 3 weeks, using 100µl of cellular suspension with the same density as McFarland standard 1. Colonies propagation was performed by 2 successive steps on mDH medium (in house prepared) at 37°C for 3 weeks each: small and medium volumes of 150 ml and respectively 1 liter were inoculated with few mDH adapted colonies (origin: glycerinated potato) and respectively ≈ 1-5 cm^2 of mDH aerobic culture by biofilm transfer.

Isolation and purification of nucleic acids and proteins. The ZR Fungal / Bacterial DNA Kit (ZymoReasearch - miniprep), whose protocol includes a glass beads lyses, was used to extract genomic DNA. For mRNA isolation and purification two different kits were used, both based on the phenol-chloroform and guanidine isothiocyanate extraction method: Ambion ™ TRIzol ™ Plus RNA Purification Kit (Fischer Scientific) and Direct-zol MiniPrep (ZymoResearch). Intracellular (membrane and cytosolubile) proteins were extracted (+4°C) and stored (-85°C) in a cell lyses buffer (total lyses buffer, TLB: 62.5 mM Tris pH 6.8, 2% (v/v) SDS; 10% (v/v) glycerol, 6M urea, 0.01% (w/v) blue bromophenol, 0.01% (w/v) phenol red, 5% (v/v) beta mercaptoethanol) starting from the bacterial sediment (centrifugation: 3500 g, 30 min.) generated by aerobic biofilm or anaerobic cell deposit. Proteins secreted in the culture medium were concentrated (+4°C) and stored (-85°C) in a preservation buffer (4x native buffer, 4x NB: 40% (v/v) glycerol, 0.5M Tris pH 6.8) after precipitation in ammonium sulfate (0.476% w/v) at +4°C overnight and sedimentation by centrifugation at 20000 g for 1 hour at +4°C of the entire proteins' suspension.

Preparation of M. bovis AN5 sensitizing antigen. The bacterial culture (age: 45 days post inoculation, medium: mDH; cultivation: 37°C) was thermally inactivated (1 h, 100°C), aseptically harvested and 3 times washed with a sterile phenol saline solution (5‰ v:v) by centrifugation at 1600 g for 30 min. The sediment was resuspended in a phenol saline solution (10‰ v:v) and mineral oil (Montanide ISA206/Seppic) and homogenized until a white homogenous suspension was obtained. The suspension represents the sensitizing antigen of guinea pigs used in the biological dilutions test of the bovine PPD products (potency test).

Preparation of bovine AN5 PPD. The bovine AN5 PPD was prepared following a protocol previously published (Patrascu et al., 1986), with minor modifications. A fresh inoculums (aerobic biofilm: 1-5 cm^2, age: 2-3 weeks, medium: mDH, temperature: 37°C) was seeded on mDH medium (flask: Roux plate, temperature: 37-39°C, time: ≈3 months).

Well-developed cultures were thermally inactivated (2 h - 100°C) and subjected to a sequential filtration: I. bacterial mass retention, II. medium clarification and III. tangential ultra-filtration, in order to obtain the exoproteins concentrate. Next, the exoproteins concentrate was mixed in a ratio of 1:1 (v:v) with a preservative diluents solution (1.64% (w/v) Na_2HPO_4; 0.36% (w/v) KH_2PO_4; 1% (w/v) NaCl; 20% (v/v) glycerol) and subjected to tyndallization (1 h, 65-70°C). The result represents the exoproteins fraction of the bovine PPD preparation. The filter retained bacterial mass was resuspended in a physiological saline solution (pH 7.2, 5-10% of the initial volume) and 2-3 times washed by tangential ultra-filtration. The bacterial mass was subjected to hydrolysis (2 h/100°C) after

homogenization in a 0.3-0.4% (v/v) HCl solution, using a mass:volume ratio of 1:10. Endoproteins' solubilization was accomplished by alkalization with 33% (w/v) NaOH by stirring till the pH reach 7.5. The liquid phase was filtered, concentrated and tyndallized by the same method as the medium filtrate. The result represents the endoproteins fraction of the bovine PPD preparation. Both fractions were pooled, homogenized and dispensed into flasks. The end product was tyndallized and stored at +4°C, light protected.

Molecular identification and characterization. Amplification of representative DNA sequences for the *M. bovis* species identification (e.g., *mtp*40, *lep*A, *lpq*T), *tuberculosis* complex membership (e.g. IS1081, IS6110, MPB70) and phylogenetic classification to the *bovis-caprae* lineage (e.g. MIRU-VNTR, RD) were performed according to the literature (Supply et al., 2005/2006; Reddington et al., 2011; Warren et al., 2006; Parsons et al., 2002; Mokaddas and Ahmad, 2007; Liebana et al., 1996; Mahmoudi et al., 2013; McNabb et al., 2004; Houben et al., 2009; Greisen et al., 1994) and the dedicated website recommendations (http://www.miru-vntrplus.org/MIRU), using a GeneAmp® PCR 9600 system (Applied Biosystem) and the High Fidelity PCR and Fast Start High Fidelity PCR System kits (Roche) as recommended by the manufacturer. The amplification products were visualized with ethidium bromide on TAE 1x agarose gels (Sigma-Aldrich) using a SubCell system (BioRad) and photographed with a ChemiDoc XRS + cabinet (BioRad).

Gene expression. Analysis of gene expression at mRNA level was performed for the main virulence factors, replication and structural markers according to the literature (Supply et al., 2005/2006; Reddington et al., 2011; Warren et al., 2006; Parsons et al., 2002; Mokaddas and Ahmad, 2007; Liebana et al., 1996; Mahmoudi et al., 2013; McNabb et al., 2004; Houben et al., 2009; Greisen et al., 1994), using a GeneAmp® PCR 9600 system (Applied Biosystem) and 2 Roche kits: Titan One RT-PCR system and Transcript One Step RT-PCR kit, as recommended by the manufacturer. The amplification products were visualized with ethidium bromide on TAE 1x agarose gels (Sigma-Aldrich) using a SubCell system (BioRad) and photographed with a ChemiDoc XRS + cabinet (BioRad).

Western-blot. Total cellular proteins and secreted proteins - after thermal (5min/95°C) and chemical (0.1% (v/V) beta-mercaptoethanol) denaturation - were separated by SDS - PAGE (denaturation gel electrophoresis) in 10% or 12% polyacrylamide and transferred to the nitrocellulose membrane (TransBlot Semi-Dry BioRad system, 18V-30 min) with 1x buffer solution (Towbin w. SDS/Santa Cruz, sc-24954). The major virulence factors highlighting was achieved with polyclonal antibodies (dilution 1:1000) against to MPB83 and ESAT-6 / CFP-10 binary system as follows: I. primary antibodies - rabbit anti CFP10 (abcam, ab45074 - IgG rabbit anti-ESAT-6 (abcam, ab45073 - IgG) and chicken anti MPB83 (LSBio, LS-C130858 - IgY); II. secondary antibodies - goat anti rabbit IgG - HRP (Santa Cruz Biotechnology, sc-2004 - IgG) and goat anti chicken IgY - HRP (antibodies-online, ABIN101022 - IgG).

Biological value control. The potency of the bovine AN5 PPD product was tested through the biological dilution method. The test compares the inflammatory reactions generated by intradermal (id) inoculation of a 0.1 ml/dilution (3 times: 1 to 5) of the AN5 PPD and the International Reference Standard (NIBSC - 58500 IU) at 28-35 days post sensitizing of 400-600 grams guinea pigs with 0.5 ml inactivated *M. bovis* AN5 culture, intramuscular administered (im)(OIE, 2017; European Pharmacopoeia, 04/2007; WHO International Standard Purified Protein Derivative (PPD) of *M. bovis* tuberculin). Dilutions were performed in isotonic phosphate buffer (containing 0.005% (v/v) Tween 80) and administered according to the Latin square principle, at 3 points on each side of the guinea pigs body (see experimental chart, Table 1). The test and control groups consisted of 10 animals for the bovine PPD product and respectively the reference standard, plus 5 uninoculated animals were used as negative control. After 24-48 h post-inoculation, lesion diameters were measured at each inoculation site and the PPD potency was calculated by the of parallel lines statistical method using the CombiStats v.5.0 logic program (CombiStats 5.0).

Table 1. Experimental chart of bovine PPD inoculation in guinea-pigs sensitized with inactivated *M. bovis* AN5 antigen. T, PPD test preparation (dilutions: T1 1/20; T2 1/100; T3 1/500); S, standard (dilutions: S1 1/32.5; S2 1/162.5; S3 1/812.5)

Guinea pig no.	LEFT			RIGHT		
	Head	Trunk	Tail	Head	Trunk	Tail
1	T_3	S_3	T_1	T_2	S_2	S_1
2	S_2	T_3	S_1	S_3	T_1	T_2
3	S_3	T_2	S_2	S_1	T_3	T_1
4	T_2	S_1	T_3	T_1	S_3	S_2
5	S_1	T_1	S_3	S_2	T_2	T_3
6	T_1	S_2	T_2	T_3	S_1	S_3
7	T_2	S_3	S_1	S_2	T_1	T_3
8	S_3	T_1	T_3	S_1	S_2	T_2
9	S_1	S_2	T_2	T_3	S_3	T_1

RESULTS AND DISCUSSIONS

Cultivation of bacterial strains and biological sampling. *Mycobacterium* strains originally grown on the solid medium (Lowenstein-Jensen and glycerinate potato) formed rough white-yellowish colonies, confluent after 3 weeks. Propagation on modified Dorset-Henley medium of these colonies allowed the formation of an aerobic biofilm (thickness about 1-2 cm). The aging of culture led to an anaerobic deposit formation and change of the culture medium pH (direct observation of the color change: yellow to brown). The biological sampling is described in Table 2 - initial analisys and Table 3 - readaptation of *M. bovis* AN5 strain from Lowenstein-Jensen onto glycerinated potato/mDH.

Table 2. Biological sampling for the analisys and gene expression experiments and for potency testing of AN5 bovine PPD

Strain	Sample labeling	Growth medium	Pasage	Biological sample
M. bovis AN5	1	Lowenstein Jensen	initial/tube P3	Aerobic biofilm
M. bovis AN5	2	glycerinated potato / mDH	initial /tube P2	Aerobic biofilm
M. avium	3	Lowenstein Jensen	initial /tube P3	Aerobic biofilm
M. avium	4	glycerinated potato / mDH	initial /tube P2	Aerobic biofilm
M. bovis AN5	5	mDH	intermediary/ Roux plate	Aerobic biofilm
M. bovis AN5	6	mDH	intermediary/ Roux plate	Cellular suspension (3ml)
M. avium	7	mDH	intermediary/ Roux plate	Aerobic biofilm
M. avium	8	mDH	intermediary/ Roux plate	Cellular suspension (3ml
M. bovis AN5	9	mDH	final/cultivation flask	Aerobic biofilm
M. bovis AN5	10	mDH	final/cultivation flask	Cellular suspension (3ml
M. avium	11	mDH	final/cultivation flask	Aerobic biofilm
M. avium	12	mDH	final/cultivation flask	Cellular suspension (3ml

Table 3. Readaptation experiment of *Mycobacterium bovis* AN5 strain from L-J medium to glycerinated potato

Pasage	Growth medium	Inoculum origin
(K)2	glycerinated potato (K)	Lowenstein-Jensen pasage 2
(L)3	Lowenstein-Jensen (L)	Lowenstein-Jensen pasage 2
TL(mDH)3	mDH (Erlenmeyer)	L3/K3
(K)4	glycerinated potato (K)	Lowenstein-Jensen pasage 4
(L)5	Lowenstein-Jensen (L)	Lowenstein-Jensen pasage 4
(K)5	glycerinated potato (K)	Lowenstein-Jensen pasage 5
(L)6	Lowenstein-Jensen (L)	Lowenstein-Jensen pasage 5

Molecular identification and characterization. MTC complex. The association of the AN5 strain to the *Mycobacterium* genus and the MTC complex (*M. tuberculosis* complex) has been demonstrated by amplifying the specific genus and complex sequences, such as the following loci: 16-23S ITS region (internal transcribed sequence) – specific to *Mycobacterium* genus (Mokaddas & Ahmad, 2007); IS1081 (IS, insertion sequence), IS6110, MPB70 - specific MTC, and *mtp*40 - specific *M. tuberculosis* (Liébana et al., 1996). Detection of an amplicon (405-408 bp), complementary to the ITS 16-23S region, demonstrated that AN5 strain belongs to the *Mycobacterium genus* (see Figure 1). The *mtp*40 sequence (396bp) was not detected (see Figure 2A - non-specific amplicons) for none of the samples used (*M. bovis* and *M. avium* - different media or passages). However, the target fragment of the MPB70 locus (372 bp) confirmed the *bovis* samples belong to the MTC complex (see Figure 2B - samples 2, 5, 9). In the case of *M. avium* extracts, there was an inconsistent amplification, present at the intermediate passage on the mDH medium, and

absent at the final passage used for the PPD preparation (see Figure 2B - samples 7 and 11). Similar results were generated by the amplification of the IS1081 sequence (see Figure 2C, amplicon 238bp). IS6110 amplification generated a 245bp product for all used DNA extracts (see Figure 2D), except for sample 1 (*M. bovis* AN5, passage on the Lowenstein-Jensen medium).

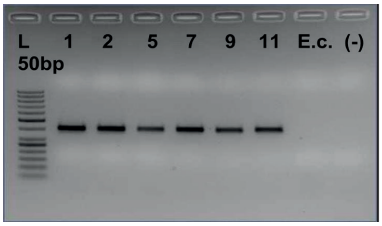

Figure 1. Identification of the *Mycobacterium* genus: 16-23 ITS / 405-408bp. The biological samples used (total genomic DNA) are numerically coded: 1 - initial passage *M. bovis* AN5 /LJ; 2 - initial passage *M. bovis* AN5 /glycerinated potato; 5 - intermediate passage *M. bovis* AN5 /mDH; 7 - intermediate passage *M. avium* /mDH; 9 - final passage *M. bovis* AN5 /mDH; 11 - final passage *M. avium* /mDH. E.C. - *Escherichia coli*; (-), only reagents; L, Ladder 50bp (Thermo Scientific); LJ, Lowenstein-Jensen medium; mDH, modified Dorset Henley medium. 2% agarose gel, 100V-30 min, TAE1x. Representative results for double experiments conducted in triplicates per biological sample

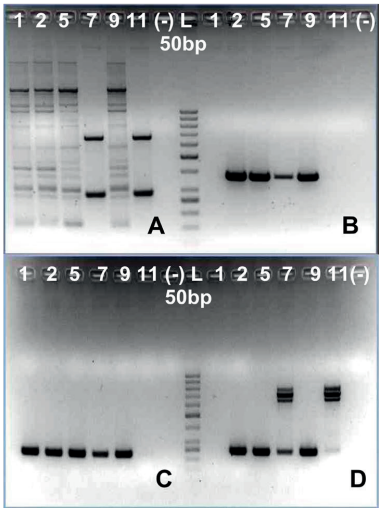

Figure 2. PCR - MTC discrimination: A - mtp40/*M. tuberculosis* (396bp) members; B - MPB70/MTC complex (372 bp); C - IS1081/MTC complex (238 bp); D-IS6110/*Mycobacterium* genus (245 bp). The biological samples used (total genomic DNA) are numerically coded: 1 - initial passage M. bovis AN5 /LJ; 2 - initial passage *M. bovis* AN5 /glycerinated potato; 5 - intermediate passage *M. bovis* AN5 /mDH; 7 - intermediate passage *M. avium* /mDH; 9 - final passage *M. bovis* AN5 /mDH; 11 - final passage *M. avium* /mDH. (-), only reagents; L, Ladder 50bp (Thermo Scientific); LJ, Lowenstein-Jensen medium; mDH, modified Dorset Henley medium. 2% agarose gel, 100V-45 min, TAE1x. Representative results for double experiments conducted in triplicates per biological sample

Bovis-caprae lineage. The differentiation between the zoonotic pathogens of the *bovis-caprae* evolutionary lineage (Reddington et al., 2011) is based on the amplification of the *lep*A (elongation factor/bacterial DNA replication), *lpq*T (essential lipoprotein/in vivo mycobacterium growth) and RD1 (region of difference 1, absence to *M. bovis* BCG strains) (Reddington et al., 2011; Qin et al., 2006; Rezwan et al., 2007; Sassetti & Rubin, 2003; Huard et al., 2006; Behr et al., 1999). The specific primers for *Mycobacterium caprae lep*A sequence determined the amplification of a 155 bp fragment for all *bovis* extracts but not for *avium* ones (negative control/species, see Figure 3A). The *bovis-caprae* lineage was confirmed for the *M. bovis* AN5 strain by usage of *lpqT* specific sequences that generated a 141 bp amplicon (see Figure 3B). The presence of the differentiation region 1 in the *M. bovis* strain genome (see Figure 3C, amplicon 117bp) indicates virulence stability reckless of the number of passages, both on solid media (LJ and glycerinate potato) or liquid media (mDH).

VNTR- RD profile. The degree of phylogenetic relatedness of the AN5 strain with *M. bovis* circulating strains has been established by amplification of 24 MIRU-VNTR regions (mycobacterial interspersed repetitive units - variable number tandem repeats) and 8 RDs (region of difference), an essential character for the diagnostic value of the bovine PPD product (Ghielmetti et al., 2017; Afaghi-Gharamaleki et al., 2017; Supply et al., 2007; Warren et al., 2006; Parsons et al., 2002). The amplification conditions of the MIRU-VNTR and RD sequences are those previously published, using the Roche PCR kits (Warren et al., 2006; Parsons et al., 2002; Supply, 2005). The designation of the MIRU-VNTR alleles took into account the amplicon size and the repeating unit length according to the known data (see Table 4). For MIRU 20 and 24 loci (as well for RD 5 and 11 regions no amplicons were obtained (see Table 4; Afaghi-Gharamaleki et al., 2017; Sun et al., 2012; Jeon et al., 2008; Iwamoto et al., 2007). The AN5 strain membership to the *bovis* virulent type has been determined based on the VNTR-RD profile according to the published algorithm: www.miru-vntrplus.org, as compared to the declared profile of registered *Mycobacterium* strains.

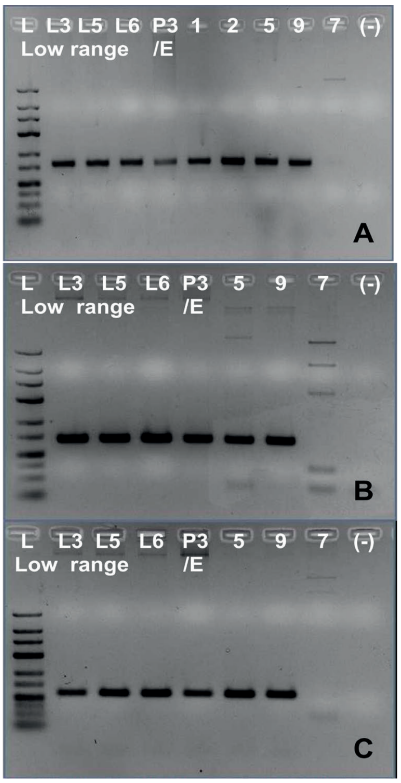

Figure 3. PCR - *Bovis-caprae* lineage: A - *lep*A/ *M. caprae* (*155bp*); B - *lpq*T/*M. bovis*, *M. bovis BCG* and *M. caprae* (*141bp*); C - RD1/complex MTC (*117bp*). The used biological samples (total genomic DNA) are encoded in letters and numbers: L3 - *M. bovis* AN5 strain/passage 3/LJ; L5 - *M. bovis* AN5 strain/passage 5/LJ ; L6 - *M. bovis* AN5 strain/passage 6/LJ; P3/E - *M. bovis* AN5 strain /passage 3/mDH; 1 - *M. bovis* AN5 primary passage/LJ; 2 - *M. bovis* AN5 primary passage/glycerinate potato; 5 - intermediary passage *M. bovis* AN5/mDH; 7 - intermediary passage *M. avium*/mDH; 9 - final passage *M. bovis* AN5 /mDH. (-), only reagents; L, ladder Low range (Thermo Scientific); LJ, Lowenstein-Jensen medium; mDH, modified Dorset Henley medium. 2.5%, agarose gel, 100V-45 min, TAE1x. Representative results for double experiments conducted in triplicates per biological sample

Table 4: MIRU-VNTRs and RDs profile of *M. bovis* AN5 strain (n.a., not applicable/not amplified)

No.	Locus	Repetitive unit (bp)	Amplicon (bp)	Allele
1	MIRU 2	53	380	2
2	Mtub 04 / 42	51	600	1
3	ETR C / 43	58	440	5
4	MIRU 4 / ETR D	77	404	3
5	MIRU 40	54	462	2
6	MIRU 10	53	550	1
7	MIRU 16	53	670	2
8	Mtub 21 / 1955	57	270	3
9	MIRU 20	77	n.a.	
10	QUB-11b	69	620	1
11	ETR A	75	630	6
12	Mtub 29 / 46	57	500	3
13	Mtub 30 / 47	58	480	4
14	ETR B / 48	57	620	5
15	MIRU 23	53	350	4
16	MIRU 24	54	n.a.	
17	MIRU 26	51	480	4
18	MIRU-27 / QUB-5	53	700	4
19	Mtub 34 / 49	54	450	2
20	MIRU 31 / ETR E	53	620	3
21	Mtub 39 / 52	58	380	2
22	QUB-26	111	200	2
23	QUB-4156 / 53	59	500	2
24	MIRU 39	53	650	2
25	RD1	n.a.	117	present
26	RD3	n.a.	500	present
27	RD4	n.a.	172	absent
28	RD5	n.a.	n.a.	absent
29	RD9	n.a.	108/206	absent
30	RD10	n.a.	202	absent
31	RD11	n.a.	n.a.	absent
32	RD12	n.a.	306	absent

Gene expression. RNA samples extracted from the aerobic biofilm or anaerobic deposit were subjected to reverse transcription in order to study the gene expression of the major structural proteins and replication markers as well as of some virulence factors. The gene expression level was normalised by amplification of a short 16S*r*DNA fragment (120bp), according to literature data (see Figure 4A) (Greisen et al., 1994). Starting from the premise of stable expression along the passages on solid or liquid media, we chose the *hsp*65 gene (heat shock protein 65 kDa) as representtative marker of mycobacteria (McNabb et al., 2004). The result of the experiment (amplicon of 441 bp), developed under the published conditions, reinforced the information obtained previously by the 16S*r*RNA fragment revertranscription (see Figure 4B). In the case of the *lepA* elongation factor (see Figure 4C) and lipoprotein *lpqT* (see Figure 4D) - essential elements in the multiplication of mycobacteria in vitro and in vivo – could be seen an accumulation of the mRNA molecules along the passages, especially on the mDH liquid medium (see sample 5 - passage 3 / mDH) (Qin et al., 2006; Rezwan et al., 2007; Sassetti & Rubin, 2003).

In the case of virulence factors, we analyzed few elements involved into pathogen dissemination (MPB70/Figure 5A), required for inhibition of phagolysosomal fusion (pknG/Figure 5B) and bacteria release from phagosome to the cytoplasm at the late stages of infection (ESAT-6/Figure 5C; CFP10 /Figure 5D).

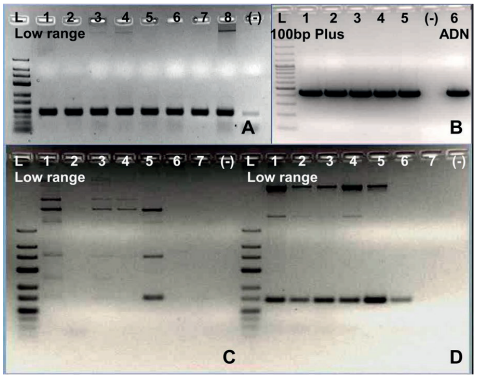

Figure 4. RT-PCR structural marker/*Mycobacterium* replication (genetic material: total RNA). A. 16S*r*RNA/eucariote bacteria (*120bp*): 1- inoculums; 2 - aerobic biofilm (passage 1/mDH);3 - anaerobic deposit (passage 1/mDH); 4 - aerobic biofilm (passage 1/glycerinated potato); 5 - aerobic biofilm (passage 2/glycerinated potato); 6 - aerobic biofilm (passage 4/ glycerinated potato; 7 - aerobic biofilm (passage 5/glycerinated potato); 8 - aerobic biofilm (passage 3/ mDH); B. *hsp65*/universal *Mycobacteria* (*441bp*): 1- inoculum; 2 - aerobic biofilm (passage 1/mDH); 3 – anaerobic deposit (passage 1/mDH); 4 - aerobic biofilm (passage 1/ glycerinated potato); 5 - aerobic biofilm (passage 3/mDH); 6 - DNA / aerobic biofilm (passage 1/LJ); C: *lep*A/specific M. caprae (*155bp*) si D: l*pq*T/ specific *M. bovis, M. bovis* BCG and *M. caprae* (*141bp*): 1- inoculum; 2 - aerobic biofilm (passage 1/mDH); 3 - anaerobic deposit (passage 1/mDH); 4 - aerobic biofilm (passage 1/glycerinated potato); 5 - aerobic biofilm (passage 3/mDH); 6 - aerobic biofilm (passage 4/ glycerinated potato); 7 - aerobic biofilm (passage 5/glycerinated potato). Inoculums - cells separated by centrifugation after washing with diluent/protocol Becton Dickinson. L, ladder Low range and 100bp Plus (Thermo Scientific); (-), only reagents; mDH, modified Dorset Henley medium; LJ, Lowenstein-Jensen medium. 2.5% agarose gel, 100V-45 min. Representative results for double experiments conducted in triplicates per biological sample.

Amplification of the messenger RNA (mRNA) corresponding to MPB70 (372bp) and *pkn*G (321bp) proteins, developed according to the previously published conditions, demonstrated a high transcription capacity of the AN5 strain regardless of passages number (1 to 3, see Figure 5A), nature of the culture medium (liquid/mDH or solid/ glycerinated potato, see Figure 5A) or the growing period (4-12 weeks, see Figure 5B), revealing its virulent feature (Liébana et al., 1996; Houben et al., 2009; Wiker 2009; Charlet et al., 2005). The mRNA sample extracted from the 4 weeks culture did not generated a *pkn*G amplicon. Reverstranscription of ESAT-6/CFP-10 heterocomplex members generated an amplicon only for the CFP-10 sequence (*300 bp*; see Figure 5D) (Mahmoudi et al., 2013). Taking into consideration that both open reading frame (ORF) are under the control of the same promoter, probably we are dealing with a false negative result (see Figure 5C) due to mutations accumulated in the primer binding sequence, data supported by western-blot heterocomplex detection.

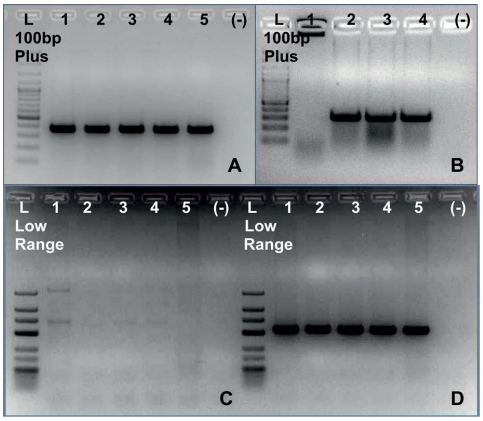

Figure 5. RT-PCR Mycobacterium virulence factors (genetic material: total RNA). A: MPB70/*Mycobacterium bovis* 70 kDa specific *M. bovis* protein (*372bp*); B: *pkn*G/serine-threonine kinase G (*321bp*); C: ESAT-6/early secretory antigenic target 6kDa (*340bp*); D: CFP-10/culture filtrate protein 10kDa (*300bp*). A, C, D: 1- inoculums; 2 - aerobic biofilm (passage 1/mDH); 3 - anaerobic deposit (passage 1/mDH); 4 - aerobic biofilm (passage 1/glycerinated potato); 5 - aerobic biofilm (passage 3/mDH); B: aerobic biofilm/mDH - 1/4 weeks culture; 2/6 weeks culture; 3/8 weeks culture; 4/12 weeks culture. L, Ladder 100bp Plus and Low range (Thermo Scientific); (-), only reagents; mDH, modified Dorset Henley medium. 2.5% agarose gel, 100V-45 min, TAE1x. Representative results for double experiments conducted in triplicates per biological sample

<u>*Translational capacity.*</u> For the correct evaluation of the protein synthesis process of the main virulence factors - especially MPB83 (M70 high structural homology) and ESAT-6/CFP-10 (heterodimer) binary system - intracellular protein extracts (concentrated in total lysis buffer/TLB) and extracellular or secreted proteins (precipitated with ammonium sulfate/$(NH_4)_2SO_4$ - data not shown) were electrophoretically separated into the denaturing polyacrylamide gel and hybridized with polyclonal antibodies specific for M. bovis immunodominante antigens (see Fig.6). Western blot experiments revealed the presence of MPB83 exclusive in the M. bovis samples (see Figure 7A) and the ESAT-6/CFP-10 complex in all Mycobacterium extracts (see Figure 6B/ESAT- 6 and Figure 6C/CFP-10).

The MPB83 lipoprotein was major identified as the intact 26 kDa form and minor as a 23 kDa N-terminal truncate product (see Figure 6A) for all tested passages (intracellular extract/ TLB, sample 9) (Wiker et al., 1991; Harboe et al., 1998). The partners of the ESAT-6/CFP-10 heteroduplex can be detected intracellular, especially in the multimeric active form at approximately 60 kDa (see Figure 6B and Figure 6C, respectively). Consistent with information gained from gene expression experiment, ESAT-6 has not been identified as a monomer or glycosylated form (see Figure 6B) and instead CFP-10 has been highlighted in all maturation stages: about 25 kDa and 30 kDa with intracellular accumulation to the high passages (see Figure 6C, samples 7 and 9). In contrast to MPB83, members of the RD1 differentiation region are detectable independently of the analyzed species (*bovis* or *avium*). The proteins expression of *M. bovis* AN5 strain at passage 1 on the Lowenstein-Jensen medium is much diminished to undetectable in contrast to the productivity of the same strains on the glycerol potato medium.

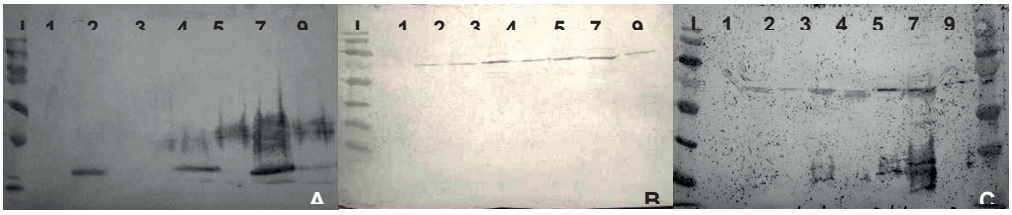

Figure 6. Western Blot of virulence factors. A. Anti-MPB83 (1:1000 dilution) - *26kDa* (intact form) and *23kDa* (N terminal truncated fom); B. Anti-ESAT-6 (dilution 1:1000) - 6kDa/monomer (undetectable) and approximately *60kDa* ESAT-6/CFP-10 hetero-complex; C. Anti-CFP-10 (1:1000 dilution) - *10kDa*/monomer (undetectable), *25kDa* and *30kDa* / probable glycosylated forms and approximately *60kDa* ESAT-6/CFP-10 hetero-complex. A, B, C: 1 - initial passage *M. bovis* AN5 /LJ; 2 - initial passage *M. bovis* AN5/glycerinated potato; 3 - initial passage *M. avium* /LJ; 4 - initial passage *M. avium* /glycerinated potato; 5 - intermediate passage *M. bovis* AN5/mDH; 7 - intermediate passage *M.avium* /mDH; 9 - final passage *M. bovis* AN5 /mDH; 11 - final passage *M. avium* /mDH. L, PageRuler Prestain Protein Ladder/Thermo Scientific; LJ, Lowenstein-Jensen medium; mDH, modified Dorset Henley medium; 10% SDS-PAGE, 120V, 90 min., Tris-glycine-SDS 1x migration buffer. Representative results for double experiments conducted in triplicates per biological sample

<u>*Biological value - PPD M. bovis AN5.*</u> The relative potency of the PPD is determined by analogy with the declared potency of the international standard (NIBSC: 58500 IU). According to the European Pharmacopoeia, this value should be between 66% and 150% when testing on guinea pigs. Following in vivo testing of the AN5 protein concentrate (origin: passage 3/mDH; filter: 0.45 µm; dilution - 1:1 with saline phosphate buffer plus 0.005% Tween 80), the diameters of the inflammatory reactions were measured at 24 hours post inoculation and a relative potency value of 72% was obtained by statistical calculation (soft: CombiStats v. 5.0, model: parallel lines, design: Latin square).

The *in vivo* testing of the bovine PPD preparation, obtained by concentration of intra- and extra-cellular proteins after sequential propagation of the AN5 strain, showed an estimated potency value of 72% by comparing to the international NIBSC standard (58500 I.U.), hovering in interval of 66-150% stipulated by the European Pharmacopoeia.

In the present study we have demonstrated that, despite the presence of major antigens as MPB70/MPB83, stably expressed, the biological value of a bovine PPD, "tuberculin B" reagent, involves the synthesis of several other virulence factors, as ESAT-6/CFP-10, influenced by some cultivation factors like culture medium type, passages number, cell density etc.

CONCLUSIONS

The genetic profile of an *M. bovis* AN5 strain based on 24 repetitive sequences and 8 differentiation regions, classifying and confirming it as virulent *M. bovis*, has been achieved for the first time, by our knowledge, in this work.

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Frz OPERON AND *R4* GENE VIRULENCE PRESENT IN ROMANIAN APEC (AVIAN PATHOGENIC *ESCHERICHIA COLI*) ISOLATES

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Abstract

Escherichia coli (E. coli) infection has a major effect on poultry production, the numerous clinical forms making it the most frequently reported diseases in commercial flocks of chickens but also in turkeys and pigeons. Some Avian pathogenic *E. coli* (APEC) strains, may share virulence factors with human extraintestinal pathogenic. The data presented in this study complete the characterization of the pathotypes of 13 APEC strains, previously isolated and characterized (Gurau M.R. et al., 2018, 2020), with the investigation over the presence of Frz operon and R4 virulence gene, by PCR method. Amplification was performed in two different runs, one for each gene, the temperature protocols being different. The Frz operon was identified in 11 isolates (84.61% or 11/13) and R4 was identified in 4 isolates (30.76% or 4/13). These results, correlated with those previously reported, highlighted that Frz operon and R4 genes are independently expressing their virulence.

Key words*: APEC, ExPEC, PCR, virulence genes.*

INTRODUCTION

In birds, as in humans, *Escherichia coli* is a member of the normal intestinal micro flora but some strains, known as APEC (Avian Pathogenic *Escherichia coli*), occur in bodily sites outside the intestinal lumen and under favorable circumstances, invade various internal organs, inducing bacteremia, respiratory tract infections, septicemia and causing fatal systemic colibacillosis (Someya et al., 2007; Kabir, 2010). Among the intestinal coliforms of chickens, 10-15% belong to pathogenic serotypes (Tabatabaei and Nasirian, 2007).

APEC (Avian pathogenic *Escherichia coli*) belong to a large and diverse group of *E. coli* strains, known as extra-intestinal pathogenic *E. coli* (ExPEC). ExPEC causes a number of systemic diseases that affect nervous system, respiratory system and urinary tract in humans, animals and birds. APEC (ExPEC strains of avian origin) has a phylogenetic relationship with human strains isolated from extra-intestinal tissues, normally sterile, and they share some of the virulence factors of the human strains. This suggests that APEC strains could pose a zoonotic risk (Moulin-Schouleur et al., 2007*)*. The scientific community is also concerned on APEC strains that are becoming an emerging pathogen in relation to food safety. One of the incriminated source of the ExPEC growing incidence in humans are the poultry products (Ewers et al., 2009).

Infections with APEC strains causes in poultry, an acute disease most often systemic, which generates significant losses in world poultry farming (Ewers et al., 2003).

APEC strains are responsible for a considerable number of clinical manifestations at different ages. Avian colibacillosis is widespread in chickens, in all age categories, with the highest prevalence in laying hens (36.73%) (Kabir, 2010). In this context, a better knowledge of phenotype and genotype characteristics of APEC strains circulating in Romanian poultry flocks was requested.

To survive and compete to the variation of the environment, bacteria have developed a complex molecular mechanisms by which they are adapting to environmental stimulus (Roquet et al., 2009). Roquet et al. 2009, identified the close association (75%) between the presence of *Frz* operon with increased virulence of avian *E. coli*.

The *Frz* operon is regulating the genes expression for the protection of the bacterium under dysgonic conditions, as the oxygen restriction or the nutritional deprivation (stationary growth phase), as well as, in promoting the bacterium development in the presence of the bird serum or in the intestinal tract, basically, by regulating the expression of adaptation and virulence genes (Patron et al., 2015).

There were identified five types of lipopolysaccharide core types in *E. coli*, known to play a crucial role in the pathogenesis of bacteraemia, sepsis and shock, marked with R1, R2, R3, R4 and K12 (Amor et al., 2000; Dissanayake et al., 2008).

In clinical, human and avian isolates, the lipopolysaccharide center R1 predominates, while R4 is found in the smallest proportion (Amor et al., 2000; Li et al., 2005). In broilers, Ozaki et al. 2017 identified the gene encoding the center lipopolysaccharide R4 in 45% of APEC strains, being the most common.

The data presented in this study complete the characterization of the 13 APEC strains, previously isolated and characterized (Gurau M.R. et al., 2018, 2020), with the investigation over the presence of *Frz operon* and *R4* virulence factors.

MATERIALS AND METHODS

There were investigated 13 *E. coli* isolates for the presence of *Frz operon* and *R4,* virulence genome determinants. The studied strains belong from farms located in Brasov, Calarasi, Dambovita, Giurgiu, Vrancea and Iasi counties. The age of poultry flocks under study ranged from 1 day to 87 weeks, breeding categories being broiler or laying hens.

The extraction of the DNA was made with the QIAamp cador Pathogen Mini Kit (Qiagen, Dusseldorf, Germany), according with the kit insert (Table 1).

The PCR amplification temperature protocol for the *Frz operon* gene of *E. coli* was: 94°C 5 minutes, 35 cycles with 94°C for 30 seconds, 63°C for 45 seconds and 72°C for 1.5 minutes. The final elongation: 72°C for 7 minutes.

The mix for the reaction of *Frz operon* gene was made in a volume of 50 µl total from which 2 µl DNA template, 2µl dNTPs 10 mM, RNase free water 35.5 µL, 2 µL of Taq platinum polymerase (5U/µL) (Invitrogen®, Itapevi, São Paulo, Brazil), 5µL of PCR buffer (50 mM KCl, 10mM Tris–HCl pH 8.0), $MgCl_2$ (1.5 mM) 1.5 µL and 1 µL of primers forward and reverse for *Frz operon* gene (10 pmol) (Table 2).

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

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

Table 2. The reagents and the quantities of the reaction mix for the *Frz operon* gene

Reaction mix	
Reagents	**µl /sample**
RNase free water	35.5 µL
PCR buffer (50 mM KCl, 10mM Tris–HCl pH 8.0)	5 µL
$MgCl_2$ (1.5 mM)	1.5 µL
dNTP solution (10 mM)	2 µL
P Frz F (10 pmol)	1 µL
P Frz R (10 pmol)	1 µL
Taq platinum polymerase (5 U/µL) (Invitrogen®, Itapevi, São Paulo, Brazil)	2 µL
DNA template	2 µL
Total	50 µL

The sequence primers for *Frz operon* gene, are described in the Table 3. The amplicons were visualized by electrophoresis in 1.5% agarose , at 90V, 1,5A, for 35 min.

Table 3. Sequence of primers-forward and reverse –used for amplification of *Frz operon* gene and expected size

Primers name	Sequence	Size (bp)
P Frz F	GAGTCCTGGCTTGCGCCGTT	843
P Frz R	CCGCTCCATCGCAGCCTGAA	

(Van der Westhuizen and Braag, 2012)

The PCR amplification temperature protocol for the *R4* gene of *E. coli* was: 94°C 4 minutes, 35 cycles with 94°C for 20 seconds, 50°C for 30 seconds and 72°C for 3 minute. The final elongation: 72°C for 7 minutes.

The mix for the reaction of *R4 gene* was made in a volume of 27 µl from which 2 µl DNA template, 1 µl dNTPs 10 mM, RNase free water 19.1 µL, 0.4 µL of Taq platinum polymerase (5U/µL) (Invitrogen®, Itapevi, São Paulo, Brazil), 2.5 µL of PCR buffer (50 mM KCl, 10mM Tris–HCl pH 8.0), $MgCl_2$ (1.5 mM) 1.5 µL and 0.25 µL of primers forward and reverse for *R4* gene (10 pmol) (Table 4).

Table 4. The reagents and the quantities of the reaction mix for the *R4* gene

Reaction mix	
Reagents	**µl /sample**
RNase free water	19.1 µL
PCR buffer (50 mM KCl, 10mM Tris–HCl pH 8.0)	2.5 µL
$MgCl_2$ (1.5 mM)	1.5 µL
dNTP solution (10 mM)	1 µL
P_A (10 pmol)	0.25 µL
P_B (10 pmol)	0.25 µL
Taq platinum polymerase (5 U/µL) (Invitrogen®, Itapevi, São Paulo, Brazil)	0.4 µL
DNA template	2 µL
Total	27 µL

The sequence primers for *R4* gene, are described in the Table 5. The amplicons were visualized by electrophoresis in 1.5% agarose, at 90V, 1.5A, for 35 min.

Table 5. Sequence of primers-forward and reverse –used for amplification of *R4* gene and expected size

Primers name	Sequence	Size (bp)
P_A	TGCCATACTTTATTCATCA	699
P_B	TGGAATGATGTGGCGTTAT	

(Amor et al., 2000)

RESULTS AND DISCUSSIONS

The *Frz operon* is rarely found in non - pathogenic strains of avian origin (5%), and his presence in the ExPEC strain increases with the increasing of the virulence in 1-day-old chicks (Roquet et al., 2009).

The *Frz operon* was detected in 8.9% of *E. coli* strains isolated from birds with colibacillosis in Zimbabwe (Mbanga and Nyararai, 2015). The frz_{orf4} is located chromosomal and belongs to the *Frz operon*. The prevalence of this operon, upon the studis, range from 53.4% of 352 APEC strains to 16.7% of 108 AFEC strains in a screening conducted by Schouler et al., 2012. In the present study, the presence of the F*rz operon* was detected in 10 (76.92%) of the 13 pathogenic avian *E. coli* strains (Table 6, Figure 1).

Table 6. The PCR-results of the tested isolates for the presence of *Frz operon* and *R4* virulence genes

Isolates	*Frz operon*	*R4*	County	Age of bird
1	**X**	**X**	Vrancea	broiler 7 day
2	**X**	**-**	Dambovita	23 weeks, layer
3	**X**	**-**	Iasi	25 weeks, layer
4	**X**	**-**	Brasov	87 weeks, layer
5	**X**	**X**	Calarasi	10 day, broiler
6	**X**	**-**	Dambovita	24 weeks, layer
7	**-**	**-**	Dambovita	1 day, broiler
8	**-**	**-**	Calarasi	7 days, broiler
9	**-**	**-**	Brasov	65 weeks, layer
10	**X**	**X**	Vrancea	11 days, broiler
11	**X**	**-**	Iasi	11 days, broiler
12	**X**	**-**	Giurgiu	7 day, broiler
13	**X**	**X**	Iasi	11 day, broiler

X = mark the strains containing the gene.

Figure 1. PCR results, *Frz gene* (843 bp). Lines 1, 2- negative control, line 3 - positive control, lines 4-7 *E. coli* strains; line 8: 100 bp DNA ladder (*Bio-Rad*)

Thus, we can note that in our study the presence of the *Frz operon* is higher (76.92%) than in the screening conducted by Schouler et al. (2012) (53.4%, 16.7%), but it should also be taken into account that in this study were tested a much smaller number of strains of *E. coli.*

In broilers, Ozaki et al. (2017) identified the gene encoding *R4* core chemoype in 45% of pathogenic *E. coli* strains, this one being the most frequent core type. In a more detailed study, Dissanayake et al. (2008) identified the core lipopolysaccharide *R4* in 13% of clinical isolates and in 4% of commensal isolates of *E. coli.* But he also noticed that *E. coli* strains with R4 core type register the lesser frequency into the group of the commensal strains, the R4 is randomly associated with other virulence genes and belong to the groups of birds` pathogens, being significantly associated with APEC strains..

In the present study, the R4 core type was detected in 4 (30.76%) from the 13 pathogenic APEC strains, sugesting that the prevalence of this determinant in the Romanian strains is higher than in Dussanayake's study (2008) (Table 6, Figure 2).

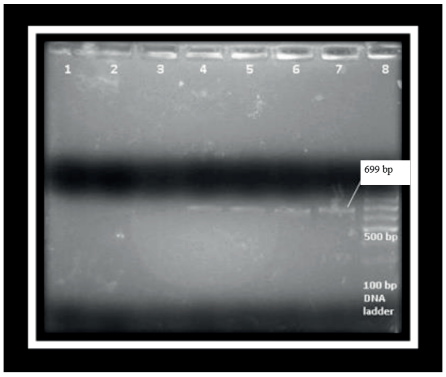

Figure 2. PCR results, *R4* gene (699 bp) Lines 1, 2 - negative control, lines 3-6 *E. coli* strains, line 7 positive control, line 8 100 bp DNA ladder (*Bio-Rad*)

The 4 APECs that house R4 are strains isolated from broilers: strains 1 from county Vrancea, broiler 7 day, strain 5 from Calarasi county, 10 day broiler, strain 10 also from Vrancea county, 11 day broiler and strains no. 13 from Iasi, 11 day broiler (Table 6). These are being in accordance with Ozaki`s study in which, R4 coretype was also identified in broilers`strains, in 45% of APEC strains (Ozaki et al., 2017).

Strains no. 7, 8 and 9 did not present any of the two studied genomic determinants, neither the *Frz operon,* nor R4. But these strains, 7, 8 and 9, according to our previously presented studies, posses other pathogenicity determinants of *E. coli*, namely the IucD, IucC and IronN genes.

CONCLUSIONS

The *Frz opreron* has a higher prevalence in our study (76.92%) than those reported by others.

We detected the R4 core type in 4 (30.76%) from 13 the APEC strains, isolated from broilers, this meaning a higher prevalence than in study of Dussanayake et al. (2008), but a lower prevalence than reported by Ozaki et al. (2017), in which R4 type was found in broilers too, but in 45% of the APECs strains.

These results came to reconfirm other results from the literature, in which, the different *E. coli* strains posses different pathogenicity genes but not all the virulence genes are present in all *E. coli* strains. According to this study and our previous ones it could be assumed that APEC pathogenicity genes are expressing their virulence in different association, without a pre-seted pattern.

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REVIEW OF THERAPEUTICAL MANAGEMENT OF EQUINE SARCOID

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Abstract

Sarcoids are reported as the most common tumour of the horse, localized of the skin, that do not metastasize and rarely regress spontaneously. Treatment described in veterinary literature includes surgical excision, cryotherapy, radiotherapy (low-dose-rate or high dose rate brachytherapy with iridium, strontium; external beam radiotherapy), chemotherapy (topical, intralesional injection, electrochemotherapy, photodynamic therapy, drug-matrix implants), hyperthermia, laser therapy, immunotherapy. The purpose of this reviewer study was to evaluate the success of various treatment methods. The information was collected from 75 scientific papers, published between of 1977-2020 and indexed in international databases (Google scholar, Web of Science - Clarivatics, Cabi). Treatments options depend by many variables, including sarcoid tumour type and location, aggressiveness of the tumour, clinical experience, client compliance, treatment costs, patient behaviour, and the availability of services, equipment, and facilities. Recent updates of the success of treatment and recurrence rate of tumour after different treatment methods are presented and discussed. Despite the many treatment options available for equine sarcoids, there is no way 100% effective in achieving healing.

Key words*: sarcoid, equine, treatment.*

INTRODUCTION

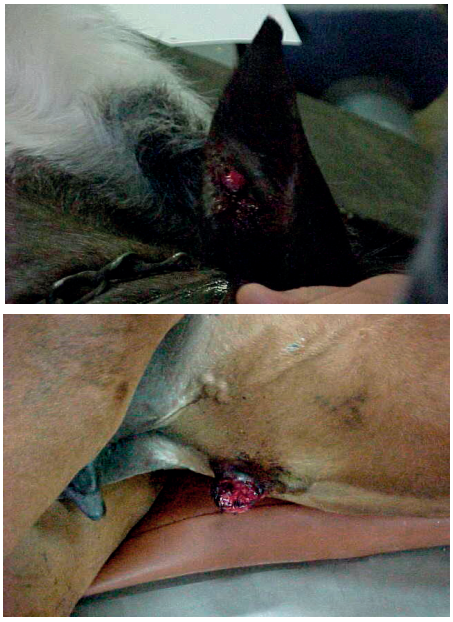
Sarcoids are reported as the most common tumour of the horse. The sarcoids are classified in six different groups according to their clinical appearance: fibroplastic, mixed, maleovant, nodular, occult and verrucous types of lesions. Common locations for these sarcoids include the periocular region, lips, ear pinnae and ventral abdomen - Figure 1. (Knottenbelt, 2005; Marti et al., 1993).

Olson and Cook (1951) identified pathological effect of papillomaviruses (PVs) at sarcoids occurring in horses could be caused by persistent infection with bovine papillomavirus (BPV). BPVs infection predominantly involves the dermis and is episomal but also affects the epidermis of equine sarcoids (Brandt et al., 2011). In studies that aimed to elucidate the pathogenesis of PV-associated sarcoids in equines, the equine cell lines were specifically transformed with BPV 1 and 2 (Gobeil et al., 2009; Kaynarcalidan and Oguzoglu, 2021).

While not life threating, sarcoids adversely affect the horse's aspect.

In the report of first international workshop on equine sarcoid Marti et al. (1993) concluded "no therapy has been found which is effective in all cases of equine sarcoids". The treatment described in veterinary literature includes more methods, permanently actualised.

The purpose of this review study was to evaluate the success of various treatment methods.



a)

b)

Figure 1. Fibroblastic sarcoids: a- nodular on the ear pinnae; b - ulcerative on the ventral abdomen (original)

MATERIALS AND METHODS

The information about the sarcoid's treatment was collected from 75 scientific papers, published between of 1977-2020 period and indexed in international databases (Google scholar, Web of Science - Clarivatics, Cabi).
For data analysis, we aimed to identify treatment methods, the factors that influenced the choice of treatment and the effectiveness of treatment.

RESULTS AND DISCUSSIONS

Equine sarcoids are challenging to treat. There are multiple treatment modalities reported, with a variety of levels of evidence to support their use. Treatment described in veterinary literature include: surgical excision, cryotherapy, radiotherapy (low-dose-rate or high dose rate brachytherapy with iridium, strontium; external beam radiotherapy), chemotherapy (topical, intralesional injection, electrochemotherapy, photodynamic therapy, drug-matrix implants), hyperthermia, laser therapy, immunotherapy etc, with variable degrees of success. The therapeutic potential of the different methods is presented in Table 1.

Immunotherapy is a treatment modality for equine sarcoid that relies on local immune stimulation to attack and kill tumour cells. The most common immunomodulator used is Bacillus Calmette and Guerin (BCG), an attenuated strain of *Mycobacterium bovis*. Success rates from 83 to 100% have been observed for periorbital sarcoids but for sarcoids found elsewhere on the body, success rates have only been approximately 50% (Caston et al., 2020; Goodrich et al., 1998; Klein et al., 1986, 1991; Knottenbelt and Kelly 2000; Lavach et al., 1985; Martens et al., 2001; McConaghy et al., 1994; Murphy et al., 1979; Newton 2000; Owen and Jagger 1987; Schwartzman et al., 1984; Vanselow et al., 1988). Severe complications, including death, anaphylaxis, severe local inflammatory reactions and septic arthritis have been reported with the use of BCG. The tumour regression may require several months of weekly therapy (Klein et al., 1986; Lavach et al., 1985; Vanselow et al., 1988).

Other immunomodulators used to treat equine sarcoid include Baypamun - inactivated parapoxovis virus (Studer et al., 1997). Topical immune modulators have also been used to treat equine sarcoid. After treatment with the immunomodulator imiquimod 5% cream (Nogueira et al., 2006; Torres et al., 2010; Pettersson et al., 2020) reported 60-80% success rate and 12,5% recurrence rate. Complications included alopecia, erythema, erosions and depigmentation of the tumour and periphery (Nogueira et al., 2006).

Vaccination with autologous vaccine (Espy 2008; Dutka, 2008; Kinnunen et al., 1999; Rothacker et al., 2015) or vaccines composed of chimeric virus-like particles (Ashrafi et al. 2008; Mattil-Fritz et al. 2008) has a complete resolution tumour rate of 50-80%, but recurrence rate is high (40-50%).

Conventional surgical excision alone has success rates of 30-50%, with most tumours recurring within 6 months (Genetzky et al., 1983; McConaghy et al., 1994; Knottenbelt and Kelly 2000). Recurrent tumours are often aggressive and regrow more rapidly than the initial tumour (Tarwid et al., 1985; McConaghy et al., 1994; Hewes and Sullins, 2009). A success rate of 20-64% has been reported (Bogaert et al., 2008; Carstanjen et al., 1997; Dutka, 2008; Funiciello et al., 2020; Genetzky et al., 1983; McCauley et al., 2002; McConaghy et al., 1994; Knottenbelt and Kelly 2000; Hewes and Sullins 2009; Ragland et al., 1970; Sala et al., 2010; Semieka et al., 2012; Tarwid et al., 1985; Taylor and Haldorson, 2013). The overall success rate was 74,9-86,8% by ***electrosurgical excision*** resulted in the highest surgical treatment success rate (Haspeslagh et al., 2016).

Laser surgical excision. A CO_2 laser or diode laser are surgical instruments that cuts and vaporises soft tissue with minimal intraoperative haemorrhage and less post-operative oedema and pain compared to scalpel blades (Taylor and Haldorson, 2013). A success rate of 60-80% has been reported (Compston et al., 2013, 2016; Carstanjen et al., 1997; Martens et al., 2001; McCauley et al., 2002). Laser surgical excision is simple to perform, suitable in majority of locations, especially where lesions are early and well circumscribed. The recurrences rate is higher

for verrucous lesions and those on head and neck.

Regardless of surgical method, the difficulty incompletely resecting sarcoid tumours, especially those in the periorbital region, often necessitates other treatment options for primary or adjunctive therapy (Taylor and Haldorson, 2013; Hollis, 2020).

Cryotherapy involves application of liquid nitrogen at -196°C, either by spray or probe, to destroy tumour cells through the formation of intracellular ice and subsequent rupture of cell membranes (Taylor and Haldorson, 2013). Three freeze-thaw cycles that decrease the tissue temperature to -20° to -30°C should be applied to each tumour (Hewes and Sullins, 2009).

The success rate of 49-100% has been reported (Lane et al., 1977; Fretz and Barber 1980; Joyce, 1976; Klein et al., 1986; Martens et al., 2001; Munroe, 1986; Newton, 2000). The highly tumour recurrence (25-60%) is more likely for periorbital sarcoids (Knottenbelt and Kelly, 2000).

Hyperthermia has been reported to induce regression for at least 7 months in 3 cases of equine sarcoid (Hoffman et al., 1983). Because of the limited reports of hyperthermia for the treatment of sarcoid tumours, it is difficult to make recommendations regarding its use (Goodrich et al., 1988; Taylor and Haldorson, 2013).

Radiotherapy has long time been considered the "*gold standard*" for the treatment of periocular sarcoids in the horse, and there are various techniques of delivering this treatment : Low-dose rate interstitial brachytherapy (LDR) iridium wires/seeds, strontium plesiotherapy, high-dose rate brachytherapy (HDR), teletherapy, and electronic brachytherapy (Byam-Cook et al. 2006; de Groot and de Groot, 1984; Hollis, 2016, 2018, 2019, 2020; Hollis and Berlato, 2018; Lewis, 1964; Theon and Pascoe, 1995; Turrel et al., 1985; Walker et al., 1991; Wyn‐Jones, 1983).

The most commonly used technique is interstitial brachytherapy. Low-dose rate interstitial brachytherapy has reported success rates of between 74 and 100%. Electronic brachytherapy is a technique which may provide an alternative to high-dose rate brachytherapy (Hollis, 2019, 2020). The requirement for general anesthesia, carefull case selection, possibility of late cataract formation and high cost and limited availability of any of the techniques are significant disadvantages.

Chemotherapy is commonly used to treat equine sarcoid (Theon et al., 1993, 1999; Hewes and Sullins, 2006; Hewes and Sullins, 2009; Funiciello and Roccabianca, 2020; Jaglan et al., 2018; Knottenbelt et al., 2018; Knottenbelt, 2019; Knottenbelt et al., 2020; Knottenbelt and Walker, 1994; Newton, 2000; Souza et al., 2017; Spoormakers et al., 2002; Tamzali et al., 2001, 2012; Tozon et al., 2016).

Chemotherapy is commonly used to treat equine sarcoid (Theon et al., 1993, 1999; Hewes and Sullins, 2006; Hewes and Sullins, 2009; Funiciello and Roccabianca, 2020; Jaglan et al., 2018; Knottenbelt et al., 2018; Knottenbelt et al., 2020; Knottenbelt and Walker, 1994; Newton, 2000; Souza et al., 2017; Spoormakers et al., 2002; Tamzali et al., 2001, 2012; Tozon et al., 2016).

Cisplatin - two approaches are available for controlled-release administration: percutaneous injection (water solution or mixing with sesame seed oil) and implantation of biodegradable beads. A retrospective analysis of long-term outcome after cisplatin injections revealed that overall tumour resolution at 4 years was 80-90% for sarcoids (Theon et al., 1993, 1999, 2007). Knottenbelt and Kelly (2000) reported a 33% success rate using injectable cisplatin and Spoormakers et al. (2002) of 78% after injectable cisplatin mixing with sesame seed oil. Intratumorally cisplatin beads have also been effective in treating sarcoids with a cure rate of 91% (Hewes and Sullins, 2006). Cisplatin electrochemotherapy (ECT) is a novel therapy, for equine sarcoid that utilises electrical field pulses to increase cell membrane permeability and thus increase cisplatin delivery to the tumour. Tamzali et al. (2001, 2012) and Tozon et al. (2016) reported a 93% success rate.

Intra-lesional *mitomycin C* are available, but 100% success rate was obtained only in a study of small case series of periocular lesions (Hollis, 2020).

Table 1. The therapeutic potential of the different methods

Treatment typs	Sarcoid localisation		Cases number	Complete resolution Time/%	Recurrence	Adverse effects	Source
	perio cular	other					
Immunotherapy imiquimod 5% cream	x	x	31	12 months/ 60-80%	12.5 %	local inflammation, exudation, alopecia, erythema, erosions, depigmentation, pain	Torres et al., 2010; Nogueira et al. 2006; Pettersson et al., 2020
Baypamun (inactivated parapoxovis virus)	unn		10	3 months/ 60%	unn	unn	Studer et all (1997)
Bacillus Calmette and Guerin (BCG)	x	x	202	8 months/ 50-100%	17-20%	Fatal and nonfatal anaphylaxis, severe local inflammatory reactions, septic arthritis	Caston et al., 2020; Goodrich et al., 1998; Klein et al., 1986, 1991; Knottenbelt and Kelly, 2000; Lavach et al. 1985; Martens et al., 2001; McConaghy et al. 1994; Murphy et al., 1979; Newton, 2000; Owen and Jagger, 1987; Schwartzman et al., 1984; Vanselow et al., 1988;
Vaccination autologous vaccine	x	x	74	90-180 days/ 59-80%	40-50%	edema and hyperemia of underlying tissue, swelling, alopecia, erythema and exudation	Espy 2008; Dutka (2008); Kinnunen et al., 1999; Rothacker et al., 2015
vaccines composed of chimeric virus-like particles	x	x	30	50%	7-30%	unn	Ashrafi *et al*. 2008; Mattil-Fritz *et al*. 2008
Surgical excision conventional	x	x	261	6-12 months/ 20-64%	40 to 72 %	dehiscence, scar	Bogaert et al., 2008; Carstanjen *et al*., 1997; Dutka, 2008; Funiciello et al., 2020; Genetzky *et al*. 1983; McCauley *et al*. 2002; McConaghy *et al*. 1994; Knottenbelt and Kelly 2000; Hewes and Sullins 2009; Ragland et al., 1970; Sala et al., 2010; Semieka et al., 2012; Tarwid et al. 1985; Taylor and Haldorson, 2013;
Electrosurgical excision	x	x	230	6 months/ 87%	unn	unn	Haspeslagh et al., 2016;
Laser removal - CO2 laser - Diode laser	x	x	172	5 months/ 60-80%	9-30 %	burns, prolonged healing, scar, corneal damage leukotrichia	Compston et al., 2013, 2016; Carstanjen et al. 1997; Martens et al. 2001; McCauley et al. 2002; Taylor and Haldorson, 2013
Cryotherapy - application of liquid nitrogen at -196°C,	x	x	68	7 months/ 49–100%	25-60%	Edema, scarring	Lane et al., 1977; Fretz and Barber 1980; Joyce, 1976; Klein et al., 1986; Martens et al., 2001; Munroe, 1986; Newton, 2000;
Hyperthermia radiofrequency 50°C	unn		3	7 months / 100%	-	na	Goodrich et al. 1988; Taylor and Haldorson, 2013; Hoffman et al., 1983;
Radiotherapy -teletherapy cobalt	unn		4	60 to 100 % success rate	unn	unn	Lewis, 1964; de Groot and de Groot, 1984;
Radiotherapy - brachytherapy iridium-192	x	x	92	12-36 months/ 60-94%		no acute adverse effect	Byam-Cook et al. 2006; Hollis and Berlato, 2018; Theon and Pascoe, 1995; Turrel et al. 1985; Walker et al. 1991; Wyn - Jones, 1983;
Plesiotherapy Strontium	x	-	8	6 - 30 months /100%	unn	No significant short or long - term adverse effects	Hollis, 2016, 2020;
Intratumorally chemotherapy – Cisplatin intratumorally injections	x	x	437	48 months/ 80-90 %	87 %	corneal damage	Theon et al. 1993, 1999; Hewes and Sullins 2009; Funiciello and Roccabianca, 2020; Souza et al., 2017
Cisplatin bed implants		x		91%	unn	unn	Hewes and Sullins 2006;
Cisplatin Oil	unn			unn / 78	unn	no systemic side-effects	Spoormakers et al., 2002;
Cisplatin electrochemotherapy	x	x	334	48 months/ 92-98%	0%	ulceration tumor	Tamzali et al., 2001, 2012; Tozon et al/. 2016;
Bleomycin	x	x	118	12 months /44%	unn	unn	Knottenbelt et al., 2018; Knottenbelt et al.,2020; Souza et al., 2017
Fluorouracil (5-FU intratumoural	x	x	14	36 months/ 61%			Stewart et al. 2006
AW3/4-LUDES % fluorouracil cream	unn		146	35%	unn	unn	Knottenbelt and Kelly 2000; Knottenbelt and Walker 1994; Newton, 2000;
Anthiomaline (lithium antimony thiomalate) + vincristine	-	x	6	50%	unn	unn	Jaglan et al., 2018;
Antiviral therapy 5% acyclovir cream	x	x	37	3 months/ 68-95%	0%	unn	Haspeslagh et al., 2016, 2017; Stadler et al. 2011;
tricyclodecan - 9 - yl - xa nthogenate	x	x	6	50%	30%	temporary oedema at the injection site	Otten et al., 1994;
Photodynamic therapy Hypericine intratumoural	unn		4	92.3%	39%	hyperaemia, edema, cyanosis and pruritus	Martens et al., 2000;
Plant extract *Viscum album* subcutaneous injections	x	x	42	12 months/ 37%	unn	edema	Christen-Clottu e al., 2010;
bloodroot ointment	x	x	49	42 moths/ 86.5%	9%	unn	Wilford et al., 2014;

X – yes; unn -unnamed

Bleomycin, non-permanent cytotoxic drug, induces DNA stand break is used in electrochemotheraphy or as intralesional injection (Knottenbelt et al., 2018; Knottenbelt et al., 2020; Souza et al., 2017). May be more effective when combined with other agents (tazarotene or 5-FU), probably due to improved penetration of bleomycin into cells (Hollis, 2020).

Five-fluorouracil (5-FU) is a topical chemotherapeutic drug. Intratumoural injection of 5-FU resulted in complete resolution of sarcoids in 61% of horses for up to 3 years (Stewart et al., 2006).

AW3/4-LUDES2 are compounded topical chemotherapy creams that contain 5% fluorouracil, heavy metals and thiouracil. Knottenbelt and Kelly (2000), Knottenbelt and Walker (1994) and Newton (2000) reported a success rate of 35% in 149 horses with sarcoids.

Antiviral therapy is a controversial method with 53-68% success rates reported for early, very superficial lesions (Haspeslagh et al., 2016, 2017; Stadler et al., 2011).

Plant extract use (*Viscum album* subcutaneous injections or Blood root ointment - extract of *Sanguinaria canadensis*) is cheap, but there are minor scientific success rates reported (Christen‐Clottu et al., 2010; Wilford et al., 2014).

To choose a proper therapeutic plan, some considerations must be evaluate: each lesion can require a specific treatment and can react in a different way compared to other sarcoids even on the same horse; the extent and location of the tumour greatly affect therapeutical response; the duration of the lesion is important as early intervention usually requires less aggressive treatments; sarcoid-affected animals can never be considered free of the disease, even after successful treatment (Funicello and Roccabianca, 2020; Hollis, 2020; Knottenbelt, 2019; Melkamu et al., 2018; Taylor and Haldorson, 2013).

From all references analysed conquered that treatments options depend by many variables: tumour type, location, aggressiveness, clinical experience, client compliance, treatment costs, patient behaviour, and the availability of services, equipment, and facilities.

CONCLUSIONS

Treatments options depend by many variables, including sarcoid type, location, and aggressiveness of the tumour.

Despite the many treatment options available for equine sarcoids, there is still no way 100% effective in achieving healing.

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ISOLATION AND IDENTIFICATION OF *Rhodococcus equi* BACTERIAL STRAIN IN A PUREBREAD ARABIAN HORSES FARM IN ROMANIA

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Abstract

Rhodococcus equi is a Gram-positive, encapsulated, pleomorphic, intracellular bacillus, which mainly affects foals. Despite often being fatal it is still a neglected pathogen. The main objective of this study is to establish the etiological diagnosis in a clinical case of pneumonia in foals using basic bacteriology techniques. The strain referred to has been collected from a 32-day-old foal with the following symptoms: fever, dyspnea, abdominal breathing, cough and a deeply altered general condition. The isolation of the bacteria has been performed on Lowenstein- Jensen selective medium and the identification has been done based on morpho-cultural and biochemical features. Consequently, an antibiogram was performed and it showed that the isolated R. equi strain is sensitive to rifampicin and azithromycin thus the therapeutic protocol was corrected.

Key words*: bacteria, foal, Rhodococcus equi.*

INTRODUCTION:

Rhodococcus equi is the most important cause of pneumonia in foals aged between 1-5 months (Johns, 2013). It is not the most frequent etiology for this age group, still has the most significant economic consequences on the account of the death rate, prolonged treatment, screening programs for early detection and relatively expensive prophylactic strategy (Rush, 2014). The disease is rarely encountered in horses older than 8 months. Epidemiological data indicate that the pulmonary infection has its origins, supposable, in the first week of life (Merck et al, 2014). *R. equi* belongs to the *Nocardiaceae* family that also comprises *Nocardia, Mycobacterium, Corynebacterium* and *Gordonia* with some similarities among the group. Due to this fact, initially is rather easy to misdiagnose, so a proper correlation between the clinical symptoms and the results of the bacteriologic examination are indispensable (Ayoade, 2020). *R. equi* (formely *Corynebacterium equi*) is a pleomorphic, Gram-positive bacterium that was first isolated in 1923 from the lungs of foals in Sweden. Though not a very frequently found pathogen, *R. equi* has proven to be one of the most important that affects foals (Manzat, 2001).

Highly effective methods for preventing *R. equi* pneumonia in foals are lacking (Cohen, 2014).

Initially the bacteria was named *Corynebacterium equi* by H. Magnusson and later, in 1977, was transferred to *Rhodococcus* genus by Goodfellow and Alderson (Vasquez-Boland, 2019).

Apart from clinical symptoms specific for pneumonia such as fever, labored breathing, cough, a rattling sound in the windpipe and depression, one of the most severe manifestation of *R. equi* infection are abscesses, mainly located pulmonary, but also can be found elsewhere in the body (Young, 2020).

Prolonged treatment is required for the affected foals because of the increased persistence of the bacteria in the lung abscesses. The elective treatment consists in a combination of the following antibiotics: erythromycin and rifampicin (Wright, 1990). The main objective

of this study was to establish the etiological diagnosis in a clinical case of pneumonia in foals using basic bacteriology techniques and the correlation of treatment with the results obtained from the bacteriological examination performed.

MATERIALS AND METHODS

The bacterial strain was isolated from a 32 day old foal that presented the following symptoms: fever, dyspnea, abdominal breathing, cough, deeply altered general condition.

Among the differential diagnosis for this clinical case were mentioned also parasite infestations such as Ascarids: Ascarid worms have a high prevalence in foals causing irritation of the digestive tract, decreased feed absorbtion and colic, but also damage lung tissue due to migrating larvae (Mitrea, 2011).

Parascaris equorum was detected mainly in foals (5/6; 83.88%) (Buzatu, 2014).

After the collection of samples for the bacteriological examination has been finalized, the foal started to receive treatment with antibiotics, gentamycin combined with cephalosporin. This treatment was followed for one week until the all the performed exams were finalized. Unfortunately, the treatment with gentamycin and cephalosporin had no significant results, on the contrary, the health status of the foal had worsened. Moreover, a ultrasonography examination was performed and it revealed that the pulmonary abscesses have increased.

Afterwards, samples were collected from the foal's nasal cavities and transported to the laboratory to further proceed with the bacteriological examination. A primary seeding on blood agar and BHI broth was performed.

Isolation of the pathogen was intiated on specific culture media (Blood Gelosis, blood agar, Muller-Hinton agar, MacConkey agar, BHI agar, Chapman agar, Lowenstein-Jensen agar, BHI broth).

Isolation and identification: the following materials were used culture media.

The obtained cultures has been investigated for the macroscopical morphology and submitted to passages from the selected colonies on both, BHI broth and agar culture media. The primary identification of the bacterial genera has been performed by bacterioscopic examination of the isolate, the catalase test and selective media seeding on MacConkey agar, Chapman agar and Lowenstein-Jensen agar. Based on this the suspicion of *R. equi* infection was issued.

The antibiogram and the lecture of the results has been performed according to Liofilchem and EUCAST standards.

The Identification of the isolated strain has been carried out on API Coryne (Biomerieux) tests, in duplicates.

RESULTS AND DISCUSSIONS

After the collection of samples for the bacteriological examination has been finalized, the foal started to receive treatment with antibiotics, gentamycin combined with cephalosporin. This treatment was followed for one week until the all the performed exams were finalized. Unfortunately, the treatment with gentamycin and cephalosporin had no significant results, on the contrary, the health status of the foal had worsened. Moreover, a ultrasonography examination was performed and it revealed that the pulmonary abscesses have increased.

Besides pneumonia symptoms, in the literature can also be found extra-pulmonary disorders that are present in the infection with *R. equi.*

Diarrhea, can be caused by pyogranulomatous typhlocolitis or caused as a result of antimicrobial treatment. Abdominal lesions are identified in approximately 50% of the foals with *R. equi* infection.

Pyogranulomatous lymphadenitis of the mesenteric or colonic lymph nodes and large intra-abdominal abscesses can be also present.

Septic inflammation of growth cartilage and osteomyelitis are less frequent localizations of the infection.

There have also been reported cases of panophthalmia, sinusitis, pericarditis, nephritis, uveitis and hepatic and renal abscesses caused by *R. equi* infection (Giguére, 2011).

The cultures made from the samples collected from the foal presented polymorphic flora, in which predominate mucoid, whitish, transparent colonies, with weak alpha-hemolysis.

In Table 1 are presented different morphological, cultural and biochemical characteristics of the isolated cultures on different types of culture media.

Table 1. Morphological, cultural and biochemical characteristics of *R. equi* on different types of culture media

Cultural Aspect Gelosis+blood	Cultural Aspect MacConkey	Cultural Aspect Chapmann	Cultural Aspect Lowenstein-Jensen	Morphologic Aspect	Catalasis
Mucoid, whitish, transparent, confluent colonies with weak alpha-hemolysis. (Figure 1)	Medium colonies, transparent, round, glossy, lactose-positive.	-	White, mucous culture, poorly developed 24 and 48 hours from seeding (Figure 2)	Coccoid forms, coccobacilary, rare chains, isolated, diplo or groups, Gram-positive. (Figure 3)	Positive

Based on the characteristics presented in the table above, the suspicion of *R. equi* infection was issued.

The antibiogram that was performed had the following results (Figure 5):

- Susceptible to: gentamicin, doxycycline, streptomycin, norfloxacin, rifampicin;
- Intermediate sensitive to: spectinomicin;
- Resistant to: cefixima, vancomycin, oxacillin,amikacin,trimethoprim+sulfomethoxazole, colistin sulfate, cephalotin, clindamycin.

For further confirmation, API Coryne tests (Biomerioux), in duplicates, were executed. The results based on the biochemical characteristics are presented in Table 2.

Table 2. Biochemical characteristics of *R. equi* strain

NIT	PYZ	PyrA	PAL	ßGUR	ßGAL	αGLU	ßNAG	ESC	URE	GEL	0	GLU	RIB	XYL	MAN	MAL	LAC	SAC	GLYG	CAT
+	+	-	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+
+	+	-	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+

Legend: NIT - Potassium nitrate; PYZ - Pyrazinamidase; PyrA - Pyrolidonylarylamidase; PAL - Alkaline phosphatase; Bgur - β-glucuronidase; βGAL - β-galactosidase; αGLU - α-glucosidase; βNAG - N-Acetyl-β-glucosaminidase; ESC - Esculin; URE - Urease; GEL - Gelatin; 0 - negative control; GLU - Glucose; RIB - Ribose; XYL - Xylose; MAN - Mannitol; MAL - Maltose; LAC - Lactose; SAC - Sacchrarose; GLYG - Glycogen; CAT - Catalase.

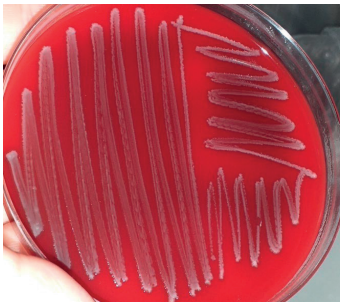

Figure 1. Blood Gelosis - pure culture of *R. equi*, after 24 hours incubation. Colonies are irregular, semitransparent and mucoid with weak alpha-hemolysis. After several days in culture, colonies produce a characteristic salmon-pink pigment

Figure 2. Cultural Aspect of *R. equi* on Lowenstein-Jensen: white, mucous culture, poorly developed 24 and 48 hours from seeding

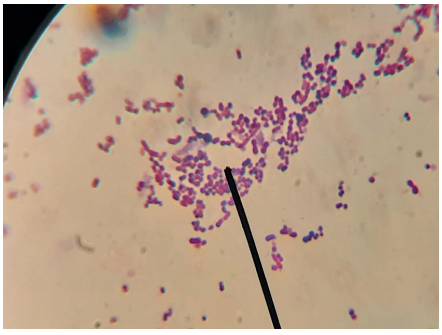

Figure 3. Morphologic aspect of *R. equi* isolate, Gram stain, 1000x, Gram-variable pleomorphic, coccoid, and bacillary bacteria

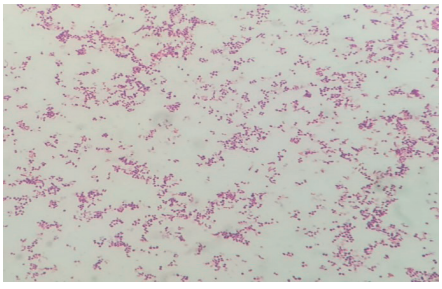

Figure 4. Microscopic aspect of *R. equi* on Lowenstein-Jensen Media (Gram stain, 700x)

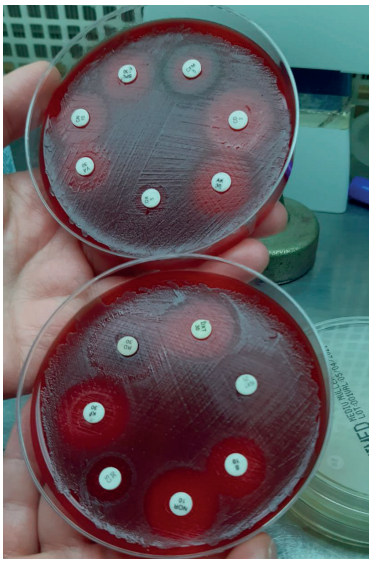

Figure 5. Antibiogram results

The successful early diagnosis and treatment of *R. equi* pneumonia in foals and management of the foals environment to reduce risks of contracting the disease are, arguably, among the most challenging experiences currently facing the equine clinician.
The incidence of the disease is increasing, as a reflection of intensification of managerial practice. High successful farms can therefore become victims of their own success. (Muscatello et al. 2007).

CONCLUSIONS

From the nasal exudate samples collected from the foal, one bacterial strain with possible involvement in the pathological process was isolated. It is presenting morphological and cultural properties characteristic for the *Rhodococcus* genus. This result was confirmed by performing API Coryne tests.
R. equi was diagnosed by correlating the results of the bacteriologic examination with the clinical symptoms presented by the foal and the anamnesis.
Currently, the treatment of choice for *R. equi* pneumonia is a member of the class of antibiotics known as macrolides combined with another antibiotic known as rifampin. Examples of macrolide antibiotics are azithromycin clarithromycin and erythromycin. This combination has been used to treat foals for about 40 years (Cohen, 2021).

After interpreting the antibiogram resulted that the isolate is for sensitive to rifampicin, norfloxacin, azithromycin and the foal has been treated accordingly by using a combination of rifampicin and azithromycin. This treatment was maintained for 5 weeks.
Positive results appeared soon after the treatment was initiated.
The foal presented improved general condition, temperature between physiological limits. By the end of the treatment, the pulmonary abscesses had gradually reduced, cough was no longer present and the appetite had returned to normal.

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ULTRASONOGRAPHIC, COMPUTED TOMOGRAPHIC, CT- ARTHROGRAPHIC DESCRIPTION OF NORMAL INTRA- ARTICULAR ANATOMY OF THE CANINE STIFLE: A CADAVERIC COMPARATIVE STUDY

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Abstract

Cranial cruciate ligament (CCL) rupture is a common orthopedic disease in canine patients. Although it is possible to diagnose this injury through an orthopedic clinical examination, imaging can increase diagnostic certainty. This comparative Ex-Vivo study allowed us to improve our knowledge of the ultrasound anatomy of intra-articular structures of the dog's healthy knee. The CCL, the patellar tendon and menisci were examined by ultrasonography, Computed Tomography (CT) and CT-arthrography. Besides performing a comparison of the ultrasound and CT results, to better understand the sonoanatomy of the CCL and to prove that it can be properly identified by sonography, the ligament was stained with methylene blue under ultrasound guidance. To overcome the limitations of the Ex-Vivo study, the ultrasound scans obtained were compared with similar ones performed in healthy non-sedated dogs. Results indicate that in large dogs, ultrasonography is a valid diagnostic method for visualizing the cranial-distal part of the CCL and also evaluating the external portion of both menisci.

Key words: *cranial cruciate ligament, dog, ultrasonography, computed tomography*.

INTRODUCTION

Rupture of the Cranial Cruciate Ligament (CrCL) is one of the main cause of lameness in dogs, also representing the major cause of knee osteoarthritis in this species. (Elkins et al., 1991; Johnson et al., 1994; Witsberger et al., 2008). The aetiology of the rupture of the CrCL is complex and multifactorial, concerning traumatic, degenerative, conformational, anatomical and autoimmune factors (Cook, 2010; Griffon, 2010; Sanchez-Bustinduy et al., 2010). Rupture of the Cranial Cruciate Ligament is ~~a~~ commonly diagnosed as a bilateral event (De Bruin et al., 2007; Cabrera et al., 2008; Buote et al., 2009; Grierson et al., 2011; Fuller et al., 2014). Most of injury occurs in the absence of a triggering traumatic event, in these cases, the lesion is the result of a chronic ligamentous degeneration (Gambardella et al., 1981; Galloway et al., 1995). The ligament gradually undergoes degenerative phenomena that they can lead to its partial rupture or, more belatedly, to a complete one (Duval et al., 1999; Griffon, 2010). Furthermore, in association with the rupture of CrCL, it is common to observe lesions affecting the medial meniscus, in particular its caudal portion (Rey et al., 2014; Ritzo et al., 2014). However, in case of partial ligament lesions or meniscal lesions in the early stage, the only clinical examination does not allow to achieve a clear diagnosis. Therefore, diagnostic imaging techniques have recently acquired an essential role in confirming the diagnosis, issuing the prognosis and planning the surgery of orthopedic diseases. In Veterinary Medicine, radiography was the first diagnostic technique for imaging used in case of suspected rupture of the CrCL. However, the X-ray exam does not allow to directly view the injured CrCL but only shows the osteoarticular degenerative lesions ensuing its rupture (Marino et al., 2010). Magnetic Resonance is the elective instrumental investigation for the study of joint pathologies, but it is an expensive tool and not so available

to be part of the common veterinary clinical practice. (Banfield & Morrison, 2000; Ohlert et al., 2001; Vande Berg et al., 2002; Soler et al., 2007; Scrivani, 2018).
Arthroscopy, which is characterized by high diagnostic sensitivity, is a common technique used for the diagnosis and treatment of rupture of the CrCL and meniscal tears, although, compared to the previously mentioned methods, it shows a certain degree of invasiveness. (Thieman et al., 2006; Pozzi et al., 2008; Plesman et al., 2013; Ritzo et al., 2014). For the reasons mentioned above, in the last few years, the attention has turned to two diagnostic techniques less used previously: Ultrasound and Computed Tomography (CT). The CT exam is an increasingly used diagnostic method in the diagnosis of knee pathologies, denoting a high sensitivity in the diagnose hard tissue diseases. On the other hand, the real usefulness of the CT examination in the study of teno-ligamentous structures is controversial (Samii& Dyce, 2004; Han et al., 2008). Knee ultrasound is a method studied since the second half of the 90s which, with the development of technologies increasingly innovative, has made significant progress in the identification and study of the main intra-articular structures. In our view, given the recent development of high-frequency ultrasound probes more advanced than those used in the past is required a new sono-anatomical evaluation of the main intra-articular structures of the healthy knee of the dog aimed to identify the limits and strengths of this diagnostic method.
The main purpose of this Ex-Vivo study was to broaden the knowledge over the ultrasound anatomy of the main intra-articular structures of the healthy knee of the dog, assessing the strengths and weaknesses of the diagnostic method. In addition, in order to understand the sono-anatomy of the CrCL, a comparison was made between ultrasound images and CT scans also with the benefit of CT-Arthrography (CTA).

MATERIALS AND METHODS

All dogs included in the Ex-Vivo study died from causes unrelated to pathologies affecting the knee joint at the Veterinary Teaching Hospital (OVUD) of the Department of Veterinary Medicine of Perugia. The presence of bone pathologies was excluded by CT examination, while the integrity of the teno-ligamentous and meniscal structures was verified by performing an arthrotomy.
A total of 14 knees from 8 dogs were examined, all of them weighed more than 25 kg. Whenever possible the corpses were evaluated through ultrasound, CT and CTA without disarticulating the limbs from the rest of the carcass. In some case only the hind limb was isolated disarticulating it at the level of the coxo-femoral joint. The corpses were stored in a cold room for a maximum of 72 hours.

Ultrasound examination

All the knees subjected to ultrasound examination undergone to trichotomy; abundant isopropyl alcohol and ultrasound gel was used as well. The corpses or individual hind limbs were placed for all ultrasound scans in lateral recumbency with the joint to be examined uppermost. A Voluson® P6 GE scanner was used. For the evaluation of the patellar tendon, the infrapatellar fat pad, the CrCL and Menisci, the linear high frequency (5-15 MHz) probe was used, while for caudal scans of the knee we opted both for a convex (2-5 MHz) or a linear high frequency (5-15 MHz). The stifle joint was investigated ultrasonographically in the sagittal and transverse planes. The acoustic windows used were obtained by cranial, medial, lateral and caudal approach. The patellar tendon, the fat pad infrapatellar and the profile of the femoral condyles were assessed by scanning the cranial window with the linear probe positioned longitudinally. In the same scan, bringing the knee to its maximum flexion, allowed the Cranial Cruciate Ligament to be seen/identified (Figure 1).

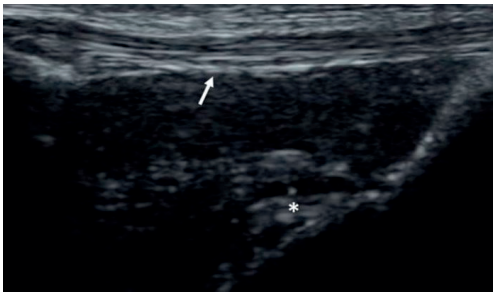

Figure 1. Cranial longitudinal ultrasonographic scan of the Cranial cruciate ligament (asterisk) and Patellar tendon (white arrow)

Sometimes, rotating the probe 20° in the proximolateral-dystomedial direction it was possible to obtain more detailed images of the cranial portion of the CrCL. The medial meniscus was visualized longitudinally from the medial knee compartment in semi-flexion; scanning the lateral compartment the lateral meniscus could be assessed. Moving the probe slightly in the cranio-caudal direction it was also possible to evaluate the medial and lateral collateral ligaments. Through the investigation of the cranio-lateral area of the knee joint, the tendon of the extensor digitorum longus muscle can be identified cranially to the lateral collateral ligament (Table 1). Caudal scans were performed both via a convex probe and via a high-frequency linear probe. The probe was placed on the joint compartment caudal and was oriented transversely or longitudinally.

Table 1. Evaluation criteria used for ultrasound to evaluate the menisci, patellar tendon and cranial cruciate ligament

Structure	***Criteria***	***Ultrasound***
Cranial cruciate ligament *Patellar tendon*	Overall visibility	0: Not visible
		1: Visible
	Visibility of the contour	0: Not visible
		1: Barely visible
		2: Moderately visible
		3: Clearly visible
	Linearity of the contour	0: Irregular outline
		1: Regular outline
	Appearance of the internal structure	0: No longitudinal echoes detected
		1: Moderate longitudinal pattern
		2: Clear longitudinal pattern
Menisci	Overall visibility of the external portion of the meniscal body	0: Not visible
		1: Barely visible
		2: Moderately visible
		3: Clearly visible
	Appearance of the internal structure	0: Non Homogeneous
		1: Homogeneous
	Shape	0: Not triangular
		1: Partially triangular
		2: Clearly triangular

CT examination

For the CT and CTA exam, all data was collected using the TC scanner HD 16 sections (Fujifilm-FCT-Speedia). The carcasses were positioned in sternal recumbency, while the individual hind limbs were placed with the cranial compartment of the knee in contact with the table. The flexion angle of the knees ranged from 110° to 130°.

To obtain the images the parameters were set at 140 kilovolts (kV) and 170 milliampere (mA). The layer thickness was 0.65 mm.

The acquired images were evaluated with a medical imaging DICOM viewer (Horos™). CT images were evaluated by applying an algorithm for both hard and soft tissue (Table 2). The CTA scans were studied using a single algorithm. The appearance and length of the CrCL were evaluated in a multiplanar reconstruction on a parasagittal plane. The width was measured in its distal third (Table 3). The lateral meniscus-femoral ligament was studied on the frontal plane. The menisci were evaluated axially. Arthrocentesis was performed using a 23-gauge needle inserted into the lateral parapatellar recess. The iodinated contrast was initially inoculated in all dogs at a volume of 3 ml (Iobitridol, Xenetix, Guerbet spa, Italy) with a concentration of 150 mg/ml.

Table 2. Evaluation criteria used for Computed Tomography (CT) to evaluate the cranial and caudal cruciate ligament, menisci and lateral menisco-femoral ligament

Structure	***Criteria***	***CT***
Cranial cruciate ligament *Caudal cruciate ligament* *Lateral menisco-femoral ligament* *Menisci*	Overall visibility	0: Not visible
		1: Barely visible
		2: Moderately visible
		3: Clearly visible
	Visibility of the contour	0: Not visible
		1: Barely visible
		2: Moderately visible
		3: Clearly visible
	Appearance of the internal structure	0: Non Homogeneous
		1: Homogeneous

Table 3. Evaluation criteria used for Computed Tomography Arthrography (CTA) to evaluate the cranial and caudal cruciate ligament, menisci and lateral menisco-femoral ligament

Structure	***Criteria***	***CTA***
Cranial cruciate ligament *Caudal cruciate ligament* *Lateral menisco-femoral ligament* *Menisci*	Overall visibility	0: Not visible
		1: Barely visible
		2: Moderately visible
		3: Clearly visible
	Distribution of the contrast medium	0: Low
		1: Moderate
		2: Good
	Appearance of the internal structure	0: Non Homogeneous
		1: Homogeneous

Inoculation of methylene blue and arthrotomy

In all the knees examined by ultrasound, after performing the CTA, a 22 G spinal needle was inserted echo-guided inside the joint cavity. After penetrating the CrCL, a minimal amount of methylene blue (0.05 ml) was placed in the ligament. An arthrotomy was subsequently performed to evaluate the CrCL and the quality of staining.

***In vivo* study**

In the comparative *in vivo* study were included 6 healthy dogs weighing more than 25 kg. No animal was sedated or anesthetized. Given the intent to exclude the presence of related pathologies of the knee, the animals were subjected to an orthopedic examination: in each patient a thorough anamnestic investigation was conducted, followed by inspection examination (gait and posture analysis) and specific orthopedic examination of the joint (external palpation, flexion-extension - specific tests for the exclusion of lesions affecting the CrCL). The same devices and scans applied for cadavers were used to evaluate the knees in these dogs. In addition, all the images were evaluated with the same criteria.

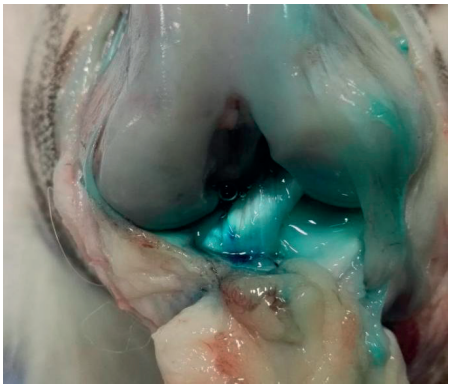

Figure 2. Arthrotomic visualization of the cranial cruciate ligament stained by methylene blue through ultrasonographic guide

RESULTS AND DISCUSSIONS

14 knees from 8 corpses were included in the cadaveric study. The corpses belonged to different breeds: Great Dane, Boxer, Czechoslovakian Wolfdog, Golden Retriever, Swiss Shepherd, German Shepherd, Hound, Mixed breed. Moreover, a total of 12 knees from 6 healthy dogs were included in the In-Vivo study. The examined subjects belonged to the following breeds: Dobermann, Labrador Retriever, American Pit Bull Terrier, Weimaraner. In this study, there were also two large mixed breeds. All subjects included in our study weighed more than 25 kg. Whenever possible, the corpses were evaluated by ultrasonography, CT and CTA without disarticulating the limbs from the rest of the carcass: the aim was to simulate the normal positioning of the animal during these examinations. We performed the US-scans with a high frequency linear probe (5-15 MHz), which has a uniform insonation angle limiting the artifacts due to anisotropy. The hypoechoic aspect of CrCL described in previous studies (Kramer et al., 1999; Soler et al., 2007) is probably associated to these artifacts.

Through ultrasonography the CrCL was visible in all cases both *ex vivo* and *in vivo* (14/14 *ex vivo*, 12/12 *in vivo*) (Table 4). This structure was seen, in all cases, as a clearly hyperechoic portion in the part closest to the tibial insertion, continued by a proximal low echogenicity portion. The CrCL showed in most cases a clearly visible and linear contour, both *ex vivo* (clearly visible 57.14%, linear 78.57%) and *in vivo* (clearly visible 58.33%, linear 78.57%). In 64.28% of cases *ex vivo* and 75% *in vivo* the hyperechoic portion showed a clear fibrillar pattern similar to the patellar tendon. *Ex vivo*, the CrCL hyperechoic distal portion showed a length ranging from 28.47% to 58.29% (average of 36.17%) of the total extension of the CrCL obtained in CTA. Moreover, the hyperechoic fibrillar portion showed a length ranging from 56.66% to 97.38% (mean of 74.17%) of the total extent of the ligament that can be ultrasound appreciated. The ultrasound visible total ligament measure ranges from 34.48% to 63.48% (average of 48.95%) of the total length visible in CTA. The estimates relating to the ultrasound visibility of the CrCL agrees with what was described by Van der Vekens et al. (2019). According to our knowledge, no scientific report has ever evaluated the *ex vivo* width of the CrCL appreciable by ultrasound. Taking as a reference the rough point linearity and width of the echoes, which is on average 5.87 mm (median 5.51 mm) from the distal ligament width limit, the maximum ligament width could be measured, which represents 52.57% (from 37.5% to 73.33%) of the total width of the CrCL measured in CTA. The total CrCL width in CTA in all cases was estimated in the distal third, location of the maximum thickness of the CrCL as we found in all the examinated scans. In CTA, the average distance between 2/3 of the ligament and the distal point of maximum visibility was equal to 6.85 mm.

Table 4. *Ex vivo* results of the ultrasonographic evaluation of the cranial cruciate ligament (CrCL) and patellar tendon (PT)

Criteria	***Ultrasound examination***	***CrCL***	***PT***
Overall visibility	Not visible	0	
	Visible	14	14
Visibility of the contour	Not visible	0	0
	Barely visible	3	0
	Moderately visible	3	0
	Clearly visible	8	14
Linearity of the contour	Irregular outline	3	
	Regular outline	11	14
Appearance of the internal structure	No longitudinal echoes detected	1	0
	Moderate longitudinal echo-pattern	4	0
	Clear longitudinal echo-pattern	9	14

The maximum CrCL width appreciable on ultrasound is a data which must be critically assessed: despite the measuring point of the ultrasound width (5.87 mm from the distal ligamentous limit) and measuring point of the CTA width (6.85 mm from the distal ligament limit) are very close to each other, it is not clear whether this data is due to a diagnostic limit, or a discrepancy between the two measurement points. Indeed, the CrCL is an anatomically complex structure, which does not have a uniform thickness along its course and its width can vary depending on the knee flexion angle (Heffron & Campbell, 1978). In addition, the measurements in ultrasound knee flexion angle showed different values from the measured in the CT study one. Consequently, it is not easy to assess if the limitation of ultrasonography to estimate only 52.57% of the CrCL width viewed in CTA is due to the inability of the method itself to view the remaining 47.43%, or is attributable to a hypothetical bias in measurements in different points of the ligament. The proximal portion of the CrCL has poor echogenicity and lacks a clear fibrillar pattern, finding in our opinion poor applicability in clinical practice. On the other hand, the distal portion of the CrCL, given its hyperechoic and fibrillar aspect, would make it easier to visualize any ligament injuries present there. To prove the ultrasound visibility of the CrCL a spinal needle was ultrasonographically introduced into the joint cavity of cadavers, inoculating blue of methylene between the fibers of the ligament. As demonstrated by the execution of arthrotomy, in all anatomical preparations in which this maneuver was performed, the CrCL was correctly identified and stained, unequivocally demonstrating the ultrasound visibility of the ligament. The portion of CrCL dyed has always been the distal one, further confirming the results obtained in the ultrasound and CTA evaluation of the CrCL.

In Ultrasound examination the medial (MM) and the lateral meniscus (LM) were identified in all cases in both the *ex vivo* and *in vivo* evaluation (Table 5). The menisci were described in all the examined cases as moderately echogenic structures. Their external portion have been appreciated as clearly visible structures in most cases. (*ex vivo* in MM 69.23% LM 61.53%; *in vivo* in MM 70%, LM 50%), with the internal one mostly heterogeneous (in *ex vivo* MM 76.92% LM 69.23%, *in vivo* MM 70%, LM 80%). In the corpses, the shape of the medial meniscus was clearly triangular in 10 knees (76.92% of cases), while the lateral meniscus was clearly triangular in 53.85% of cases.

Table 5. *Ex vivo* results of ultrasound evaluation of the menisci

Criteria	***Ultrasound examination***	***Medial Meniscus***	***Lateral Meniscus***
Overall visibility of the external portion of the meniscal body	Not visible	0	0
	Barely visible	2	2
	Moderately visible	2	3
	Clearly visible	9	8
Appearance of the internal structure	Non Homogeneous	10	9
	Homogeneous	3	4
Shape	Not triangular	1	2
	Partially triangular	2	4
	Clearly triangular	10	7

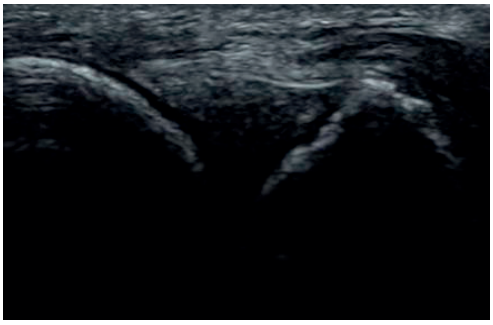

Figure 3. Lateral longitudinal scan of the lateral meniscus

In vivo, the shape of the medial meniscus was clearly triangular in 60% of cases (6 knees), while the lateral meniscus was so in 40% of cases. The medial meniscus showed overall visibility, both *ex vivo* and *in vivo*, slightly higher than that of the lateral meniscus;

moreover, the typical triangular meniscal aspect was also more easily assessed in the medial meniscus. These observations are in agreement with what Van der Vekens et al. (2019) described. The superior ultrasound visibility of the medial meniscus compared to the lateral one represents a significant diagnostic advantage, as the medial meniscus represents the most commonly affected structure by CrCL rupture (Rey et al., 2014; Ritzo et al., 2014). Unlike what has been published by various authors (Kramer et al. 1999; Mahn et al., 2005; Soler et al. 2007), who attributed to the menisci a homogeneous aspect, in our paper, in line with Van der Vekens et al. (2019), menisci often presented a typical heterogeneous aspect.
The hypoechoic focal areas we considered to be the ultrasound representation of the radial meniscal fibers transversely from the ultrasound beam (Kambic & McDevitt, 2005; Van der Vekens et al., 2019). The patellar tendon, in agreement with Soler and colleagues (2007) was visible in all cases both *ex vivo* and *in vivo*, presenting a hyperechoic aspect in all cases comparing to the infrapatellar fat pad, with a clear hyperechoic and a fibrillar pattern and a linear and clearly visible contour.
In the *in vivo* study an innovative ultrasound approach to the knee was proposed: the caudal femoral-tibial scan. The idea of using this method was born with the aim of investigate the menisci as fully as possible, in fact the Lateral sagittal scan allows us to evaluate only a limited portion. By positioning the probe oriented transversely on the caudal surface of the knee, it was possible to appreciate, in all the joints taken into consideration, the profile of the femoral condyles. Moreover, by orienting the probe longitudinally it was possible to visualise in all the joints a small caudal portion of the menisci. Caudal scans were carried out with a convex or linear probe according to the animal examined. In the Labrador, in Dobermann, in Pitbull and in a large mixed breed, was used a low frequency convex probe. In the Weimarainer and in the medium-sized mixed breed was used the linear probe. In all knees scanned (12/12, 100%) the medial meniscus and the lateral meniscus were correctly identified by longitudinal scan. Furthermore, in all the dogs (12/12, 100%) the profile of the femoral condyles was correctly highlighted in the transverse scan. Through this method, the caudal portion of the meniscal body was visible, allowing to evaluate a part of the menisci usually not distinguished by ultrasound. The caudal scan therefore, expanding the portion of the meniscus visible ultrasonographically, could facilitate the diagnosis of secondary meniscal lesions, which commonly occur in the caudal portion of the medial meniscus (Pozzi et al., 2006). This new ultrasound approach, allowing to evaluate also the profile of the femoral condyles, could be used, in the future, as an aid in the diagnosis of osteochondritis dissecans (OCD) in the knee. It is now known that similar pathologies affecting the cartilage profile, such as the OCD of the caudal humeral head, can often also be diagnosed by ultrasound (Cook, 2016).
According to our knowledge, this is the first study in Veterinary Medicine that described accurately the differences between the visualization of knees CT scans based on the different algorithm chosen (Tables 6 and 7).

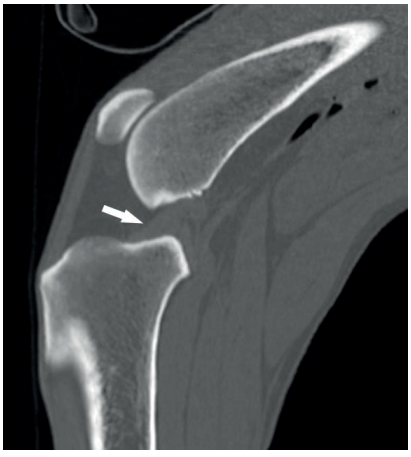

Figure 4. Tomographic aspect of the CrCL (arrow) in para-sagittal scan with hard tissue algorithm

For this reason, after evaluating the CT images both with an algorithm for hard tissues and one for soft tissues we described the imaging differences of the two settings. In previous studies (Samii & Dyce, 2004; Soler et al., 2007) the lateral meniscus-femoral ligament (LMFL) was only identified and never thoroughly described. In our work the LMFL was described using the same evaluation scales used for cruciate ligaments. By applying an

algorithm for hard tissues, the bone was represented with a high image detail, making the demarcation between medullary and cortical. Quite the opposite, using a soft tissue algorithm the bone was represented in all cases exuberant and indefinite. Using a soft tissue algorithm cruciate ligaments, meniscus-femoral ligaments and menisci showed a radio-density similar to each other but higher than the infrapatellar fat pad. These observations agree with what previously described (Soler et al., 2007). By applying a hard tissue window, the intra-articular ligament structures were less visible than those assessed by using the soft tissue algorithm. The ligaments described by the hard tissue algorithm also exhibited a lower visibility of the ligamentous contour than those described with the soft tissue setting. Furthermore, the LMFL resulted, both using the algorithm for hard tissue than soft tissue, less visible than the cranial and caudal cruciate ligaments. However, in neither case the ligamentous structures appeared clearly visible and well contoured overall. Using a hard tissue algorithm, the internal structure of the ligaments always appeared homogeneous, while using a soft tissue setting in some cases the internal ligament structure appeared heterogeneous. It is important to underline that the ligamentous structures with neither of the two algorithms presented an anatomical detail such as to be able to investigate any injuries. The menisci were better visible through the application of a soft tissue algorithm. Despite the soft tissue algorithm providing superior meniscal detail, through both algorithms the menisci were less visible than the ligaments, underlining the obvious limitations of the method in investigating these structures. The menisci, as well as the ligaments, also presented a more inhomogeneous appearance when examined using the soft tissue algorithm. In our opinion, the visualization of an inhomogeneous radio-density pattern is related to the superior anatomical detail architecture obtained with the soft tissue algorithm. In CT scans following the inoculation of the contrast medium, the ligamentous structures showed an overall visibility higher than the one described in the pre-arthrographic scans. The contrast medium showed a variable distribution based on the intra-articular structure taken under examination: the CrCL and the CaCL were well contoured by the contrast medium being always visible, in most cases having a good or moderate visibility. The LMFL has been surrounded less by the contrast medium, being not visible in 2 anatomical preparations. The worst distribution of the contrast medium occurred at the meniscal level; despite this, the menisci were found however visible in all anatomical preparations, showing higher overall visibility than the pre-arthrographic scan. Despite the fact that the volume and concentration of inoculated iodinated contrast medium concurred with what is described in the bibliography (Gielen & Van Bree, 2018), the fluid distribution was not uniform throughout the joint cavity. This phenomenon is partly associated with the onset of post-mortem alterations. In addition, it is possible that the more caudal structures do not receive the same amount of contrast as the cranial ones. The poor arthrographic delineation of the menisci is instead in agreement with what described by Samii et al. (2009), according to which CTA is a diagnostic method that presented gaps in the identification of meniscal tears.

Table 6. *Ex vivo* results of the tomographic evaluation with Hard tissue algorithm scans of Cranial (CrCL) and Caudal (CaCL) cruciate ligament, lateral meniscus-femoral ligament (LMFL), medial (MM) and lateral meniscus (LM)

Criteria	***TC-HAlgo***	***CrCL***	***CaCL***	***LMFL***	***MM***	***LM***
Overall visibility	Not visible	3	3	5	3	2
	Barely visible	5	5	3	8	9
	Moderately visible	3	3	3	0	0
	Clearly visible	0	0	0	0	0
Visibility of the contour	Not visible	3	3	5	3	2
	Barely visible	7	7	5	8	9
	Moderately visible	1	1	1	0	0
	Clearly visible	0	0	0	0	0
Appearance of the internal structure	Non Homogeneous	0	0	0	0	0
	Homogeneous	8	8	6	8	9

Table 7. *Ex vivo* results of the tomographic evaluation with Soft tissue algorithm scans of Cranial (CrCL) and Caudal (CaCL) cruciate ligament, lateral meniscus-femoral ligament (LMFL), medial (MM) and lateral meniscus (LM)

Criteria	***TC-SAlgo***	***CrCL***	***CaCL***	***LMFL***	***MM***	***LM***
Overall visibility	Not visible	2	1	1	2	2
	Barely visible	3	4	6	6	6
	Moderately visible	6	6	4	3	3
	Clearly visible	0	0	0	0	0
Visibility of the contour	Not visible	2	1	1	2	2
	Barely visible	4	6	7	7	7
	Moderately visible	5	4	3	2	2
	Clearly visible	0	0	0	0	0
Appearance of the internal structure	Non Homogeneous	1	2	2	4	4
	Homogeneous	8	8	6	5	5

Table 8. *Ex vivo* results of the computed tomography arthrography (CTA) evaluation of Cranial (CrCL) and Caudal (CaCL) cruciate ligament, lateral meniscus-femoral ligament (LMFL), medial (MM) and lateral meniscus (LM)

Criteria	***CTA***	***CrCL***	***CaCL***	***LMFL***	***MM***	***LM***
Overall visibility	Not visible	0	0	2	0	0
	Barely visible	3	3	2	7	5
	Moderately visible	5	5	4	4	6
	Clearly visible	3	3	3	0	0
Distribution of the contrast medium	Low	0	0	2	1	0
	Moderate	5	6	3	8	9
	Good	6	5	6	2	2
Appearance of the internal structure	Non Homogeneous	1	2	1	0	1
	Homogeneous	10	9	8	11	10

Still according to Samii et al. (2009), CTA represents a sensitive and specific method in the identification of CrCL injury. However, a clarification must be made: as described by Vande Berg et al. (2002) and Han et al. (2008), most of the partial ruptures of the CrCL are not acute but chronic; in addition, the knee affected by this pathological condition generally presents abundant effusion and fibrosis of variable degree that could alter the interpretation of the images. For this reason, the use of CTA in order to diagnose a partial rupture of the CrCL is a controversial procedure still under investigation. The internal structure of ligaments and menisci appreciated in CTA, unlike what was seen using a soft tissue algorithm was generally homogeneous. In our opinion, the high peripheral radio-density of the ligamentous structures and meniscal ligaments conferred by the medium contrast may have reduced the anatomical detail of the ligamentous and internal meniscal surface.

CONCLUSIONS

The goal of this *ex vivo* study is to deepen the knowledge on the ultrasound anatomy of the main intra-articular structures of the dog's knee, assessing the strengths and criticalities of the diagnostic method.

From the results obtained, ultrasonography is a valid diagnostic method in visualizing the cranial-distal part of the CrCL in dogs weighing more than 25 kg. Moreover, the ultrasound examination is capable of evaluating the internal meniscal structure with great anatomical detail. Given the scientific progress associated with the orthopedic diagnostic imaging, it is likely that in the next few years, particular interest will be focused on the ultrasonographic diagnosis of lesions affecting the CrCL and menisci.

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TRICHOGRAM - A HANDLE AND VALUABLE TOOL IN DERMATOLOGY PRACTICE

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Abstract

In dermatology practice, hair-loss along with pruritus represents a very common and challenging problem. Persistent or transient hair-loss is associated to a lot of skin disorders, being of secondary origin in the most cases. The evaluation of alopecic patient for diagnostic assessment should include a complete history, a general and dermatological examination followed by suitable tests. Among them, trichogram is recognized as an easy and fast aid for investigation of troubles in hair anatomy and hair growth. The present study describes some distinct trichography aspects from alopecic dogs, cats and buffaloes which were examined at Dermatology Service of Veterinary Medicine Faculty from Bucharest. In summary, trichogram offers a definitive diagnostic especially for parasitic, fungal-associated and self-induced alopecia, but also an indicative data for many other skin and hair disorders.

Key words*: hairs, alopecia, microscopy.*

INTRODUCTION

Alopecia is partial or complete lack of hair in areas where it is normally present. It is basically classified into congenital and acquired alopecia, and etiologically divided into functional, structural and traumatic events. A hair-loss patient needs a systematic approach for identification of inflammatory or non-inflammatory origin of alopecia, starting with a complete history and identification of clinical pattern of lesions: solitary, multifocal or patchy hair-loss, symmetrical or diffuse alopecia; presence of papules, pustules, seborrhea; presence/absence of pruritus (Moriello & Mason, 1995). The clinically formulated differential diagnosis should be argued by several simple to complex tests, including trichogram, acetate tape preps, scraping, Wood's lamp exam, cytology and even biopsy.

The simplest and non-invasive way to investigate hair disorders consists in microscopic examination of plucked hairs from the periphery of alopecic areas (Medleau & Hnilica, 2006). This test can provide information about abnormalities of hair cycle (telogen/anagen ratio), acquired hair shaft defects (traumatic damage, hair casting, abnormal shapes), genetic hair shaft defects (pigmentary disorders) and also the presence of parasitic or fungal elements. This paper aimed to reconfirm the real support of trichogram in routine veterinary dermatology practice.

MATERIALS AND METHODS

Our study included 20 dogs, 10 cats and 10 buffaloes (hair samples), exhibiting persistent pruritus and hair loss with different distribution. In dog and cat patients, hairs were gently sampled from the periphery of alopecic lesions, in the direction of hair growth, using a hemostatic forceps. The plucked hairs were then placed on a glass slide containing a drop of mineral oil and covered by a coverslip and thereafter examined under a low power (x4, x10, x20). Trichography consisted in the microscopic examination of the whole hairs involving hair root (for anagen/telogen phase), hair shaft (for integrity of cuticle, cortex, medulla, and pigment distribution) and hair tip (for signs of damage).

RESULTS AND DISCUSSIONS

The microscopic findings of hairs from examined dogs, cats and buffaloes were grouped according to the following criteria:

1. Traumatic damage of hair shaft, very suggestive for pruritic dermatitis, especially allergies (Figures 1, 2, 3).
2. Genetic and acquired unusual shapes of hair shaft (Figures 4, 5).
3. Loss of cuticle, cortex and/or medulla integrity (Figures 6, 9).
4. Diffuse cuticle defects (Figure 7).
5. Pigmentary abnormalities (Figure 8).
6. Hair casting (Figures 10, 11, 16).
7. Presence of different parasitic and fungal elements (Figures 9, 10 and 12 to 16).

In dogs, self-induced alopecia was seen with atopy and sarcoptic mange, and consisted in traumatic damage of hair shaft (broken hair tips, hair shaft fractures) due to chronically and severe pruritus (Figures 1, 2, 3). In cats, similar findings have been associated with flea allergic dermatitis (FAD) and eosinophilic syndrome.

We have also been found unusual shaped hairs, both in dog and cat with different pathology. Thus, in a kitten, twisted hairs indicated a congenital, structural hair abnormality known as *pili torti* (Figure 4) and in parallel, abnormal hair shapes with the loss of hair cuticle and cortex integrity were detected in a geriatric dog with hepatocutaneous syndrome caused by underlying metabolic disorders (Figures 5, 6, 7).

Furthermore, severe damage of hair's structure was noticed in fungal infections produced by *Microsporum* and *Trichophyton*, especially in long haired cats, with active infection. First step of fungal infection was proved by the adherence of the arthroconidia to corneocytes from cuticle hair followed by the germination with production of hyphae which penetrated the anagen hair shaft (endothrix invasion - Figure 9). Following hyphal invasion of the hair shaft, sleeves of infective spherical microspores were observed on the surface of the hairs (ectothrix invasion - Figure 10).

Hair pigmentary abnormalities could also been detected by trichoscopy, both in puppy and kitten, reflected by abnormal clumping of melanin granules in the hair shaft and root (Figure 8). Pigmentary disorders are generally associated with congenital alopecia and commonly encountered in dogs with color dilution alopecia (CDA) and black hair follicular dysplasia (BHFD).

Trichogram was also very useful in the diagnosis of demodicosis in juvenile dogs, particularly for difficult areas (around the eyes, nail beds) and very painful lesions. Trichogram is considered to be 75-80% more sensitive than skin scrapings in demodicosis diagnosis. We recommend it for solitary alopecia and paronychia due to *Demodex* mites, which appear as adults, nymphs, larvae or eggs (Figures 12, 13). In dry form of demodicosis, but also in dermatophytosis with *Microsporum canis*, trichoscopy showed a lot of follicular casts or sheats of sebum and keratin accumulation around hair shafts (Figure 11).

In hair samples from buffaloes occurring diffused alopecia, focal leukotrichia, severe seborrhea and pruritus, we identified a concomitant pediculosis with *Haematopinus trunculatus* and chorioptic mange (Figures 14, 15, 16). Louse infestation is not a commonly condition in dogs and cats, but more often in farm animals leading to skin irritation, anemia, anorexia, restlessness and reduced productivity (Butler, 1985; Veneziano et al., 2003).

In veterinary dermatology, a lot of hair loss conditions are recognized, either of primary or secondary origin, such as, genetic/congenital hair defects, endocrine/metabolic diseases, immunologic, parasitic and fungal hair disorders, psychogenic induced alopecia, drug-induced alopecia, traumatic or paraneoplastic alopecia (Colombo, 2010; Paterson, 2002). Acquired alopecia is usually of higher incidence compared to congenital alopecia (Geary, 1986).

In dermatology practice, post-inflammatory alopecia commonly found in dog and cat (Guaguère, 2008) is mainly caused by infectious folliculitis with *Staphylococcus*, *Demodex* mites and *Dermatophytes* fungi. Before trichogram, clinical pattern of alopecia may provide useful diagnosis clues. Thus, symmetrically generalized alopecia without inflammation implies the alteration in hair cycling (functional alopecia), but non-symmetrically localized alopecia with inflammation indicates the loss of the follicular structure (structural alopecia).

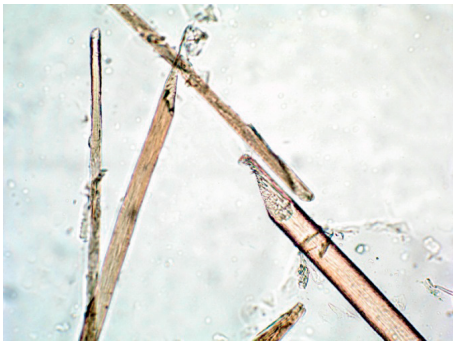

Figure 1. Dog hairs - broken off hair tips

Figure 2. Dog hairs - middle and tip fracture of hair shaft

Figure 3. Dog hairs - fractured hair shaft

Figure 4. Kitten hairs - unusual shaped hairs in pili torti (genetic defect)

Figure 5. Dog hair - unusual shaped hairs in hepatocutaneous syndrome (metabolic defect)

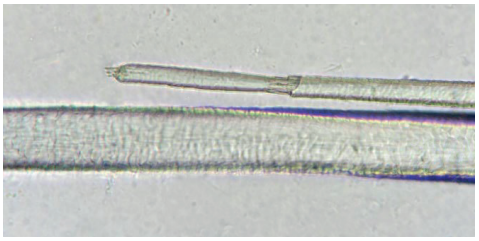

Figure 6. Dog hairs - loss of cuticle and cortex integrity in hepatocutaneous syndrome (metabolic defect)

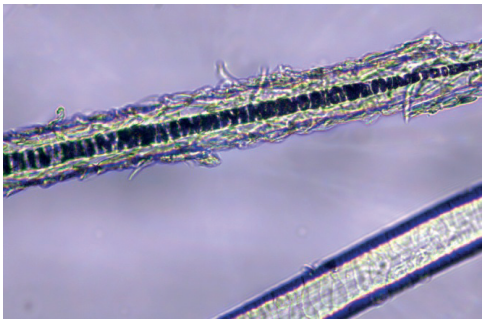

Figure 7. Dog hairs - diffuse cuticle defects

Figure 8. Dog hairs - pigmentary abnormality (melanine clumping)

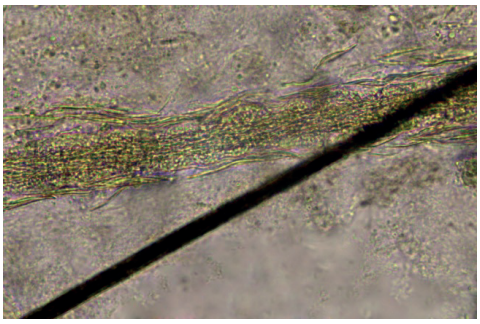

Figure 9. Cat hairs - severe damage of hair shaft structure: hyphae and spores (dermatophytosis)

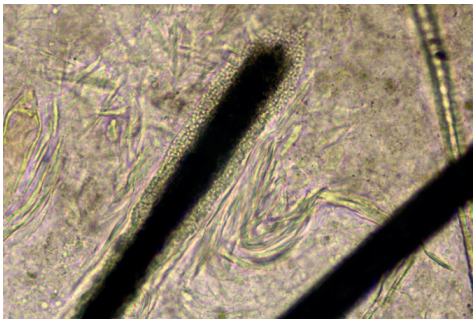

Figure 10. Cat hairs - sleeves of microspores around hair shaft (dermatophytosis)

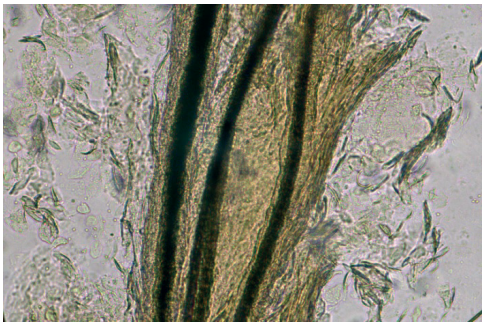

Figure 11. Cat hairs - large hair castings (dermatophytosis)

Figure 12. Dog hair - one adult of *Demodex* mite hanging on the hair bulb in anagen phase

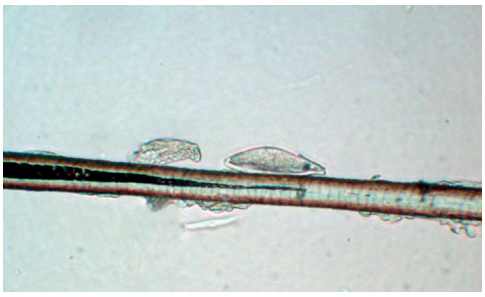

Figure 13. Dog hair - a spindle shaped egg of *Demodex* mite attached to hair shaft

Figure 14. Buffalo hairs - an egg of *Chorioptes* mite attached to hair shaft

Figure 15 Buffalo hairs - an operculated louse egg (*Haematopinus* spp.) cemented to hair shaft

Figure 16. Buffalo hair - an adult of *Chorioptes* mite attached to hair shaft and hair casting

CONCLUSIONS

Acquired alopecia was of higher frequence compared to congenital alopecia. The present study emphasized the valuable aid of trichogram in definitive diagnostic of parasitic and dermatophytosis - induced alopecia. Trichogram was also indicative for self-induced alopecia (in pruritic conditions), keratinization and pigmentary disorders of various origins.

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SYSTEMIC PATHOLOGIES IN A CAPTIVE HUMBOLDT PENGUIN (*Spheniscus humboldti*) - STUDY CASE

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Abstract

The present case study was represented by a captive, adult, female Spheniscus humboldti penguin submitted for post mortem investigations at the Pathological Anatomy Department, from the Faculty of Veterinary Medicine of Bucharest. The diagnostic methods included necropsy, microbiology, cytology, histopathology and PCR examination.
Necropsy revealed poor body condition and obvious thickened air sacs along with multifocal, coalescing, yellow nodules in multiple organs. Cytology revealed necrotic and inflammatory cells, detritus, bacteria and fungal hyphae and microbiologic examination isolated Aeromonas hydrophila and Candida krusei. Histopathology revealed old and developing multifocal granulomas with a central oxyphilic material and circular disposition surrounded at the periphery by multinucleated giant cells and cellular reactivity. Other lesions identified were interstitial nephritis with glomerulosclerosis, lymphohistiocytic hepatitis. and splenic lymphocyte depletion. Also, protozoan cysts (50-80µ in diameter) were identified in all major tissues, but PCR examination was negative for Toxoplasma gondii.
The case of the Humboldt penguin presented multifocal granulomatous inflammations, associated with emaciation, immunosuppression and parasitism and the cause of the death was respiratory insufficiency.

Key words*: Humboldt penguin, granulomatous systemic inflammation, respiratory insufficiency.*

INTRODUCTION

Penguins are highly specialized birds, with morphologic and functional particularities induced by living in, both terrestrial and aquatic habitats (Schneider et al., 2014). This is the reason why raising in captivity this type of birds represents a challenge and requires specific notions regarding environment, feeding, resting and reproduction (Schneider et al., 2014).
Pathologies diagnosed in captivity penguins are frequently associated with inappropriate housing and feeding conditions (Schneider et al., 2014). Also, it is taken into account the fact that penguins are prey animals and instinctively they will tend to mask signs of disease until it becomes severe or critical for the body (Schneider et al., 2014).
Post-mortem examination is a valuable tool for lesion evaluation, causes of morbidity and mortality and also, overall evaluation of general health status and group pathologies in both cases of captivity and wild penguins (Schneider et al., 2014).
The present paper presents a detailed evaluation of lesions, both macroscopic and microscopic, encountered in a Humboldt penguin kept in captivity, the first of this kind in Romania, regarding the species and pathological findings.

MATERIALS AND METHODS

The present case study involved an adult, female penguin, belonging to *Spheniscus humboldti,* owned and kept in captivity by a private owner. After death, the bird was submitted to multiple investigations at the Faculty of Veterinary Medicine of Bucharest in March, 2014. Little information regarding housing conditions and feeding were obtained, only that it was the only bird of this species and was kept in an individual enclosure. Regarding clinic evolution, the information provided was that during the last week the bird had poor appetite and was lethargic with no medication administered. The following examinations were performed in the study: necropsy, citopathology (M.G.G. stain), microbiology,

histopathology (H.E., H.E.A., PAS stain and Giemsa stain) and PCR testing for *Toxoplasma gondii.*

RESULTS AND DISCUSSIONS

Gross examination in the necropsy procedure revealed an adult *Spheniscus humboldti* penguin in cadaveric resolution. Examination of the exterior revealed full plumage with a dirty aspect, due to droppings from the cloaca up to neck and even wing region. This aspect is suggestive for prolonged decubitus, lack of bathing and possibly, presence of digestive disorders that affected the bird before the moment of death (Figure 1). The legs were affected by discrete lesions of plantar hyperkeratosis and medium to long, not-worn claws.

Figure 1. External examination: dirty plumage with greenish watery droppings on the entire ventral surface

These aspects were associated in penguins with improper floor or bedding and lack of extensive movement due to lack of enrichment in the environment (Blay & Cote, 2002; Clarke, 2003).

After skinning, pectoral and leg muscles were observed as reduced, with a prominent sternum keel and obvious subcutaneous highly fibrous conjunctive tissue.

The extended breastbone with a large basal plate and a membrane, is a normal feature in diving birds, including the present case, since these animals require protection of the intestines from high water pressure while diving (Johnsgard, 1987).

The examination of coelomic cavity revealed white-yellow nodules, with variable dimensions, from 0.2-3 cm in the air sacs (Figure 2). In addition to the nodular structures, air sacs presented thick, white-yellow deposits, that resulted in a rigid structure of the wall. Similar structures were observed on the surface and in-depth in both lungs and surrounding serous structures. In addition, the lungs presented a red colour, increased volume and a foaming reddish fluid on fresh cut section, suggesting inflammatory pulmonary oedema.

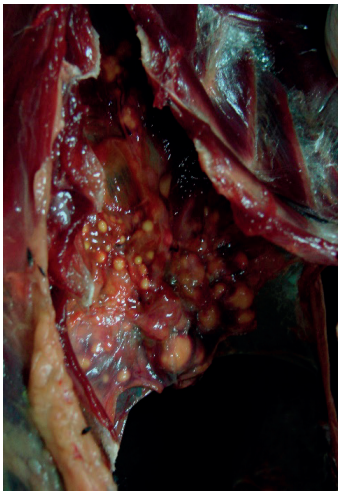

Figure 2. Abdominal air sac with thick walls and multiple yellow nodular structures with varying dimensions

Occasionally, white nodules were observed in the liver, kidney and spleen. On cut section, the nodules presented a dry, multilayer, concentric yellow material, suggestive for a granulomatous inflammation.

The digestive tract was generally empty and a white-green liquid content was observed in the large intestine.

The liver was large and black. The colour of the organ is specific (Hocken, 2002), but the size and the fact that dark, red blood was expressed on cut surface, sustained the diagnosis of stasis in the liver.

The fat tissue stored in the subcutaneous pelvic region and peritoneal cavity was reduced and also, the one from the base of the heart that had a transparent, slightly yellow colour, lesion diagnosed as serous atrophy of the fat tissue. It is known that penguins have particularities regarding lipid storage, appearing to be an obligatory prerequisite of the periods of fast,

mostly before molt and the breeding cycle (Cherel et al., 1993; Hocken, 2002; Schneider et al., 2014). Both, Blem (in 1990) and Lewden (in 2017), suggest that penguins have different body sites and mechanisms for body fat usage. While subcutaneous fat is the first to be deposited and last to be used, the peritoneal fat may be the first to be mobilized during stress. Another aspect regards the relationship between subcutaneous fat tissue deposits and thermoregulation. Less the fat tissue deposit, higher peripheral body temperatures in order to restore fat deposits and, in consequence, less capacity of insulation (Lewden et al., 2017)

In the present case, reduction of internal and subcutaneous fat tissue deposits, along with pectoral muscle emaciation, are strong indicators of poor body condition (Schneider et al., 2014).

Cytopathologic examination was performed by imprint and scraping from the nodular lesions previously observed and the internal layer of air sacs. The result obtained was a cellular population composed of macrophages and heterophils, erythrocytes, thrombocytes, cellular detritus, rare epithelial cells, along with intra and extracellular bacteria and fungus (Figure 3).

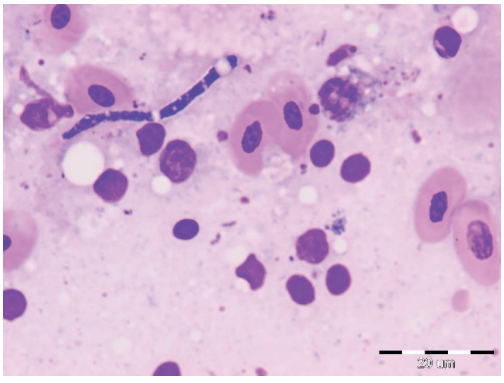

Figure 3. Air sac smear with a slightly eosinophilic background, bacillar and coco-bacillar bacteria extra and intracellular, nude nuclei, few erythrocytes and a fungus (M.G.G., 40x)

Microbiologic examination of samples of freshly cut granulomas from the lung and liver isolated *Aeromonas hydrophilia* and *Candida krusei. Aeromonas hydrophilia* is widely known as an opportunistic pathogen that affects debilitated organisms and has the capacity of infecting through aquatic mediums (Hazen et al., 1978). In 2005, a study concerning penguins belonging to species *Spheniscus demersus*, kept in a zoologic garden, identified a clinic episode of anorexia, lethargy, vomitus and diarrhoea with green droppings, that ended by death of four from the total of seven individuals. Microbiologic examination of affected tissues and the food, isolated a bacterial population among which *Aeromonas hydrophilia* had the pathogenic potential to cause the outbreak of the disease (Kim et al., 2005). In another case study, a captivity spectacled caiman which was exposed to stressful conditions, developed an infection with *Aeromonas hydrophilia* that was involved in the death mechanism. Captivity conditions are stressful events are involved in infections with potential pathogens for exotic animals (Kim and Kwak., 2013).

Mycologic examination isolated *Candida krusei,* a potential pathogen for immunosuppressed individuals. Donnelly et al. isolated and studied non-albicans Candida species in gastro-intestinal pathologies for birds demonstrating the possibility of transformation from a commensal species to a pathogenic one (Donnelly et al., 2019). Nevertheless, most studies indicate that Aspergillus spp. is the main fungus encountered in respiratory, chronic, granulomatous lesions in birds and, in particular, in penguins (Khan et al., 1977; Xavier et al., 2007; Beernaert et al., 2010). As a matter of fact, for wild Humboldt penguins (*Spheniscus humboldti*) as a part of ongoing ecological study, health surveys were carried and comprised blood tests, bacterial and virusologic tests and serology for Aspergillus sp. (Smith et al. 2008). This gives the fungus a central position in incidence and in screening health status for penguin population.

Cytopathology and histopathology using PAS stain, from respiratory tract lesions revealed long fungi with a septate hypha and sharp-angular branching suggestive for Aspergillus spp., although it was not positive for microbiologic examination.

Histopathology revealed mainly chronic lesions. Multiple granulomatous lesions, with different stages of evolution and extension were evident in lungs, air sacs, liver, spleen and kidneys.

The normal structure of the lungs and air sacs was severely compromised. The air sacs presented thick layers of amorphous material, formed by cellular detritus and fibrine along with granulomatous lesions (Figure 4).

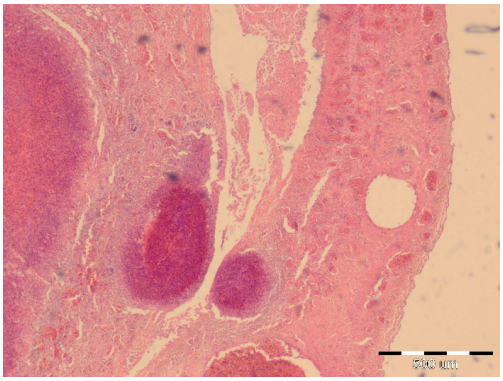

Figure 4. Air sac with a thickened wall due to inflammation, congestion and fibrin deposits and multiple granulomatous lesions (H.E., 4x)

The lungs were affected by focal to coalescing areas of chronic granulomatous inflammation, congestion and inflammatory oedema. The microscopic aspect of the granulomatous lesions revealed a central region, represented by an oxyphilic, unstructured necrosis surrounded by concentric layers of detritus and inflammatory cells, mainly lymphocytes and macrophages, epithelioid cells and, less frequent, gigantic multinucleated cells. Often, marginal hyperaemia and congestion was observed in lung and liver. Due to severe affection of the air sacs and the lungs, with acute and chronic lesions, respiratory insufficiency was the main cause of death for the present case of Humboldt penguin.

The liver presented a lympho-histiocytic hepatitis, with groups of inflammatory round cells frequently observed in perivascular areas, along with uneven distribution of hepatic stasis. Also, hepatocyte degeneration and necrosis, chronic inflammatory reaction and discrete proliferation of conjunctive tissue were observed in the liver and quantified as possible incipient granulomatous lesions.

The spleen was affected by lymphocytic depletion, represented by rare or degenerated lymphocytes in the white pulp and a histiocytic infiltrate in the red pulp along with circulatory disturbances suggestive for immune suppression (Figure 5).

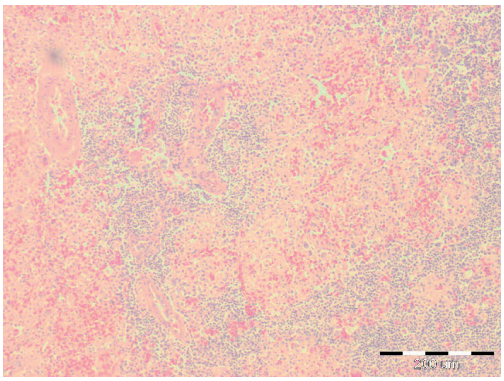

Figure 5. Lymphocytic depletion and hyperaemia in the spleen (H.E., 10x)

In birds, direct immune suppression can occur since fungal toxins interfere with protein synthesis, which affects both B and T-cell immunity. Secondary suppression can be encountered with high plasma corticosterone when birds have an acute feed restriction or are fastening. Nutrition plays a critical role in maintaining the immune system since poor protein and calorie diets supress antibody responses and enhance evolution of diseases (Schmidt et al. 2003). The penguin from the present study was affected by the presence of fungi, that could have delivered fungal toxins and produce primary suppression. The other mechanism was fastening before death and potentially inappropriate feeding during the time in captivity, that lead to lack of response of the immune system to immunogens.

Histopathology of the kidney revealed multiple lesions such as tubular degeneration and tubular necrosis, glomerular degeneration with reduced capillaries and few mesangial cells, along with interstitial mononuclear inflammatory cells. Some areas in the kidneys presented unstructured oxyphilic necrosis with granulomatous peripheral reaction, similar to those observed in the lungs and air sacs.

In addition, protozoan cysts were observed both in cytopathologic and histopathologic examinations, in all major tissues examined, including brain. They presented elongated form with a 50-80µ diameter, had multiple round structures inside, with discrete inflammatory or degerenative ractions in the tissues (Figure 6).

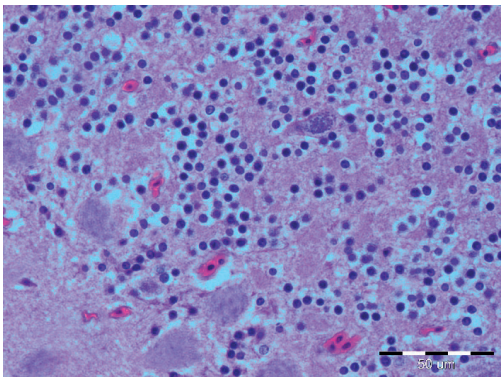

Figure 6. Cerebellum with necrotic neurons, mild lymphocytic inflammatory reaction and a parasitic cyst in the granular layer (H.E.A, 40x)

The morphologic aspects resemble for infection with *Toxoplasma gondii*. Still, PCR examination on paraffin embedded tissues gave a negative result, further studies being needed for sure exclusion of this parasite, such as immunohistochemistry. Several studies on wild and captivity penguins revealed the presence of *Toxoplasma gondii* in several organs, such as lung, liver and brain and was confirmed by PCR or immunohistochemistry or using antibody test surveillance (Ploeg et al., 2011; Bartova et al., 2018). In chronic cases, inflammation around the cysts is discrete in the tissues affected. In the brain, the inflammatory response consists of perivascular cuffing of blood vessels and mild lymphocytic reaction and gliosis (Zachary, 2017). This type of response was observed in the present case, with mild lymphocytic reaction and perivascular cuffing. The presence of the parasitic infection adds in the overall diagnosis of chronic, debilitating, systemic pathologies for the captive penguin.

CONCLUSIONS

The external gross examination in the necropsy procedure of the captive Humboldt penguin (*Spheniscus humboldti)* revealed a poor body condition.

Subcutaneous fat tissue can be used as a good indicator of body condition in penguins.

Necropsy of the coelomic cavity and organs revealed systemic granulomatous lesions on serous surfaces, air sacs, lungs, liver, spleen and kidneys.

Multiple investigations, such as microbiology, cytopathology, histopathology and PCR investigation indicated the presence of potentially pathogenic microorganisms represented by bacteria, fungi and parasitic cysts.

Poor body condition, the presence of granulomatous lesions and spleen lymphocytic depletion lead to the diagnosis of polyfactorial systemic disease in the case of the Humboldt penguin.

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FIRST OCCURRENCE OF SHEEP DEMODICOSIS IN SERBIA

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Abstract

Demodicosis of sheep are parasitic infection caused by Demodex spp. Infection is not common like other type of parasitic dermatitis and there are not many cases of this infection described. In most cases no clinical symptoms are cusing and has little or no economic impact on sheep flocks. Demodex spend all life under host skin and present a normal skin habitat. Mainly are resent at hair folicules where its complete transformation from eggs to adult is performed. There are two types of diseases, local and general. Local demodicosis were present like small reddish places without hair on skin. Main topic are skin on head, around eyes and mouth, and on the back legs. Most important role to clinical demodicosis presents a immunological status of animals. Hereditary against demodicosis were important role too. During our examination of parasitoses in one flock of sheep in the south of Serbia during the spring of 2018, demodicosis was diagnosed. Established clinical signs are nonpruritic papules and nodules which develop over the face, neck, shoulders, and sides and udder. At a later stage, there was a pustular eruption that gradually merged, while later there was a thickening of the skin and loss of wool. Itch rarely occurred. The nodules contain a thick, waxy, grayish material that can be easily expressed and mites can be found in this exudate. To diagnosis we performed microscopic examination of deep skin scrape which revealed adult parasites, larval forms and lemon-shaped eggs. Lesion were spontaneous loos around few months without therapy. In the area of the Western Balkans, only one case of ovine demodicosis was recorded in Bosnia and Herzegovina, and this is the first case described in Serbia.

Key words*: Demodex spp., sheep, demodicosis.*

INTRODUCTION

Mange represents one of the most costly nuisances for stock-breeders. The heavy losses for which they are responsible occur throught two mechanisms: directly, due to discomfort or reduction in milk and meat production and damage to leather or wool (Chapman, 1975; Pavlović et al., 2011; Jańczak et al., 2017). The latter is due to bacterial and viral diseases with severe lesions due to mange-causing acarid (Pavlović et al., 1997).

Essentially, four types of mange based on the genus of mites may affect sheep, sarcoptes mange, psoroptes mange, chorioptes mange, and demodicosis (Kaufmann, 1996).

Demodectic mites are considered normal inhabitants of hair follicles and sebaceous glands of sheep and represent a normal skin habitant (Kibeb et al., 2016). These parasitoses cause pruritus of varying severity, as well as skin lesions (Priselkova and Zorina, 1955).

In sheep, three demodex mite species have been described: *D. ovis, D. aries* and one more species not completely specified. All three species live in hair follicles (Jańczak et al., 2017).

Demodicosis in sheep is not common as infection with other mites agents. Demodectic mange seems to be of little clinical significance sheep. It causes a follicular dermatitis rather than a scab-forming dermatitis. It does not cause pruritus and is usually manifested by nodules, crusts and small scabs. Because there is not as much inflammation as caused by other mange mites, and since the animals do not itch and therefore do not scratch and rub, the skin and hair are not damaged as much.

In our paper we describe a case report of first occurence of *Demodex* spp. in Serbia.

MATERIALS AND METHODS

During 2018 in one herd of 27 sheep in south Serbia it have been observed some changes in the skin that were followed by relegation wool. In 15 animals the skin had a pinkish-red color and wool fell off in those places. At the same time in those places was found non-pruritic papules and nodules. Each animal were clinically examined for the presences of skin lesions like scales, crust, alopecia and clinical signs of itching. Established clinical signs are develop over the face, neck, shoulders, inner and outer side of the leg and udder (Figures 1 and 2).

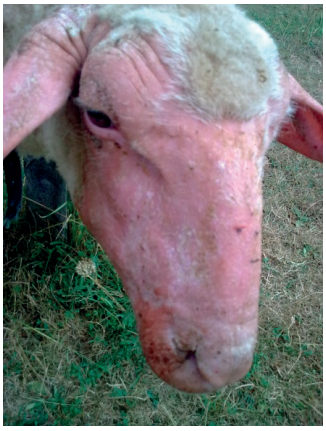

Figure 1. Non-pruritic papules on sheep head

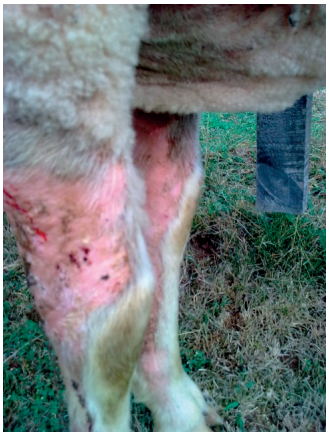

Figure 2. Alopecia and clinical signs of itching

At a later stage, there was a pustular eruption that gradually merged, while later there was a thickening of the skin and loss of wool. Itch rarely occurred. The nodules ranging between 3-8 mm in diameter and contain a thick, waxy, grayish material that can be easily expressed and mites can be found in this exudate.

To diagnosis we performed with microscopic examination of deep skin scrape (suspected skin nodules were incised with a scalpel and the contents collected and sent for further checking), and reveals of adult parasites, larval forms and lemon-shaped eggs. Determination of genus of found parasites was based on the morphological characteristics (Pavlović and Rogožarski, 2017).

RESULTS AND DISCUSSIONS

In the examined samples, we determined the presence of adult and developmental stages of *Demodex* spp. (Figure 3).

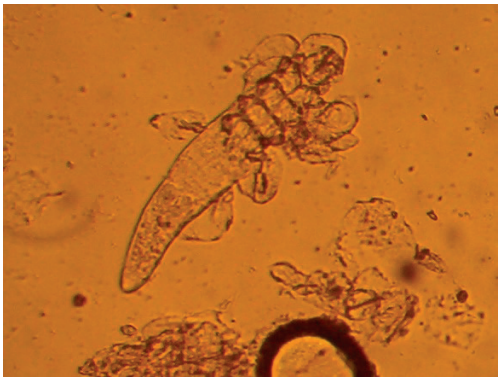

Figure 3. *Demodex* spp. collected from sheep

Demodex spp. are unique among parasitic mites, because they are elongated with short, stumpy legs. We also noticed these changes during our research. Their distinct morphology is a presumed adaptation to living in hair follicles and sebaceous glands of their hosts forming nodules (Clifford and Desch, 1986; Soulsby, 1977).

Nemeseri and Sezeky (1966) reported the occurrence of this mite in different habitats including meibomian glands, hair follicles and sebaceous glands and epithelial tissue of the sensory hairs of the same host. Some authors claim that skin areas particulary rich in well developed sebaceous glands are most frequently parasitized (Brownlee, 1935; Clifford and Desch, 1986; Yeruham et al., 1986). These mites feed on sebum, protoplasm, and epidermal debris. Transmission of

demodex parasites occurs through close contact of infested and naive hosts, with the transfer of mites from infested dams to neonates being the primary route (Ivanović and Pavlović, 2015).
Infection is not common like other type of ectoparasites infection and there are not many cases of this infection described.
First description of *Demodex* infection of the sheep are given by Griffiths (1915) who describes the disease like slight pustular eruptions which gradually coalesce are usual, while later there is thickening of the skin and loss of wool. Later, Hirst (1919) considers that such mites are probably common parasites of this animal, he records their presence only in the Meibomian glands of one recently slaughtered sheep.
Mainly are resent at hair folicules where at hair folicules where its complete transformation from eggs to adult is realized (Brownlee, 1935). Most important role to clinical demodicosis present a immunological status of animals (Ivanović and Pavlović, 2015). Hereditary against demodicosis were important role too.
During our research we occured localized lesions of infection. Local demodicosis were present like small redish places withouth hair and with the presence of nodules on the skin. Itch rarely occured. Main topic are skin on head, around eyes and mouth, and on the back legs. In our cases, lesion are spontaneously stopped around few months without therapy.

CONCLUSIONS

Demodex ovis infests sheep but demodicosis in sheep is not common. In the area of the Western Balkans, only one case of ovine demodicosis was recorded in Bosnia and Herzegovina (Pavlović et al., 2011) and this is the first case described in Serbia.

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IATROGENIC THERMAL INJURY MANAGEMENT IN A GERIATRIC DOG - A CASE REPORT

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Abstract

This case report aims to propose a wound healing protocol for iatrogenic thermal wounds in small animals. Thermal injuries in small animals are most frequently caused by domestic accidents, as a result of contact with hot surfaces, liquids or fire. Burns can also be inflicted during surgery by the use of faulty heating pads or grounding plates. An 11 years old female dog was presented for a post-surgical check-up after undergoing mastectomy. At this check-up, two-weeks after surgery, extensive burn wounds on the dorsal cervical and thoracic areas were discovered. Over 80% of the affected area was covered by eschars. The injured area was still covered with hair but nociception was absent. Wound management aimed to promote secondary intention healing by using a complex protocol that included an initial escharectomy followed by the use of honey dressings, hydrocolloid dressings, paraffin-impregnated tulle and sulfadiazine ointment. The wound healed in 8 weeks, mainly by contraction; a small percent healed by epithelialization. We concluded that during each phase the dressings should be adapted to the specific phase of wound healing and that combining multiple products offers a superior result.

Key words*: iatrogenic, burn wound, thermal injury, wound management, secondary intention healing.*

INTRODUCTION

The management of burn wounds is one of the most active areas of research that is constantly devising new and improved treatment protocols. It is widely accepted that burns, especially severe burns, are not limited to the local injury, but also inflict a systemic imbalance known as "burn disease". In veterinary medicine, the frequency of burn wounds is considerably lower than in human patients, and injuries are often not life-threatening.

A burn injury is defined as tissue (most frequently, the skin) damage inflicted by thermal, electrical, chemical or radiation exposure. Thermal injuries are a consequence of direct contact with fire, boiling water, hot steam, and hot objects, or secondary to prolonged contact with a source of moderate heat. In small animals, burns usually occur consecutive to domestic accidents. They can also be associated with various heating devices used during anaesthesia or cage rest to increase the body temperature in low mobility patients.

The classical system used in human medicine classifies the severity of burn wounds into four degrees. However, in veterinary medicine, burns are more commonly classified according to the depth of the injury, as follows (Pavletic, 2010):

- Superficial burns (first degree) – when lesions only include the epidermis;
- Partial-thickness burns (second degree) – both epidermis and superficial dermis are affected;
- Full-thickness burns (third degree) – the epidermis, full dermis and subcutaneous tissues are affected;
- When the injuries extend to deeper tissues such as muscle or bone, a fourth degree can be described.

The severity of a burn wound is not only determined by the depth of the lesion, but also by the percentage of the total body surface that was affected. Jackson's burn model describes three concentric zones in full-thickness burns: a central area of coagulation and necrosis, an intermediate area of stasis and an outer zone of hyperemia (Hettiaratchy & Dziewulski, 2004).

The therapy of burn patients has two directions: patient stabilization and monitoring (fluid therapy, multimodal analgesia, nutritional support) and wound management.

Superficial and partial thickness burns can heal through re-epithelialization either from surviving germinal epidermal cells or from the cells of the root sheath of the hair follicle. In injuries accompanied by loss of the entire skin structure, healing can be obtained either through contraction and epithelization or secondary to surgical reconstruction.
This article aims to draw attention to the importance of adapting the wound management protocol to the healing phase, and emphasize that proper wound closure can be obtained exclusively by secondary intention, even in burns that involve large body surfaces. Care should be taken when using warming devices, given that associated iatrogenic injuries occur with a significant frequency.

MATERIALS AND METHODS

The patient presented in this case study is a 10-year-old mixed breed medium sized (12 kg) intact female dog.
The history revealed that the dog underwent mastectomy for a solitary ulcerated necrotic mammary mass (4th mammary gland on the left side) with secondary infection, 10 days before it was presented for skin lesions. The mass had been carefully resected with clean margins and the skin closure posed little tension on the wound margins.
The pre-anaesthetic work-up had been normal (blood work within the reference range, no cardiac pathology and no visible metastasis on the thoracic and abdominal imaging). The patient had been discharged the following day after the mastectomy, and two evaluations of wound healing had been performed on the third and 10th day at the first clinic. Wound dehiscence, behavioural changes, loss of appetite or malaise were not observed during this period. However, at the second check-up on the postoperative day 10, the skin on the dorsal trunk and lateral side of the neck appeared modified and the patient was referred.
At the first consultation of the animal, 10 days after the surgery, the temperature, heart and respiratory rates were within physiologic limits. No pain behaviour was exhibited during manipulation of the animal and palpation of the affected area. Once the hair was clipped, a burn-like lesion was observed, involving a large area of the dorsal and lateral part of the thorax and neck. This area was mostly covered by a black leathery eschar, and surrounded by small areas of stasis and hyperemia.
Blood tests at this time revealed low albumin, mild hyperkalemia, leukocytosis, neutrophilia and lymphocytosis, all consistent with the pathophysiological process of a burn lesion, accompanied by extensive cell death and ongoing inflammation. A bacterial wound culture was not performed at that point because the skin was either intact or covered by the eschar. No signs of secondary infection such as exudate, redness not correlated to the burn injury or pain could be observed. Although the patient had no cardiac, pulmonary or other associated diseases that would impede the anaesthesia or surgery, the owner opted for a conservative approach.

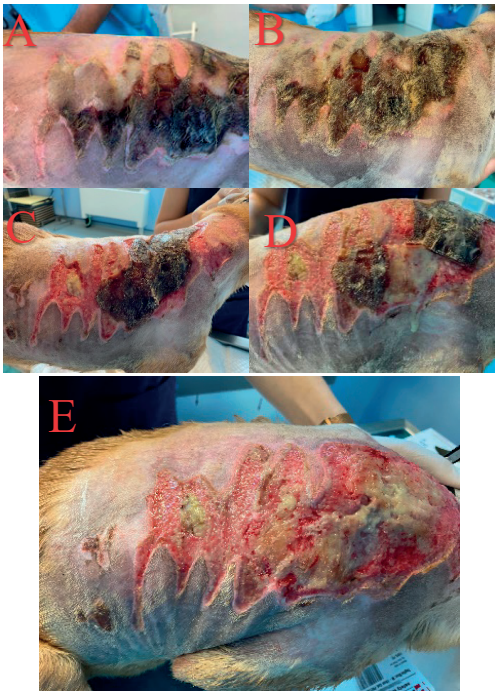

Figure 1. Inflmatory and debridement phase. A. day 1- initial aspect. B. day 2; C. day 3; D. day 4; E. day 4.

The wound could not be properly assessed while the eschar covered the affected area. Thus, the first purpose of local therapy was to promote autolytic debridement and facilitate escharectomy. To avoid further bacterial conta-

mination and multiplication and simultaneously aid debridement, raw honey was used for the first four days (Figure 2-A). Secondary and tertiary layers were applied to finish the thoracic bandage. The bandage was applied in such a manner as to prevent excessive movement of the skin, ensure moisture and absorb the exudate while the separation between the eschar and the healthy tissue occurred. Sedation was not required as the animal exhibited no discomfort or aggressive behaviour.

The entire eschar was gradually eliminated over the following four days, by removing the areas where the eschar was loosely attached to the tissue underneath and had no vascular connection.

As the injury had occurred two weeks prior and the animal was not in a hypermetabolic state or affected by systemic alterations associated with the wound, limited general support was initiated. In the first few days, patient management included fluid therapy and opioid analgesia; systemic anti-infective drugs were not used. This decision was guided by the the clean aspect of the wound, the absence of fever, the frequent change of bandages, and low exudate production.

The animal had a good appetite and its vital signs were within physiologic limits at each bandage change. This was not considered a patient with complex burn disease, so once the eschar was removed, the skin lesion was treated as any other large open wound.

Once the escharectomy was performed, the subcutaneous fat was exposed on the dorsal area of the trunk. This tissue, due to the lack of vascular supply, was expected to become necrotic and undergo autolysis and treatment was continued with topical honey (Figure 2-B). However, the tissues that presented healthy granulation tissue were treated with silver sulfadiazine cream (Dermazin).

The bandage was changed daily for the first week and every other day for the following two weeks. Starting on day 6, a combination of tulle dressing impregnated with chlorhexidine (Bactigras) and silver sulfadiazine cream was used (Figure 2-C). Each of these dressings was conceived for use as the primary layer of a bandage. In this case, the tulle dressing was used as a non-adhesive network to hold the cream in place. Both of them promote healing and have a satisfactory antibacterial effect. This combination was used during the second week.

Although at the first assessment of the wound, the hair on the surrounding area was clipped properly to accurately assess the extent of the lesions, a week later new necrotic foci were encountered. At this point, to confirm the diagnosis, biopsies were taken from this tissue and from the margins of the largest defect. The pathology report supported the initial clinical diagnosis and excluded other differential diagnoses such as toxic epidermal necrolysis, vasculitis, that had been previously considered. Histopathology identified necrosis, inflammation and signs of tissue regeneration.

Once a healthy granulation tissue was established across the entire wound surface, a hydrocolloid dressing was used to further promote healing by assuring a moist environment and to act as a barrier for external contamination (Figure 2-D). The use of this dressing allowed a reduction of the frequency of bandage change. However, as this dressing can impede contraction, its use was alternated with the Bactigras-Dermazin combination.

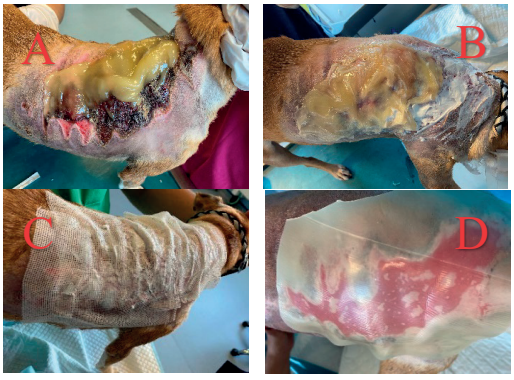

Figure 2. Types of dressings.
A. Honey dressing. B. Honey dressing+ Silver sulfadiazine cream; C. Tulle dressing+Silver sulfadiazine cream; D. Hydrocolloid

The use of these dressings as part of a bulky bandage allowed the patient's discharge and subsequent outpatient therapy. The progression of healing was monitored every two to four days at each bandage change.

Although at home the patient continuously wore an Elizabethan collar and the bandages were kept in place between check-ups, there was a small setback on day 34, when the dog

rubbed against various surfaces due to pruritus (Figure 3-G). The perilesional skin was hyperemic and the wound surface was larger compared to the previous evaluation.

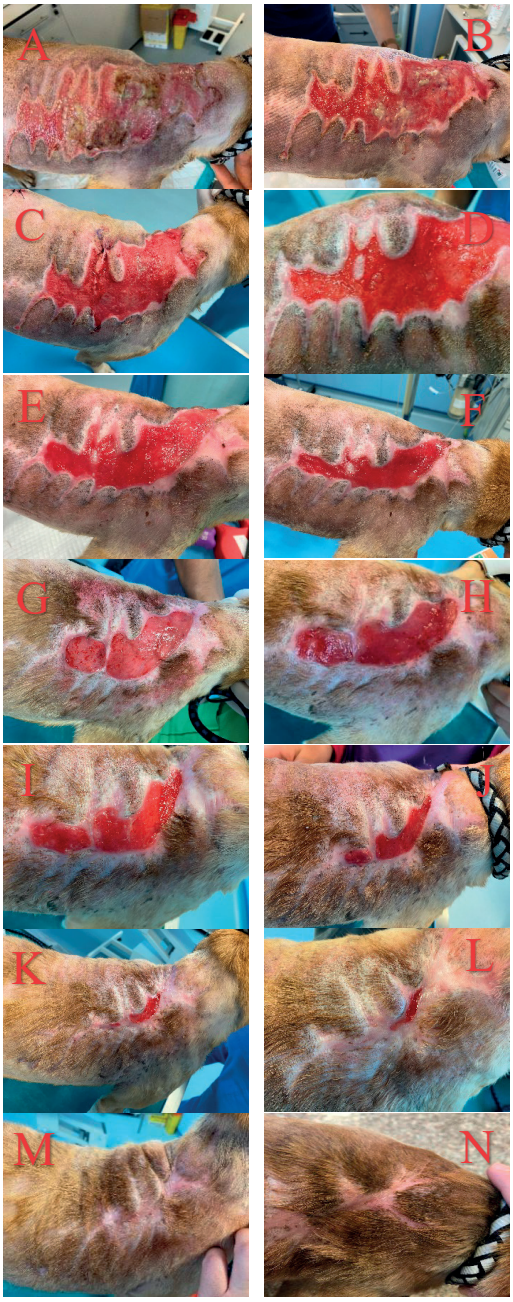

Figure 3. Healing process.
A. Day 7; B. Day 10; C. Day 15; D. Day 18; E. Day 21; F. Day 32; G. Day 36; H. Day 38; I. Day 43; J. Day 46; K. Day 50; L. Day 53; M. Day 56; N. Day 76

To prevent other accidents from occurring and properly monitor wound healing, the bandages were changed every other day from that day onward.

On day 56, the wound was entirely closed (Figure 3 - M). After a skin defect is entirely covered by epithelium, the wound healing continues. Due to this consideration, we advised the owner to apply silver sulfadiazine cream every two or three days to maintain skin moisture. To avoid any trauma to the fragile new scar tissue, the owner was advised to keep the area covered with light shirt.

RESULTS AND DISCUSSIONS

Radiant heat injuries in small animals have a low rate of occurrence. Although some of the warming systems used in veterinary medicine are known to be a burning hazard, accidents related to them continue to happen.

Case series and articles on this topic date back more than 50 years, but they are not numerous (Crino & Nagel, 1968; Dunlop et al., 1989). Since thermal injury is not frequent in veterinary medicine, the existing data regarding burn management is scarce compared to the body of literature from human medicine (Johnson & Richard, 2003; Pretorius et al., 2011; Mullally et al., 2010; Sheridan, 2012; Rowan et al., 2015). Most guidelines for burn wound management in use are veterinary adaptations of the protocols used in human hospitals (Pavletic, 2010; Pavletic & Trout, 2006; Vaughn & Beckel, 2012; Vaughn et al., 2012; Vigani & Culler, 2017).

The severity of a burn is dictated by the temperature of the heat source, duration of contact, and size of the affected area. In this patient, the heat source had a low temperature (most heat pads reach ~35-40ºC) and the duration was estimated at 1-2 hours (surgical intervention and recovery from anesthesia). As previously mentioned, the degree of severity of a burn lesion is also correlated with the depth of the lesion. In this case, depth could not be described initially, due to the fact that the wound was covered by dry necrotic tissue.

Sometimes, radiant heat injuries represent a diagnostic challenge. The evolution of this case is similar to data found in scientific literature

for this type of burn injury. The skin changes can go unnoticed for long periods (up to 7-10 days) if the affected area is covered by a thick haircoat that conceals the initial signs. In our patient, the lesions were observed once the scab began to develop, ten days after contact with the heat source.

Small animals are more vulnerable to heat exposure and prone to radiant heat injuries due to the lack of a well represented superficial vascular plexus of the skin that is well defined in humans (Wohlsein et al., 2016).

When uncertainties exist related to the aetiology of a lesion, like in this case, biopsies may establish diagnostic certainty. In this case, the biopsy was performed late in the course of disease, when the wound was already in the healing phase. Biopsies can identify anatomic depth precisely and even describe the presence and extent of microbial infection (Gross et al., 2005). However, burn wounds often have an irregular surface and variable depth, therefore the pathology report may not be valid for the entire wound. In this case, the biopsy report described a deep partial-thickness wound, eliminated other differential diagnoses such as toxic epidermal necrolysis, vasculitis, and confirmed the clinical diagnosis.

Clinically, burns unlike other skin injuries, do not progress beyond the fifth day and often have irregular shapes. The burn wound presented here had a "drip" configuration, a pattern that is typical for a burn lesion (Figure 1). This dog, like other patients with similar lesions described in the literature, presented with a good clinical state. This differentiates burn patients from those with other types of skin disease that are associated with severe systemic disorders.

The initial focus when managing a burn patient is stabilization. The importance of this step varies with different types of burn wounds. In this case, the patient was stable throughout the entire postoperative period and stabilization only included fluid therapy and analgesia. Since the burns were observed 10 days after the injury, cooling was not required.

Current recommendations in human medicine focus on controlling the infection, preventing sepsis, providing enteral nutrition and analgesia, and locally, on rapid escharectomy. This approach is often applied to small animals. When the process is acute, fluid therapy, analgesia, oxygen supplementation and assessment and management of metabolic imbalances should be rapidly initiated. In this case, the mild hyperkalemia, anaemia, hypoalbuminemia and neutrophilia were secondary to the massive cell destruction, inflammation and protein loss. Anaemia is expected to occur 1-2 weeks after the burn, secondary to cell destruction, blood loss and also due to a reduced cell lifespan associated with the burn (Efimova et al., 2019). Occasionally, loss of plasma can lead to an increased hematocrit. The increased packed cell volume in this patient was corrected using fluid therapy over the first couple of days. Although severe systemic complications have been described, small animals are often stable without fluid therapy. Enteral nutrition also played an important role in the management of this burn patient. A high-protein high-calorie diet was recommended to promote wound healing.

Another major goal in burn wound therapy is wound closure. A correct assessment of the burn has to be made to develop an appropriate treatment strategy. In most burn wounds, early assessment can be difficult due to the presence of the eschar. When evaluating a burn, one should note the aspect of the wound (colour, size), depth, surface affected, the presence of pain, unpleasant odour and/or hair.

To accurately approximate the percentage of the total body surface affected, various charts and formulas are used in human medicine. The most frequently employed of these is called the "rule of nines". This chart divides the body surface into areas of 9% or a multiple of 9 to estimate the surface area based on the various body regions. The "rule of nines" was adjusted for veterinary medicine, as follows (Pavletic, 2010):

- ✓ Each forelimb: 9%;
- ✓ Each hind limb: 18%;
- ✓ Head and neck: 9%;
- ✓ Dorsal half of the trunk: 18%;
- ✓ Ventral half of the trunk: 18%.

Using this rule, we estimated the burn surface area in our patient to be around 15%. This formula does not correlate well with age and body surface changes; thus, to assess burn

injuries in children, the scientific literature quotes the Lund-Browder chart as more accurate (Murari & Singh, 2019). In small animals, it is easy to overestimate the affected surface, due to the higher elasticity and looseness of the skin as compared to humans.

In this dog, the appearance of the lesion was characteristic for an eschar secondary to a full-thickness burn. Since this injury was inflicted by prolonged contact with a low-temperature heat source, hair was still present on the affected skin. The fissure that delineated necrotic from viable tissue that was present in this case is frequently associated with third-degree burns.

The history and signalment of the animal, such as the age of the dog, the recently performed mastectomy, the anatomical location of the burn, the animal's general state, the cost of multiple surgical interventions and postoperative care were taken into account before opting for a conservative approach. The strategy used to close this wound involved only non-invasive and minimally invasive techniques (Figure 3). Literature often advises early escharectomy for a positive outcome. Since most of the necrotic tissue in this patient was represented by the eschar, debridement focused on removing the black, leathery, thick escharotic tissue.

In injuries such as the one presented, two main approaches can be used. Conservative debridement relies on the physiological process of autolytic debridement as part of the initial phase of wound healing. To promote this process and accelerate the removal of non-viable tissue, enzymatic debriding agents and wetting agents in combination with antibacterial substances can be used. An escharotomy may be useful to facilitate the penetration of the debriding agents underneath the eschar. While this process helps obtain a selective debridement, when there is no clear demarcation, it is a slow process.

Aggressive debridement envisions the removal of the entire burn or eschar through surgical excision. The arguments that support this type of non-selective approach are that necrosis impedes granulation tissue development and increases the risk of infection. Spontaneous separation (delamination) may occur after long periods, even weeks. In the case presented here, the eschar was removed over 4 days by selective debridement, a result considered satisfactory. Scientific data mentions that if no other impeding factors intervene post-escharectomy, the granulation bed develops in 5 to 7 days (Pavletic, 2010). Once the granulation tissue develops (in our case 1-2 days after eschar removal) the type of wound closure has to be chosen. From this point onward, the approach is similar to wounds with other etiologies.

The option used here was secondary intention healing, where healing occurs through contraction and epithelialization from bordering skin. The other option is reconstructive surgery, which is invasive but offers a faster closure (eg. flaps, grafts). An important factor in the decisional process is the location of the lesion. Patients with burns in areas with loosely attached, highly elastic skin (trunk, abdomen) represent good candidates for secondary intention healing. In this case secondary closure occurred mainly by contraction with a satisfactory cosmetic outcome and no major complications or need for long term care. The conservative approach was chosen due to the size of the patient (that made bandaging manageable), the recently performed mastectomy and the potential need to remove the other mammary chain, which diminished the possibility to perform large flaps involving the neighbouring skin. These considerations, as well as the invasive nature of reconstructive surgery, the risk of dehiscence, the more demanding postoperative care in order to prevent necrosis, and the higher cost discouraged the owner from opting for a surgical approach.

The use of dressings and bandages as described previously made possible the evaluation of the gradual and visible reduction of the affected surface during healing. The animal had an optimal level of comfort throughout this process. When choosing secondary intention healing as a closure option for large wounds, certain complications have to be taken into account. Wound contraction may result in a poor aesthetic outcome and a lack of hair over the scar that the owner must be informed about. Similar to the case described here (Figure 3-M.), skin creases can develop, which can lead to dermatological lesions. The scar tissue undergoes further remodelling during the maturation phase, partially correcting these

defects after wound closure. Since the new epithelium is scar tissue and doesn't have the same characteristics as the skin, it is more prone to injury due to its higher fragility. In this case, only in a small percentage did the wound heal by epithelialization, so long term management is not expected to be demanding. Complications are not only encountered after wound closure, but throughout the healing phase if the animal is not properly monitored, precautions are not taken to protect the wound and the injury is not properly managed.

There are a few case reports describing similar wounds that healed by secondary intention, but the high variability of the patients makes it difficult to compare protocols and healing time (Sogebi et al., 2017; Lima & Bahr Arias, 2015; Maravelis et al., 2015). Most of the cases identified in the literature healed over a period of 60 to 100 days and were approached using a combination of medical and surgical treatments. Delays were reported due to complications such as dehiscence, infection, or the patients' general status. Maravelis and his team obtained a one-month healing time using medical-grade honey in a similar case to the one presented.

Many articles regarding burn wound management mention that systemic antibiotics are not entirely effective against microorganisms that multiply at the level of the avascular burn necrosis (Salyer et al., 2021). Systemic broad-spectrum antibiotics are unable to penetrate the eschar or reach any affected area where the vascular supply was compromised (Sevgi et al., 2014). They should be introduced in the burn management protocol when there is evidence of infection (eg. pneumonia or sepsis) or immunosuppression (Barajas-Nava et al., 2013). This case had a positive outcome using only topical antimicrobials. One of the most commonly used topical antibiotic is silver sulfadiazine (Momoh et al., 2009; Boekema et al., 2013). Its use is recommended in partial and full-thickness burns to prevent infection. This sulfonamide has a broad antibacterial spectrum which includes the most common bacteria found at the wound level. The silver ions released inhibit bacterial growth and multiplication. Silver sulfadiazine has the ability to penetrate necrotic, escharotic tissue. Additionally, throughout the debridement phase, the antiseptic properties of honey were also employed (Eroglu et al., 2018; Rozaini et al., 2004).

Improper use of most heating devices can inflict a thermal injury. Studies have shown that one of the safest devices used is the forced air system, which evenly disperses heat across the body's surface, prevents loss of warm air heat, and gives superior results compared to other systems, while decreasing patient burn risk (Clark-Price et al., 2013; Waterman, 1975).

CONCLUSIONS

Although thermal injuries are the topic of numerous studies in human medicine, little has been published regarding treatment guidelines in veterinary medicine.

Further research is needed to better understand the pathophysiology of the burn disease and its systemic and local implications in animals, to serve as a starting point for novel therapeutic protocols.

When promoting secondary intention healing in a wound, the treatment has to be adjusted and altered for each phase of the healing process, to obtain the best closure time. In this case, a satisfactory time was obtained (56 days) to close a wound that initially affected approximately 15% of the total body surface.

Raw honey used as a debriding and antimicrobial agent may be a proper dressing for full-thickness burns during the initial healing phase.

The combination of chlorhexidine impregnated tulle dressing and silver sulfadiazine cream may be superior to the individual use of these products. This combination provides the non-adhesive characteristic of the dressing while keeping the cream in place for longer periods.

Radiant heat injuries may be a diagnostic challenge, thus biopsies may help confirm the diagnosis.

Full-thickness burns of the neck and trunk can be managed exclusively by a minimally invasive approach.

Maintaining normothermia during anaesthesia remains challenging but special attention should be paid when using heating devices during surgery, due to the risk of burn injury to the patient, even at low device temperature.

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STUDY REGARDING THE NUTRITIONAL AND TECHNOLOGICAL QUALITY OF WHEAT

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Abstract

In the bakery industry, the wheat quality is essential for flour processing. Thus, the main objective of this paper is to present the results of the research on yield quality of seven winter wheat samples collected from Prahova County farms. To determine the wheat quality, the following analysis were performed: thousand grain weight (TGW g), hectolitre mass (HLM kg/hl), moisture content (%), protein content (%), carbohydrates content (%), lipid content (%), ash content (%), wet gluten content (%), gluten deformation index (mm) and falling number. For the physical parameter determined at wheat seeds the following data were obtained in average: 41.47g for TGW (g), 76.84 kg/hl for HLM and 12.72% for moister content. In average, the chemical composition of wheat seeds was the following: 14.22% proteins, 1.95% lipids, 65.98% carbohydrates, 1.78% ash. For the whole wheat flour the following results were obtained: the wet gluten content was on average of 28.02%, with variation between 25.5% for sample 2 and 30.5% for sample 5; the gluten deformation index was on average of 8.47% and the value of falling number varied between 227 s and 258 s. Those results show that all samples of wheat are suitable for use in the bakery industry.

Key words*: yield quality, wheat, nutritional value.*

INTRODUCTION

Wheat is considered the most important cultivated plant, being used by the 40% of the world population for bread and staple food. The wheat flour can be used for obtaining various bakery products and pasta (Toader et al., 2019). The wheat grains are usualy included in the *cereal mixtures* used for breakfast, also are used *in animal feed* or to obtain *gluten, starch, alcohol, spirits (vodka, whiskey), beer, biofuel like ethanol* etc. According to Roman et al. (2011) "straws can be used as raw material in the pulp and paper industry, for bedding, roughage, as an organic fertilizer or for producing energy" (Roman et al., 2011).

Grain quality can be defined by a series of physical and compositional properties where threshold requirements are set according to the end-use requirements. For grains like wheat, the physical properties of grain (i.e. size and shape) can influence the milling yield, which also determine the efficiency of processing and the grain value (Nuttall et al., 2017).

According to Dumbrava et al. (2012) "there are several factors like growing practices, time and type of harvesting, postharvest handling, storage management and transportation practices that can affect grain quality. Also, the quality of wheat used for bakery is influenced by the environmental conditions, the interaction between varieties, the applied crop technology as well as by the effect of some climatic accidents" (Dumbrava et al., 2012).

The following indicators can determine the quality of wheat grains: moisture content, hectolitre mass, foreign matter, percentage of coloured, damaged and broken grains, milling quality, protein content, vigour, mycotoxins, presence of insects and fungi (Lusse J., 2006).

Wheat flours baking potential is influenced by several factors, especially the protein content (MacRitchie F., 1987; Toader et al., 2019). Based on the solubility in different solvents, the major flour protein types are classified into albumins, globulins, gliadins and glutenins (Shewry P.R., 2002; Xue et al., 2019). The gluten proteins (i.e. gliadins and glutenins) can determine the baking quality of wheat flour: gliadins mainly contribute to dough extensibility and viscosity and glutenins contribute to dough strength and elasticity (Wieser H., 2007). The gluten content can be influenced by the cultivar, climatic conditions, nitrogen fertilizer rate and time of application, residual soil nitrogen and the available moisture during grain filling (Vita P. et al., 2007; Dupont F. et al., 2003).

The wheat quality has a critical importance throughout the grain value chain. The wheat delivered by the producers must comply with the required specifications and the producers will be remunerated in accordance with its quality. The storage facility must determine the grain quality in order to establish if it complies with the required standards and then to store it until it can be processed. The processor requires grains that complies with his needs, to obtain a specific product that meets the consumer's demand (Lusse J., 2016).

MATERIALS AND METHODS

Wheat quality is essential for flour processing in the bakery industry. So, the main objective of this paper is to present the results of the research on yield quality of seven wheat samples collected from Prahova County farms. To determine the quality of wheat, the following analysis were performed: thousand grain weight (TGW g), hectolitre mass (HLM kg/hl), moisture content (%), protein content (%), carbohydrates content (%), lipid content (%), ash content (%), wet gluten content (%), gluten deformation index (mm) and falling number.

TGW was determined by weighing 8 samples of 100 seeds which were taken from the pure mass and HLM by using the Hectolitre Measuring System - Chondrometer with 0.5 l capacity.

The chemical analyses were made within the Yield Quality Laboratory from the Faculty of Agriculture, USAMV of Bucharest, using a spectrophotometer infrared NIR Inframatic 9200 Product Instalab-Analizer.

The energetic value was determined by the relationship between the daily necessary nutrients and the contribution of these substances provided per product unit (usually 100 g).

The formula for calculating the energetic value (kcal) for the wheat seeds was:

Energetic value = % proteins x 4.1 + % lipids x 9.3 + % carbohydrates x 4.1.

Wet gluten content of the wheat samples was determined manually according to SR ISO 21415-1:2007 methods and the gluten deformation index according to SR ISO 90/2007.

The method used for gluten determination consist in the separation of proteinic substances such as gluten, using NaCl solution for dough washing (dough is obtained from wheat groat), and after drying the obtained gluten. For gluten deformation index, the method was to leave a wet gluten sphere rest for one hour at 30°C, to measure the initial and final diameters and to calculate the differences (Figure 1).

Figure 1. Aspects from gluten deformation index analysis
(Yield Quality Laboratory within Field Crop Department)

Falling Number test, according to ISO 3093-2005 standard, it was determined in laboratory were was use a Falling Number device.

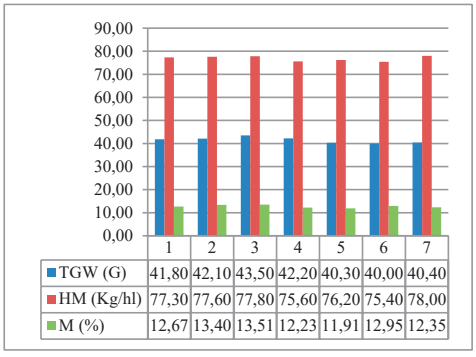
All chemical analyses were performed in three replicates and the results were statistically analysed by Fisher's least significant differences (LSD) test.

RESULTS AND DISCUSSIONS

Physical quality parameters

Analyzing the data in Figure 2 regarding the physical quality indicators of wheat seeds it can be observed that the thousand grains weight of the wheat seeds was, on average, 41.47 g. This value shows a good quality of the wheat used for bakery industry.

According to Dumbrava et al. (2012), the thousand grain weight can be influenced by the specific soil and climatic conditions of the cultivation area, the crop technology, the presence of foliar diseases, the pest attack, the grain chemical composition and the state of the grains upon harvesting (impurities content, the percentage of broken grains).



	1	2	3	4	5	6	7
TGW (G)	41,80	42,10	43,50	42,20	40,30	40,00	40,40
HM (Kg/hl)	77,30	77,60	77,80	75,60	76,20	75,40	78,00
M (%)	12,67	13,40	13,51	12,23	11,91	12,95	12,35

Figure 2. Physical quality parameters of wheat samples

The hectolitre mass is currently an important indicator for the milling industry, because in the milling units, the total flour extraction is determined based on its value.

In our research, the hectolitre mass of wheat samples ranged between 75.4 kg/hl at sample 6 and 78.0 kg/hl at sample 7, the average being 76.84 kg/hl. Those results show that all wheat samples can be included in the "good for bakery" category.

The moisture content of wheat seeds is one of the most important criteria for assessing its quality. The optimum state of ripeness upon harvesting is also characterized by wheat moisture content, which must have a maximum of 15%. The storage of wheat largely depends on its moisture. At a normal temperature, wheat can be stored in good condition only if the moisture value is below 13%. If the moisture exceeds 14%, a series of chemical processes related to the acceleration of respiration with the production of heat and water will take place, followed by complex fermentation processes that will lead to the alteration of the grain mass (Toader et al., 2020).

Our research showed that for all samples, the moisture values did not exceed the STAS values of 14% (SR ISO 13548:2013).

The moisture values ranged between 11.91% and 13.51%, the average being 12.71%.

Biochemical parameters

The analysis of the chemical composition of the wheat seeds showed that the highest protein content was registered at sample 6 (14.81%) which significantly exceeded the average protein content. This was followed by sample 5 with 14.70% protein, while the lowest value was determined at sample 2 i.e. 12.70% (Table 1). According to the protein content analysis, it can be seen that most of the wheat samples are included in the "very good for bakery" category, except for sample 2 which is included in the "good for bakery" category.

Table 1. Chemical composition of wheat varieties (% d.m.)

Wheat samples	Quality parameters*			
	P (%)	L (%)	C (%)	A (%)
1	14.20	1.89	66.53	1.89*
2	12.70^{000}	2.02	65.60	1.72
3	13.98	2.16**	66.31	1.56^{000}
4	14.51	1.91	64.90^{0}	1.91*
5	14.70	1.94	67.50*	1.74
6	14.81*	2.14**	66.82*	1.98**
7	14.62	1.65^{00}	64.21^{000}	1.64^{0}
Average	14.22	1.95	65.98	1.78

Note: P (% d.m.) - protein content; L (% d.m.) - lipid content; C (% d.m.) - carbohydrates content (% d.m.); M - moisture content (% d.m.); ^{000}- very significant negative differences; ^{00}- distinct significant negative differences; ^{0}- significant negative differences; **- distinct significant positive differences; *- significant positive differences.

The lipids content of the studied samples ranged between 1.65% and 2.16%. Higher lipid contents were observed at sample 3 (2.16%) and sample 6 (2.14%), which exceeded the average with a distinctly significant value (Table 2).

The lowest lipid values were recorded in samples 7 (1.65%), 1 (1.89%), 4 (1.91%) and 5 (1.94%), respectively.
Regarding the carbohydrates content of the wheat seeds, in table 1 a higher content (over 67%) can be observed at sample 5.
Samples 1, 3 and 6 had a carbohydrates content which ranged between 66.31% and 66.82%, compared to samples 4 and 7 which registered lower values.
The values of ash content ranged between 1.56% (in sample 3) and 1.98% (in sample 6).
Seeds from samples 2 and 5 had a medium ash content of 1.72% and 1.74%, respectively.
All results regarding the chemical composition and statistical processing are included in Table 1 and Table 2.
The level of significance (DSL5%, 1%, 0.1%) from Table 1 has been based on the values of Table 2.

Table 2. Statistical results of the chemical composition

Statistical index	DM (%)	P (%)	C (%)	L (%)	Ash (%)
Average	87.30	14.22	65.98	1.87	1.78
Sample variance	0.19	0.29	0.72	0.02	0.01
Standard deviation	0.43	0.54	0.85	0.13	0.11
Sd	0.18	0.22	0.35	0.05	0.05
CV (%)	0.49	3.79	1.28	6.53	6.40
DSL 5%	0.387	0.484	0.760	0.115	0.102
DSL 1%	0.548	0.684	1.074	0.162	0.144
DSL 0.1%	0.782	0.976	1.533	0.232	0.206

The energetic values of wheat seeds (Figure 3) ranged from 338.54 kcal at sample 7 and 355.06 kcal at sample 5.

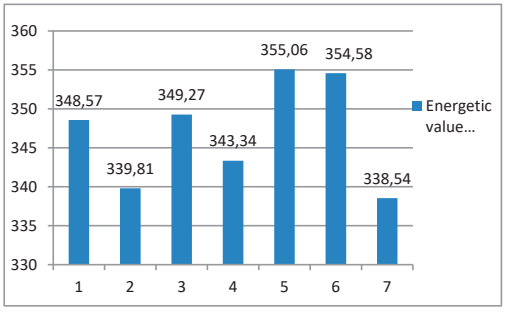


Figure 3. The energetic value of wheat seeds

Falling Number characterizes the carbohydrates activity in the wheat seeds, which is given by the activity of alpha-amylase, that influences the quality of the wheat used in bakery. The optimum value for baked wheat is between 180 and 260 seconds.
In our research the value of the falling number ranged between 227 s and 258 s, the average being 243 s (Figure 4). Those results show that all flour samples are suitable for use in the bakery industry.

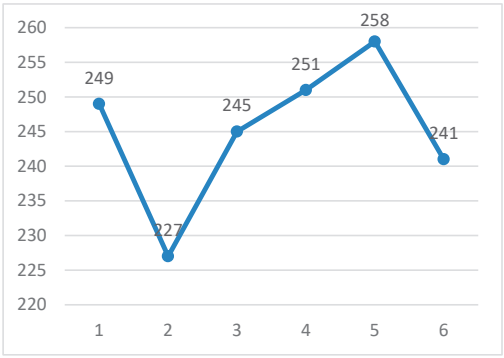


Figure 4. Falling number of wheat samples

Gluten is the main responsible factor for the extensibility and elasticity of the dough. Thus, in our research, *the wet gluten test* offers information on the quantity and quality of gluten from the analyzed wheat samples. According to Wheat Marketing Center (2004) "in the food industry the wet gluten reflects the protein content of the flour and represent a common flour specification required by the end-users" (Wheat Marketing Center, 2004).
The wet gluten content of the studied wheat samples was on average of 28.02% (Figure 5).

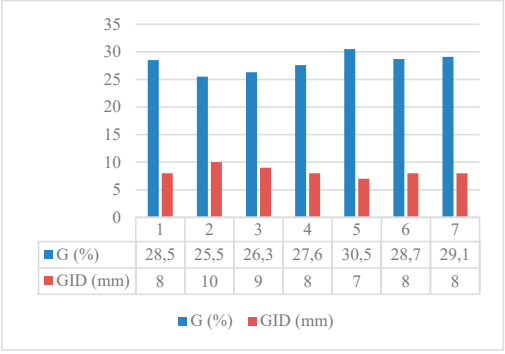


Figure 5. Wet gluten content and gluten deformation index for wheat
Note: G (% d.m.) - wet gluten content; GID (mm) - gluten deformation index

Seeds from sample 5 registered the highest value of wet gluten content, i.e. 30.5%, this being followed by samples 7, 6 and 1, with a wet gluten content above 28%.
The lowest values of the wet gluten content were registered at samples 2 and 3, i.e. 25.5% and 26.3%.
According to SR 90/2007 standard, wheat that is good for baking must have a wet gluten content of at least 24%. So, the analyzed samples comply with the quality requirements.
In the bakery, the gluten deformation index reflects the "proteolytic activity" of the wheat flour. So, if values exceed 15 mm, the gluten deformation is high, and if values are below 5 mm, the gluten is highly elastic, and the flour requires amelioration because the proteolytic activity is very low. According to standards, for the baking flours, gluten deformation index ranges between *3 to 25 mm* (Toader et al., 2019).
Regarding the gluten index deformation, figure 5 shows that all samples are "very good for bakery industry". The gluten index deformation was on average of 8.47 mm, the variation limit being of 7 mm for sample 5 and 10 for sample 2.
Figure 6 shows that there is a *positive correlation* between the wet gluten content and seeds protein content, the value of R^2 being 0.691.

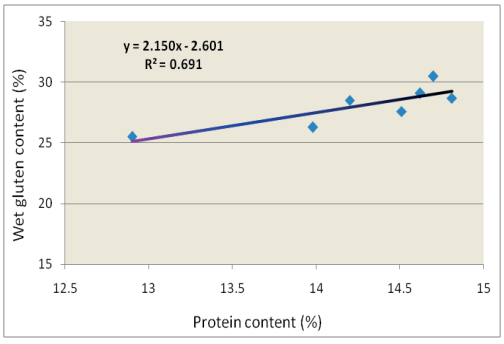


Figure 6. Correlation between protein content and wet gluten of wheat

Research show that there is a direct correlation between gluten content and the grain protein content, which is influenced by the "pedoclimatic conditions" from Prahova County area. The main factor which influences the qualitative characteristics of gluten is the "wheat genotype" (Mariani B.M. et al., 1995; Simic G. et al., 2006; Ionescu et al, 2010), and the increase at the total protein content in the flour determines an increase of the gluten content (Perten et al., 1992; Ionescu et al., 2010).

CONCLUSIONS

One of the main factors that influences the quality of bakery products is the quality of the raw material, i.e. the quality of the wheat seeds.
All the quality indicators that were analyzed in our research, i.e. the physical quality indicators, the biochemical indicators, the wet gluten content, the gluten deformation index and the falling number, showed a "good quality" of the wheat for bakery.
In our research, there was a "*direct correlation*" between the *gluten content* and the *grain protein content*, and the factor that influenced this correlation was the *pedoclimatic conditions*.
We can conclude that all wheat samples meet the quality requirements imposed by the Romanian standards.

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SOME INDICES OF CONTAMINATION OF POULTRY PRODUCTS WITH BACTERIA OF THE GENUS *Salmonella* spp. AND *Listeria* spp.

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Abstract

The scope of the proposed research was to establish the presence of bacteria of the genus Salmonella spp. and Listeria spp. in the samples of poultry meat and eggs sold in the commercial food network of the Central Agricultural Market from m. Chisinau. The bacteriological investigations were performed in the laboratory of Microbiology of the Department II of the Faculty of Veterinary Medicine, SAUM. The serotyping of the isolated bacteria forms was performed at the Republican Center for Veterinary Diagnosis. The following culture media were used for isolation and identification: RVS broth, MKTTn broth, XLD agar, BSA, semi-Fraser broth, ALOA agar, Ottaviani and Agosti agar, Oxford agar. In total, 120 samples including 60 egg samples, 20 samples of frozen poultry carcasses and 40 samples from refrigerated broiler carcasses were collected and examined. As a result, the bacteriological investigation on the eggs of current consumption has established the presence of bacteria of the genus Salmonella typhimurium. Also, in the samples of frozen meat were isolated the serotypes Salmonella enteritidis and Listeria monocytogenes and finally, in the samples of refrigerated poultry carcasses was established the presence of serotypes Salmonella typhimurium and Listeria monocytogenes. The bacteriological investigations of poultry products (eggs and meat) showed the presence of their contamination with bacteria of the genus Listeria monocytogenes in 2.5% of the examined samples and with Salmonella bacteria in 8.3%, which may lead to the transmission of these bacteria to consumers by means of contaminated products.

Key words*: samples, contamination, poultry products, Salmonella, Listeria.*

INTRODUCTION

In recent years, poultry production as a branch of the livestock sector in the Republic of Moldova remains one of the most developed branch with a high priority share in the consumption of animal products at the national level. Therefore, obtaining safe poultry products in terms of microbial contaminants is an essential component of commercial poultry production.

The protection of poultry flocks against contamination with undesirable micro-organisms requires constant monitoring by the veterinary health service, as well as the maintenance of high-level biosecurity measures for bird populations. Nonetheless, the application of daily biosecurity procedures can contribute to reduce the possibility of contacting zoonotic microbial infections with an important impact on public health such as: salmonellosis, campylobacteriosis, listeriosis, etc. (Sexton, 2018; Youn, 2017) Compared to other bacteria, the genus *Salmonella* spp.bacteria has been determined as a frequent cause of food poisoning. In addition, some *Salmonella* serotypes (*S. gallinarum, S. pullorum, S. enteritidis*, etc.) which have been detected in chickens raised for meat, or in laying hens can cause serious clinical symptoms in birds; thus, providing an increased risk of poultry meat and eggs contamination with these bacteria (Hardie, 2019; Nidaullah, 2017).

Currently, at European level, approximately 200 *Salmonella* serotypes are associated with foodborne infections in humans, of which two serotypes are considered more dangerous for public health: *Salmonella enteritidis* and *Salmonella typhimurium.* However, these two serotypes have successfully been reduced in many countries due to the introduction of strict biosecurity measures, effective surveillance of poultry flocks and vaccinations against salmonellosis (Asma, 2018; Webber, 2019).

There are various mediums where the horizontal transmission of the above-mentioned infections occurs: in the contaminated feed, in the incubation stations' equipment, in the buildings for birds raising, in the process of slaughtering and storage of poultry products.

Another, important route of contamination with the bacteria of the genus *Salmonella* spp., *Listeria* spp. etc. remains the elements of the poultry product marketing chain. Hence, good management of production processes and bio-safety of technological processes, as well as systematized control within different segments of the production chain are critical aspects that can minimize the risk of contact and persistence of infections (Bourassa, 2019; Li, 2018).

The goal of our research was to establish the degree of presence and the diversity of the genera *Salmonella* spp. and *Listeria* spp. in poultry meat and eggs for common consumption, given the highly negative health impact that these bacteria can have over humans and poultry flocks.

MATERIALS AND METHODS

The researches were conducted at the Department II of the Faculty of Veterinary Medicine of State Agrarian University of Moldova. Serotyping of isolated bacteria *Salmonella* spp. and *Listeria* spp. were performed at the Republican Center for Veterinary Diagnostic and at the National Agency for Public Health. As research materials served the samples from the poultry carcasses and eggs of current consumption from some units for the production of poultry meat and eggs such as: SRL "Silver Bird", v. Ciorescu, mun. Chisinau, SRL "Codim Com", v. Sadaclia, district Basarabeasca, "IM PB Nord" SRL, district Edinet, v. Blesteni, SRL "Intervetcom", district Cimisliea, SRL "Redi Agro", district Dondușeni, v. Tirnova, SRL "Dant Agro", district Ungheni, v. Pirlița, SRL "Solar Nord" district Edinet, v. Gordinesti, Avicola "Riscani", district Riscani, v. Corlateni.

The isolation and identification of bacteria of the genera Salmonella spp. was performed according to the methodology SM EN ISO 6579-1: 2017 - Microbiology of the food chain. The horizontal method was used for the detection, counting and serological typing of bacteria such *Salmonella*, as well as bacteria of the genera *Listeria* spp., according to the methodology SM EN ISO 11290-2: 2017 - Microbiology of the food chain, horizontal method was used for the detection and enumeration of Listeria spp. The samples were subjected to classical microbiological tests using the national standards methods and the confirmation was made using "Microbact" tests according to the manufacturer's instruction for Listeria spp. - ATCC 19118 and for *Salmonella* spp. ATCC 14028. For the isolation and identification of the bacterial forms were used ordinary, selective and special culture media (peptone water buffered, XLD agar (Xyloze Lysine Deoxycholate), BSA (Brilliance Salmonella Aga), Semi-Fraser broth, ALOA agar, Agar Oxford), monoreceptor sera for serotyping. Totally for examinations were taken 60 samples of eggs and 60 poultry carcasses samples (40 samples from refrigerated carcasses and 20 samples from frozen carcasses).

RESULTS AND DISCUSSIONS

Some investigations of the incidence of salmonellosis in laying birds have been performed based on pathomorphological observations specific for avian salmonellosis. Attention was drawn to the presence of enterocolitis, vetellin peritonitis, salpingitis, and ovaries. The incidence of mortality caused by this changes ranged from 2 to 4%. In broiler chickens, symptoms and pathomorphological changes specific for salmonellosis (diarrhea, enterocolitis and liver miliar necrosis) ranged from 3 to 5% of growing chickens. No specific clinical symptoms and macroscopic changes were detected for listeriosis in flocks of birds. In order to establish the presence of pathogenic serotypes of bacteria of the genera *Salmonella* spp. and *Listeria* spp., samples of eggs and meat were taken from the poultry enterprises mentioned in the material and methods.

The results of bacteriological investigations showed an increased number of *Salmonella* spp. colonies in over 75% of the samples taken from bird carcasses, which demonstrates the presence of *Salmonella* serotypes on objects that come in contact with poultry products.

For the isolation and identification of bacteria of the genera *Salmonella* spp., samples were taken from poultry carcasses with a weight of 25 g, which were subsequently inoculated into 225 ml APT and incubated. After incubation, the culture obtained in RVS broth was inoculated on the surface of the Petri dish with

the selective environment XLD (Xyloze Lysine Deoxycholate) and BSA (Brilliance Salmonella Aga) to obtain isolated colonies. Colonies with a typical morphological structure of Salmonella grown on the XLD medium have a black center and a bright transparent area of red color (Figure 1), and on the Brilliance Salmonella Agar medium, the *Salmonella* spp. colonies are purple (Figure 2).

Salmonella H2S serotypes negative on XLD medium are dark pink with a dark center. Lactose-positive *Salmonella* on XLD are yellow with or without a black center.

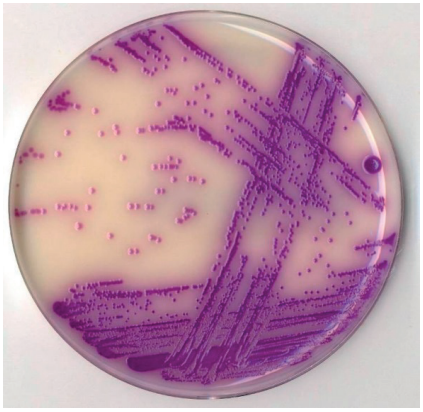

Figure 1. Brilliance Salmonella Agar, (purpury colonies of *Salmonella*)

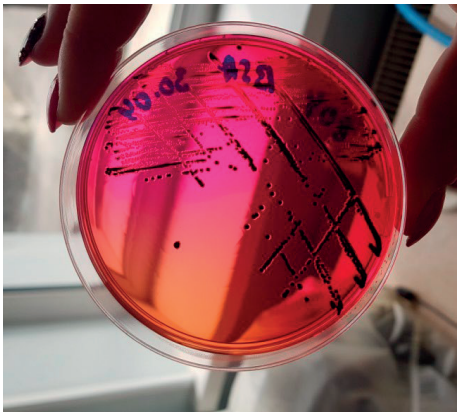

Figure 2. Typical *Salmonella* colonies on XLD medium with black center and transparent area and red color

The level of contamination with *Salmonella* bacteria was determined by counting *Salmonella* colonies on the surface of Petri dishes. Inoculations performed on Brilliance Salmonella Agar medium from samples taken from frozen meat carcasses ranged from 54 to 127 colonies. In the case of inoculations from refrigerated carcass samples, the number of *Salmonella* colonies ranged from 89 to 187.

In the case of inoculations from lavages taken from the surface of egg samples, the number of *Salmonella* colonies varied from 69 to 114 colonies, but in the case of inoculations performed from egg contents, the number of colonies varied from 6 to 17.

In the case of inoculations performed on XLD agar from samples taken from frozen carcasses, the number of *Salmonella* colonies ranged from 31 to 135, and from refrigerated carcasses the number of colonies ranged from 77 to 205.

Inoculations from egg surface washings confirmed the presence of *Salmonella* colonies with variations from 43 to 127 colonies, and in the case of inoculations from egg contents the number of colonies ranged from 8 to 25.

For the isolation and identification of bacteria of the genera *Listeria* spp., a primary enrichment was performed with 25 g from the meat sample in 225 ml of selective liquid of enrichment medium, with low concentration of selective agents (Semi-Fraser broth) and subsequent incubation according to the established method. From the culture obtained by enrichment by striation, it was applied on the surface of the first selective medium - ALOA agar to obtain isolated colonies, and later on the second selective striation medium - Oxford agar.

On the nutrient medium Agar ALOA, the typical colonies of *Listeria* spp. are green-blue surrounded by an opaque halo (Figure 3). On the nutrient medium Agar Oxford, the typical colonies of *Listeria* spp. after 24 h incubation are small (1 mm), gray, surrounded by a black halo. After 48 hours they become darker in color, with a possible greenish tint, with a diameter of 2 mm, with a black halo and a concave center (Figure 4).

In the case of inoculations taken from frozen poultry carcasses, there was one positive sample out of 20 examined, but in the case of inputs taken from samples from refrigerated carcasses, from 40 examined samples two samples were contaminated with *Listeria* (Table 1). The number of *Listeria* colonies in the case of inoculations performed from

samples of frozen carcasses varied from 4 to 28, and from refrigerated samples the number of colonies varied from 33 to 62.

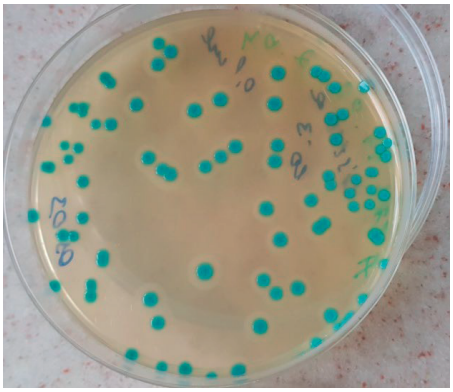

Figure 3. ALOA agar - the tipical colonies of *Listeria monocytogenes*

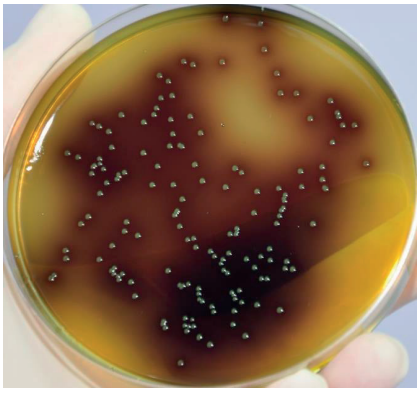

Figure 4. Oxford agar - the tipical colonies of *Listeria* spp.

Subsequently, smears were prepared from colony cultures and examined under a microscope (ob.10x100). Figure 5 shows the morphological structure of bacteria of the genus *Salmonella* spp. which is placed in the field of the microscope in separate rods or in piles with oval heads. The microscopic examination of the smears prepared from the colonies of bacteria from the genus *Listeria* (Figure 6) are represented by sticks having the shape of short thin form or boomerang shape.

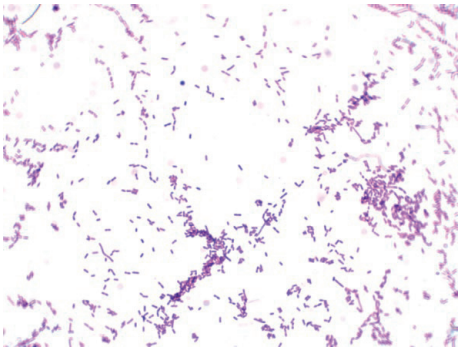

Figure 5. *Salmonella* spp. (separate sticks or ples, gr -)

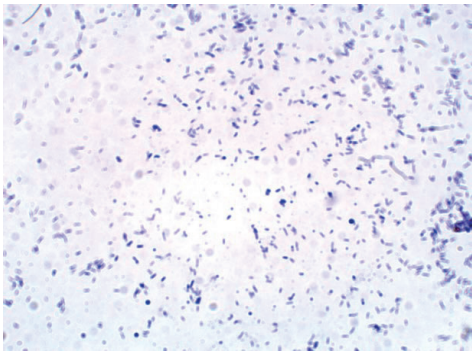

Figure 6. *Listeria* spp. (short, thin sticks, gr +)

Salmonella spp. Colonies were serotyped sing monoreceptor sera (Figure 7), biochemical investigations, commercial putties (Micobact test, Figure 8) with the use of positive controls.

Figure 7. Monoreceptoric sera for serotyping of *Salmonella* spp.

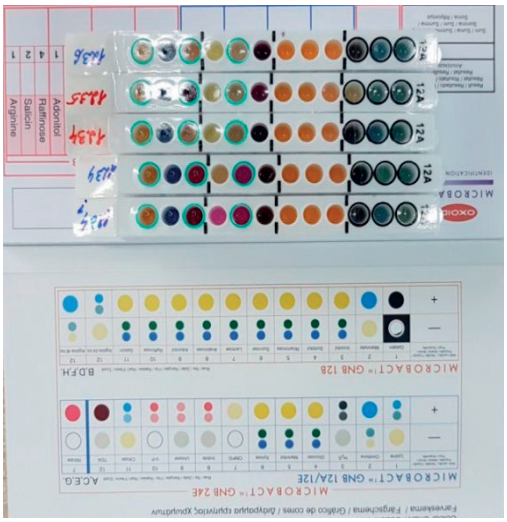

Figure 8. "Micobact" test for serotyping of *Salmonella* spp.

As a result of serotyping the colonies of bacteria of the genera *Salmonella* spp., and *Listeria* spp., were established the results that are presented in the Table 1.

The results of the performed serotyping confirmed the presence of 13 positive samples with pathogenic serotypes of *Listeria* spp. and *Salmonella* spp. Out of 120 examined samples, from the poultry carcasses were isolated and serotyped only 3 positive samples of the bacteria from genera *Listeria* or 2.5% of the examined samples. Positive samples with bacteria of the genus *Salmonella* pathogenic to humans and birds (*S. enteritidis* and *S. typhimurium*) were isolated from 10 samples, which constituting 8.3% of the total number of examined samples of which 4 samples were isolated from eggs for current consumption, and 6 samples from meat carcasses.

Table 1. Results of serotyping of bacteria of the genera *Salmonella* spp. and *Listeria* spp.

Nr.	Product name	Number of examined samples	Bacterial genera			Nr. of positive samples	% of contamination
			L. mono citogenes	*S. enteritidis*	*S. typhi murium*		
1	Eggs for current consumpion	**60**	-	-	4	4	6.6
2	Frosen poultry carcasses	**20**	1	1	-	2	10
3	Refrigelated poultry carcasses	**40**	2	3	2	7	17.5
Total		**120**	**3**	**4**	**6**	**13**	**10.8**

CONCLUSIONS

1. The research on the circulation of pathogenic serotypes of bacteria of the genera *Salmonella* spp. and *Listeria* spp. showed that the veterinary measures currently taken are not sufficient to prevent the incidence of contamination of poultry products with these bacteria; therefore, a perspective program that will have as priority to monitor and analyze the risks of contamination for poultry units and for humans has to be implemented.

2. The pathomorphological analyses confirmed the presence of specific changes for salmonellosis in laying hens such as: enterocolitis, vetellin peritonitis, salpingitis and enterocolitis, liver infarction in broiler chickens, with an incidence from 2 to 4%.

3. Bacteriological investigations of poultry carcasses showed that from the total number of examined samples, the incidence of positive samples with the presence of *L. monocytogenes* was 2.5% and the incidence of pathogenic serotypes of *Salmonella* spp. was detected at 8.3% of examined samples of eggs and carcasses with the predominance of serotypes *S. enteritidis* and *S. typhimurium* which implies a major risk for contamination of consumers of poultry products with these pathogenic bacteria.

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RESULTS ON THE IDENTIFICATION OF THE HONEYBEE SUBSPECIES FROM SOME SOUTH AND SOUTH-EAST ROMANIAN COUNTIES USING A SEMIAUTOMATIC SYSTEM FOR ANALYZING WINGS

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Abstract

The geometric morphometric analysis proved to be an efficient and rapid method for the identification of honeybee subspecies. This analysis involves several steps but takes less than classic morfometric methodes being easier to apply. The aim of this study was to identify the honeybee subspecies from the Romanian South and South-Eastern area, using the semiautomated french system ApiClass, system that allows to identify the subspecies based on the wing's image of Apis mellifera honeybee worker. This program is analyzing the wing considering the 19 points corresponding of the intersections of the main veins of the honeybee forewings. Using ApiClass we analized 13 samples from Tulcea, Calarasi, Giurgiu, Olt, Dambovita, Prahova and Ialomita counties. Each sample was composed from wings of 20 honeybees. Five of the analysed samples were identified as hybrids without being possible to specify the type or the level of the hybridization and 8 samples had been identified as being Apis mellifera carnica presenting a homology degree over 90%.

Key words: *honeybee wing, Apis mellifera, geometric morphometric analysis.*

INTRODUCTION

Apis mellifera honey bees are found throughout Europe, Africa and Western Asia (Alattal et al., 2014). *Apis mellifera* is one of the most studied invertebrates. This species is distributed all over the world and has been distributed throw humans to numerous countries (Arias et al., 2008). Honeybees are usually classified into about 30 subspecies (Leno da Silva et al., 2015) and are grouped into four phylogenetic lines: A, C, M and O. Every line is associated to a geographical area (Rinderer, 2008). The O line (the Middle East group) includes the *Apis mellifera: anatolica, adami, cypria, syriaca, caucasica, meda.* The line M (western and northern European Mediterranean group) includes the *Apis mellifera: mellifera, iberica* subspecies. The line C (Central and Southern European Group) includes the *Apis mellifera: sicula, ligustica, carnica (the Carniolian bee), macedonica, cecropia* and line A includes African breeds of *Apis mellifera* (Tropical Africa, North Africa): *scutellata*, *adansonii, litorea*, *monticola, lamarckii*, *capensis, unicolor*, *yemenitica, sahariensis*, *intermissa*, *major* (Ruttner, 1988; Rinderer, 2008). This sub-species are ussualy described as - geographic sub-species - because their distributions correspond to a distinct geographic areas. Commercial breeding and also migratory beekeeping have affected the genetic variability of local honey bee populations (Bouga et al., 2011).

The honeybees express an important geographical variation, resulting in adaptation to regionally factors of climate and vegetation being particularly sensitive to inbreeding (Meixner et al., 2013).

The honeybee subspecies. The honeybee subspecies could be classified using morphometric characters, being available a lot of studies on this issue. The main morphological featurescan be classified into three major groups: length measurements, color measurements, and wing vein characteristics. The morphology characteristics is used to characterize honey bee races and individuals, to determine the degree of hybridization with foreign races and/or for the discrimination between honey bee subspecies (Aboushara et al., 2013). Wings are considered the most

reliable morphological feature for honey bees identification, so lately, the measurements of the fore wings have proven to be reliable for the honeybees' classification (Leno da Silva et al., 2015; Koca and Kandemir, 2013). There were developed different methodes for automatic honeybee classification based on images of the wings. Also, the geometric morphometry demonstrated to be a useful tool in terms of time consuming and reliability of the results (Leno da Silva et al., 2015).

Different software packages were developed for automated identification of the honeybee subspecies. Some of this software are: ABIS (Automated Bee Identification System) (Leno da Silva et al., 2015); ID-BEES; FABIS (Nawrocka et al., 2017); DrawWing system (Tofilski, 2008); ApiClass system (Nawrocka et al., 2017; Baylac et al., 2008). DrawWing and ApiClass software are available to the general public (Nawrocka et al., 2017).

MATERIALS AND METHODS

The aim of this study was to identify the honeybee subspecies from some South and South-East Romanian counties area, using the geometric morphometric method. For this purpose it has been used the semiautomated french system ApiClass. This system allows to identify the honeybees subspecies based on wings' picture of the *Apis mellifera* honey bee worker and is using the recent advances of geometric morphometry. ApiClass, analyzes the wing comparing the 19 points corresponding of the intersections of the main veins of the bee forewings (http://apiclass.mnhn.fr).

The coordinates are processed before being analyzed by the system. After that, the Apiclass system returns the probability of belonging to one of the honeybee subspecies from it reference system. This system has more than 5000 honeybee wings of the honeybee main lines and subspecies. Using the ApiC
lass system we analized samples from Tulcea, Calarasi, Giurgiu, Olt, Dambovita, Prahova and Ialomita counties. Each sample was composed from wings of 20 honeybees.

We used a slide scanner OpticFilm 7400 Plustek, pipettes, forceps, glass blades and small glass blades, Petri dishes, distileted water, collector tubes and alchool 70°.

Every honeybee forewings was scanned and then transformed in to jpg image being analyzed with ApiClass system. We select the automatic measuring system, the wing image was inserted into the system, this positioning points A, B, C on the wing in three steps. After this steps, the system is processing the , is placing the 19 points on the wing, is validating and analyzing the imageand its comparing it with the database of the system.

The analyzed honeybees came from the following counties/localities: Tulcea (Jijila, Luncavita, Pecineaga), Calarasi (Oltenita and Cocon), Giurgiu (Gostinu), Dambovita (Gura Ocnitei and Pucioasa), Olt (Visina, Bals), Prahova (Sinaia, Breaza) and Ialomita (Marsilian).

RESULTS AND DISCUSSIONS

To consider that a honeybee belongs to a particular subspecies of *Apis mellifera* the homology must be over 90%. Eight of the analysed samples had been identified as being *Apis mellifera carnica* (homology degree over 90%). Five of the analysed samples were identified as hybrids without being possible to specify the type or the level of the hybridization. Analysing the wings with ApiClass system the following were observed:

- in Tulcea county, the samples from Jijila, Luncavita and Pecineaga were identified as hybrids without being possible to specify the type or the level of the hybridization (see Tables 1 to 3);

Table 1. Analysed wings sample from Jijila - Tulcea County

Wing	Line	Jijila - Tulcea							
		Carnica	Ligustica	Cecropia	Buckfast	Mellifera	Caucasica	Cypria	Anatolica
1	C	45	24						15,23
2	C	98,09	2				0,79		
3	C	92,11	1,4				44,68		
4	C	98,05	0,85				0,88		
5	C	91			7,8	0,8			
6	O		10,22		84				
7	C	53	12,76			10,11			
8	C	64			54,78				
9	C	79,45				8,44			
10	C	83	11				31,14		
11	C	34		29					
12	C	23	2,01			29,92	20,35		
13	C	84					31,55		4
14	C	46	18,02		10,34				
15	C	89					31		
16	C	69			22,33		10,12		
17	C	53				34			
18	C	79,98	15,01		11				
19	O	90	2,67						
20	O			15,1			71		

**Line O - *Apis mellifera* - the Middle East group: *anatolica*, *adami*, *cypria*, *syriaca*, *caucasica*, *meda*;

*Line C - *Apis mellifera* subspecies Central and Southern European Group: *sicula*, *ligustica*, *carnica* (the Carniolian bee), *macedonica*, *cecropia*.

Table 2. First analyzed wings sample from Luncavita - Tulcea County

Wing	Line	Luncavita - Tulcea							
		Carnica	Ligustica	Cecropia	Buckfast	Mellifera	Caucasica	Cypria	Anatolica
1	C	33,04	73				18,74		
2	C	95	0,64				1		
3	C	10	52						1,19
4	C	89,11	25				0,88		
5	C	34	76						
6	O		15		3,24				79
7	C	62	26			10,11			
8	C	36			61,2				
9	C	80			8,99				
10	C		59		36				
11	C	50			12,99		29,43		
12	C	33,78	11		29,92		20,35		
13	C	77			3,56		30		
14	C	48,98			11		20		
15	C	82			26				
16	C	68	29				11		
17	C	51,41	37						
18	C	81	19		14				
19	O	81,98	4,5						
20	O			17	72				

**Line O - *Apis mellifera* - the Middle East group: *anatolica, adami, cypria, syriaca, caucasica, meda*;
*Line C - *Apis mellifera* - Central and Southern European Group: *sicula, ligustica, carnica* (the Carniolian bee), *macedonica, cecropia*.

Table 3. Second analyzed wings sample from Pecineaga - Tulcea county

Wing	Line	Pecineaga-Tulcea							
		Carnica	Ligustica	Cecropia	Buckfast	Mellifera	Caucasica	Cypria	Anatolica
1	M	42				20,01	22		
2	C		12,45			31,65		13,91	13,46
3	C	76,12		23,03					
4	O	34,28					70,11		
5	C	49,76					47		1,89
6	C	60		10,54		15,2			11
7	O	34,11					50		10
8	C	48		11,6			42		
9	C	98					0,27		
10	O	97,54			1,21		43		
11	M	19,65				41			
12	C	93,14	2,43						0,5
13	C	66			8,77		11		
14	C	93,41	10				4,55		
15	C	49,65			16,98		5,98		
16	C	92			0,31		0,56		
17	O	25			6,61		66,31		
18	C	90			10.00				
19	C	96	13,54		9,1				
20	C	91				5,43			

**Line O - *Apis mellifera* - the Middle East group: *anatolica, adami, cypria, syriaca, caucasica, meda*;
*Line C - *Apis mellifera* - Central and Southern European Group: *sicula, ligustica, carnica* (the Carniolian bee), *macedonica, cecropia*.
***Line M - *Apis mellifera* - Western and Northern European Mediterranean group: *mellifera, iberica* subspecies.

- in Calarasi county, the samples from Cocon (17 wings from 20) presented a homology degree over 90% with *Apis mellifera carnica,* instead the samples from Oltenita were hybrids (see Tables 4 and 5);

Table 4. Third analyzed wings sample from Oltenita - Calarasi County

Wing	Line	Oltenita - Calarasi							
		Carnica	Ligustica	Cecropia	Buckfast	Mellifera	Caucasica	Cypria	Anatolica
1	C	89			4				6
2	O	14							88
3	C	77					19		7
4	C	89				7			2
5	C	60		2			21		
6	O	11							79
7	M					82	4		10
8	M					69		14	
9	C	35				18	15		
10	C	56				20			
11	C	91			14				1
12	M					79			19
13	C	49					22		11
14	O	14							76
15	C	85			23			5	
16	M	17		5		69			
17	C	71			3		17		
18	O	14							84
19	C	91	12						
20	O	10							77

**Line O - *Apis mellifera* subspecies the Middle East group includes the *Apis mellifera*: *anatolica, adami, cypria, syriaca, caucasica, meda*;
*Line C - *Apis mellifera* subspecies Central and Southern European Group includes the *Apis mellifera*: *sicula, ligustica, carnica* (the Carniolian bee), *macedonica, cecropia*;
***Line M - *Apis mellifera* subspecies western and northern European Mediterranean group includes the *Apis mellifera*: *mellifera, iberica* subspecies.

Table 5. Analyzed wings sample from Cocon - Calarasi County

Wing	Line	Cocon - Calarasi									
		Carni	ca	Ligus	tica	Ceer	opia	Buck	fast	Melli	fera
1	C	89		21							
2	C	90				5		11			
3	C	90		11							
4	C	98						16			
5	C	92		9							
6	C	90		15							
7	C	91						4			
8	C	87		16							
9	C	99						8			
10	C	96		3						5	
11	C	90		12							
12	C	91								6	
13	C	92		9						7	
14	C	94									
15	C	95		12,34							
16	C	90		17				6			
17	C	91		7							
18	C	97								12	
19	C	88		14,89							
20	C	93		16							

Wing	Line	Cocon - Calarasi									
		Caucasica		Cypria		Anatolica					
1	C		2								
2	C										
3	C										
4	C										
5	C										
6	C										
7	C		3								
8	C										5
9	C										
10	C										
11	C										
12	C										7
13	C										
14	C										
15	C										
16	C										
17	C										9
18	C		12								
19	C										
20	C										

**Line O - *Apis mellifera* subspecies the Middle East group includes the *Apis mellifera*: *anatolica, adami, cypria, syriaca, caucasica, meda*;
*Line C - *Apis mellifera* subspecies Central and Southern European Group includes the *Apis mellifera*: *sicula, ligustica, carnica* (the Carniolian bee), *macedonica, cecropia*;
***Line M - *Apis mellifera* subspecies western and northern European Mediterranean group includes the *Apis mellifera*: *mellifera, iberica* subspecie

- in Giurgiu county, 16 wings from 20 showed a homology over 90% with *Apis mellifera carnica* (see Table 6);

Table 6. First analyzed wings sample from Gostinu - Giurgiu county

Wing	Line	Gostinu - Giurgiu							
		Carnica	Ligustica	Cecropia	Buckfast	Mellifera	Caucasica	Cypria	Anatolica
1	C	89	23			14,34			
2	C	90		2			21,05		
3	C	92	10		7				
4	C	90				11	6		
5	C	90				12	8		4
6	C	91				22			
7	C	96,12	11						
8	C	88			25				3
9	C	93,21					11		2
10	C	98	12						
11	C	98	11,2			12			
12	C	98			10				1
13	C	95		3			12		
14	M	12				62,33			
15	C	90	16						
16	C	88				21			
17	C	94					6		
18	C	97,11	2						
19	C	95	9		6				
20	C	94			1	5			

**Line O - *Apis mellifera* subspecies the Middle East group includes the *Apis mellifera*: *anatolica, adami, cypria, syriaca, caucasica, meda*;
*Line C - *Apis mellifera* subspecies Central and Southern European Group includes the *Apis mellifera*: *sicula, ligustica, carnica* (the Carniolian bee), *macedonica, cecropia*;
***Line M - *Apis mellifera* subspecies western and northern European Mediterranean group includes the *Apis mellifera*: *mellifera, iberica* subspecies.

- in Olt county, the sample Visina could not be classified in any subspecies, most wings expressing variable relatedness to one of the subspecies, without being possible to assess the type or level of hybridization (see Table 7);the sample from Bals (18 wings from 20) presented a homology over 90% with *Apis mellifera carnica* (see Table 8);

Table 7. Analyzed wings sample from Visina - Olt county

Wing	Line	Visina - Olt							
		Carnica	Ligustica	Ceeropia	Buckfast	Mellifera	Caucasica	Cypria	Anatolica
1	C	35					20		21
2	C	99	6						
3	C	51,71	43						
4	C	98,05					3		
5	C	72					23		7
6	O		18				5		83,52
7	C	61,91	11						
8	C	79	19	14					
9	C	79,45				14			3
10	C	59					33		
11	C	48	3	7			30		
12	C	65	2,01				30,35		
13	C	72,95	23						3,56
14	C	47			17	12			
15	C	90					11		
16	C	69	21	11					
17	C	52					33		
18	C	77	15,01		3				
19	C	83	8		7				
20	O			19			70		

**Line O - *Apis mellifera* subspecies the Middle East group includes the *Apis mellifera*: *anatolica, adami, cypria, syriaca, caucasica, meda*;
*Line C - *Apis mellifera* subspecies Central and Southern European Group includes the *Apis mellifera*: *sicula, ligustica, carnica* (the Carniolian bee), *macedonica,cecropia*;
***Line M - *Apis mellifera* subspecies western and northern European Mediterranean group includes the *Apis mellifera*: *mellifera, iberica* subspecies.

Table 8. Analyzed wings sample from Bals - Olt county

Wing	Line	Bals - Olt										
		Carni	ca	Ligus	tica	Ceer	opia	Buck	fast	Melli	fera	Cauc	asica	Cypri	a	Anat	olica
1	C		90									12					
2	C		90						8			10					
3	C		98		7												
4	C		97								22						
5	C		98		15,47												
6	C		89		16,29				4								
7	C		94									3,87					
8	C		93				8				11						
9	C		93								3,44						4
10	C		90				1				8						
11	C		98								13,4						
12	C		97									15					
13	C		99									20,55					
14	C		90		18												
15	C		90								10	5					
16	C		91									19					
17	C		96									23					
18	C		96						8,48			3					
19	C		97		13												
20	C		88				9										12

**Line O - *Apis mellifera* subspecies the Middle East group includes the *Apis mellifera*: *anatolica, adami, cypria, syriaca, caucasica, meda*;
*Line C - *Apis mellifera* subspecies Central and Southern European Group includes the *Apis mellifera*: *sicula, ligustica, carnica* (the Carniolian bee), *macedonica, cecropia*;
***Line M - *Apis mellifera* subspecies western and northern European Mediterranean group includes the *Apis mellifera*: *mellifera, iberica* subspecies.

- in Dambovita county: the samples from Gura Ocnitei (17 wings from 20) and Pucioasa (17 wings from 20) presented a homology over 90% with *Apis mellifera carnica* (see Tables 9 and 10);

Table 9. Second analyzed wings sample from Gura Ocnitei - Dambovita county

Wing	Line	Gura Ocnitei - Dambovita							
		Carnica	Ligustica	Ceeropia	Buckfast	Mellifera	Caucasica	Cypria	Anatolica
1	C	99	9						
2	O					12			89
3	C	90	6						
4	O		2		5				77
5	C	92	17			1			
6	C	93			4			3	
7	C	97	7		2				
8	C	91	1				2		
9	C	96				5,6			
10	C	96			9				
11	C	98			7				
12	C	98	9						
13	C	91		2				7	
14	O		7			1			79
15	C	91					10		
16	C	96				12			
17	C	91			12			2,07	
18	C	91	11						
19	C	93	9		10				
20	C	90	8,21		3				

**Line O - *Apis mellifera* subspecies the Middle East group includes the *Apis mellifera*: *anatolica, adami, cypria, syriaca, caucasica, meda*;
*Line C - *Apis mellifera* subspecies Central and Southern European Group includes the *Apis mellifera*: *sicula, ligustica, carnica* (the Carniolian bee), *macedonica,cecropia*;
***Line M - *Apis mellifera* subspecies western and northern European Mediterranean group includes the *Apis mellifera*: *mellifera, iberica* subspecies.

Table 10. Analyzed wings sample from Pucioasa - Dambovita county

Wing	Line	Pucioasa - Dambovita							
		Carnica	Ligustica	Ceeropia	Buckfast	Mellifera	Caucasica	Cypria	Anatolica
1	C	90							
2	C	90	22						
3	C	97	11						
4	O					17			88
5	C	90			5	12			
6	C	93					8		
7	C	98			11				
8	C	90	1		6				
9	C	89				5,6	12		
10	C	95					3		
11	C	97,1			6				
12	C	91	12						
13	C	91		5				10	
14	C	96			8,01				
15	C	88			1		14		
16	C	91,88				16			
17	C	94						3	
18	C	99						12	2
19	C	98			10				
20	C	95	8		1				

**Line O - *Apis mellifera* subspecies the Middle East group includes the *Apis mellifera*: *anatolica, adami, cypria, syriaca, caucasica, meda*;
*Line C - *Apis mellifera* subspecies Central and Southern European Group includes the *Apis mellifera*: *sicula, ligustica, carnica* (the Carniolian bee), *macedonica,cecropia*;
***Line M - *Apis mellifera* subspecies western and northern European Mediterranean group includes the *Apis mellifera*: *mellifera, iberica* subspecies.

- in Ialomita county, 17 wings from 20 showed a homology over 90% with *Apis mellifera carnica* (see Table 11);
- in Prahova county, the samples from Sinaia and Breaza (19 wings from 20) presented a homology over 90% with *Apis mellifera carnica* (see Tables 12 and 13).

Table 11. Analyzed wings sample from Marsilieni - Ialomita county

Wing	Line	Marsilieni - Ialomita							
		Carnica	Ligustica	Cecropia	Buckfast	Mellifera	Caucasica	Cypria	Anatolica
1	C	98	7						
2	C	90	25						
3	C	92	20						
4	O					26		11	68
5	C	89				18			21
6	C	91,38					6		
7	C	90	11		7				
8	C	98,98	0,3						
9	C	91					7		2
10	C	96,6	9						
11	C	92			4				
12	C	93	15				3		
13	C	90		2					10
14	C	91,3			9				
15	C	98					3		
16	C	91,98				12			
17	C	96,42						6	
18	C	91	1						9
19	C	89,67			13				
20	C	97	2	4					

**Line O - *Apis mellifera* subspecies the Middle East group includes the *Apis mellifera*: *anatolica, adami, cypria, syriaca, caucasica, meda*;
*Line C - *Apis mellifera* subspecies Central and Southern European Group includes the *Apis mellifera*: *sicula, ligustica, carnica* (the Carniolian bee), *macedonica,cecropia*;
***Line M - *Apis mellifera* subspecies western and northern European Mediterranean group includes the *Apis mellifera*: *mellifera, iberica* subspecies.

Table 12. Analyzed wings sample from Sinaia Prahova county

Wing	Line	Sinaia - Prahova							
		Carnica	Ligustica	Cecropia	Buckfast	Mellifera	Caucasica	Cypria	Anatolica
1	C	98,6							
2	C	93	12						
3	C	95	11						
4	O					2,17		23,15	71,51
5	C	91,38				16,68			21,77
6	C	90,24					1,45		
7	C	98			1,56				
8	C	98			0,2				
9	C	97					5,6		1,02
10	C	92							
11	C	91			3,4				
12	C	90					11,9		
13	C	94		3,04					10,7
14	C	91,3			7				
15	C	94					9,11		
16	C	91,01							
17	C	95					11,22		
18	C	91							12
19	C	90			11,56				
20	C	92,12		8,23					

** Line O - *Apis mellifera* subspecies the Middle East group includes the *Apis mellifera*: *anatolica, adami, cypria, syriaca, caucasica, meda*;
*Line C - *Apis mellifera* subspecies Central and Southern European Group includes the *Apis mellifera*: *sicula, ligustica, carnica* (the Carniolian bee), *macedonica,cecropia*;
***Line M - *Apis mellifera* subspecies western and northern European Mediterranean group includes the *Apis mellifera*: *mellifera, iberica* subspecies.

Table 13. Analized analyzed wings sample from Breaza - Prahova county

Wing	Line	Breaza - Prahova							
		Carnica	Ligustica	Cecropia	Buckfast	Mellifera	Caucasica	Cypria	Anatolica
1	C	95							
2	C	93,11	22						
3	C	91	9,8						
4	O					1,87			73
5	C	90,2				17			
6	C	93					3,89		
7	C	93			2				
8	C	98,04	0,2						
9	C	94				5,6			
10	C	96,44							
11	C	97,1			3,4				
12	C	94,11	11,3						
13	C	90		5,89				10,67	
14	C	91,65			8,01				
15	C	90					9		
16	C	91,88				16			
17	C	95						2,07	
18	C	90						10,98	
19	C	90			12				
20	C	93,14			2,43				

**Line O - *Apis mellifera* subspecies the Middle East group includes the *Apis mellifera*: *anatolica, adami, cypria, syriaca, caucasica, meda*;
*Line C - *Apis mellifera* subspecies Central and Southern European Group includes the *Apis mellifera*: *sicula, ligustica, carnica* (the Carniolian bee), *macedonica, cecropia*;
***Line M - *Apis mellifera* subspecies western and northern European Mediterranean group includes the *Apis mellifera*: *mellifera, iberica* subspecies.

CONCLUSIONS

Based on the results of geometric morphometry upon the analyzed wings, the honey bee populations studied has hybrids but also pure breed identified as being *Apis mellifera carnica* based on the homology over 90%. The pure breed were identified in samples originating from Calarasi (Cocon), Giurgiu, Olt (Bals), Dambovita (Gura Ocnitei, Pucioasa), Ialomita and Prahova (Sinaia and Breaza).

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METHODS FOR DETERMINING OSSEOINTEGRATION OF ENDOSSEOUS DENTAL IMPLANTS - REVIEW

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Abstract

The dental implant is currently the treatment of choice for dental restoration in edentulous patients. The success of implantation is represented by the dental implant osseointegration. An important indicator of osseointegration is the primary stability (represents the direct mechanical contact between the dental implant and the bone tissue) and secondary stability (appears when regenerative processes and bone remodeling around the implant occur). The gold standard for determining the stability of the implant is histomorphometric analysis, this being a direct and objective method. The method is mainly used in experimental animal studies. Currently, non-invasive methods are used to monitor human subjects. They have the disadvantage that they are indirect methods (e.g.: radiography, cutting torque test, periotest) or they are subjective (e.g.: Percussion test). Till now, no universally accepted non-invasive method has been discovered to directly and objectively quantify the stability of the dental implant.

Key words: *dental implants, implant stability, RFA (Resonance Frequency Analysis), osseointegration.*

INTRODUCTION

Currently, the dental implant is the treatment of choice for dental restoration in edentulous patients (Antonio et al., 2011). The success of implantation is represented by the ability of osseointegration of the implant in the body (Satwalekar et al., 2015).

Branemark and associates first promoted, in 1969, the concept of osseointegration, defining it as the direct structural and functional connection between living bone tissue and the implant surface (Branemark, 1985), and in the following years they published study reports on dental implants and osseointegration (Branemark, 2001).

In 1987 Albrektsson and Jacobsson stated that tests are generally used to indicate, not to verify, the osseointegration process.

Several factors participate in the osseointegration process, each of them having an important role. These factors refer to: the type of implant and its external architecture, the roughness of the implant, its surface or coating, the biocompatibility of the implant, the mechanical properties and physical characteristics of the implant, the place of insertion of the implant, the quality and quantity of bone in which the implant will be inserted, the surgical technique, long-term implant maintenance (Parithimarkalaignan & Padmanabhan 2013)

The stability of the implant has an important role to assure the success of implantation and osseointegration.

Two stages of implant stability can be identified: primary stability and secondary stability (Sennerby & Meredith, 1998) (Table 1).

Table 1 - Factors that may influence the stability of the dental implant

Type of stability	Factors
primary stability	- bone quality - the surgical technique - the design and the surface of the dental implant (the external architecture, the material, the length, the diameter, the surface characteristics of the dental implant)
secondary stability	- primary stability - the surface of the dental implant - the reactivity of the peri-implantar tissue

Primary stability refers to the lack of mobility when establishing the mechanical contact

between the implant and the bone cortical area and depends on certain factors such as: bone quality, surgical technique, the implant design and the surface of the implant (the external architecture, the material, the length, the diameter, the surface characteristics of the implant) (Sennerby et al., 1991, Quesada - Garcia et al., 2009, Konstantinovic 2015).
Secondary stability occurs when regenerative processes and bone remodeling around the implant occur. Secondary stability usually refers to a period of 3-6 months postimplant-tation, although the response of peri-implantar tissue to the trauma produced can take place over a period of up to 18 months (Sennerby & Meredith, 1998; Huang et al., 2002; Nedir et al., 2004; Lang, 2007). Secondary stability depends on factors such as: the primary stability, the implant surface and the reactivity of the peri-implantar tissue which takes into account the period of bone remodeling and healing (Cannizzaro et al., 2007; Huwiler et al., 2007; Atsumi et al., 2007).
Liubavinac- Hack N. and colab. and L. Molly appreciate that primary stability is essential in determining secondary stability and further in determining the correct operating time of the implant (Lioubavina et al., 2006; Molly, 2006).
It is important that in order to predict the long-term evolution of osseointegration, the stability of the implant can be quantified at certain periods of time, by measurable procedures. (Atsumi et al., 2007).
In order to be able to clinically evaluate the patient, certain mechanical indicators of implant stability and less histological criteria are used (these being used especially in animal studies being the gold standard for determining osseointegration), as stated by Meredith, in 1998 (Meredith, 1998).
This article aims to analyze/expose/identify invasive, non-invasive methods that can directly or indirectly demonstrate, subjectively or objectively, the presence of the phenomenon of osseointegration.

METHODS OF EVALUATION OF THE IMPLANT STABILITY

Currently, both invasive and non-invasive methods are used to determine osseointegration. (Swami et al., 2016) as listed in Table 2.

Table 2 - Methods used in assessing implant stability

Invasive methods	Noninvasive methods
1. *histomorphometric analysis*	1. the implantologist's perception
2. *tensional test*	2. percussion test
3. *push-out/pull-out test*	3. radiographic analysis
4. *removal torque test (RTT)*	4. cutting torque resistance analysis (CRA)
5. *circularly polarized light microscopy*	5. periotest
6. *reverse torque test /implant loosening test*	6. resonance frequency analysis (RFA)
	7. insertion torque resistance measurement
	8. measurement of the lateral mobility

Invasive methods
Invasive methods refer to a series of tests generally performed on experimental animal model, some of these tests being rarely used on human patients due to the invasiveness of the method. (Atsumi et al., 2007; Brunski, 2006).
Histomorphometric analysis - represents the gold standard for determining the stability of the implant. The method consists of biopsy of bone tissue and microscopic examination by specific techniques.
Currently it is made only on experimental animal model. For human patients, this method is no longer accepted due to the invasive nature necessary to perform the biopsy that provides data on the stability of the implant (Kastala 2018).
Tensional test - at the beginning, the measurement was performed by detaching the implant from the bone support in which it had been inserted. Afterwards, the methodology was modified, applying lateral loads on the implant. Even though improvements have been made to the method, there are difficulties in interpreting the results, these being directly influenced by the characteristics of the insertion substrate. (Meenakshi et al., 2013)
Push-out/pull-out test is the method that evaluates the healing capacity of bone tissue at the interface with the cylindrical implant without a threaded surface (Brunski, 2006). It is a method that measures shear strength by applying a load parallel to the bone tissue/implant interface (Meenakshi et al., 2013).
Removal torque test is the method that was proposed by Roberts and his collaborators in

1984, and will later be developed and experimented by Johansson and Albrektsson (Atsumi, 2007). The method consists in removing the implant after the healing period to determine osseointegration (Atsumi et al., 2007).
Sullivan et al., have speculated, following their experiment, that any removal torque value (RTV) above 20Ncm may indicate implant osseointegration (Sullivan et al., 1996). This method is limited to animal studies (Atsumi et al., 2007).
Circularly polarized light microscopy can provide information about the inorganic and organic structure of different materials, information that cannot be provided by other methods (Bromage et al., 2003).
A number of studies use this method on an experimental animal model to evaluate osseointegration in the early stages of osseointegration (7, 21 and 42 days), following the collagen fibers orientation patterns. A certain type of orientation of these fibers has been observed which at a certain moment may indicate the maturation of collagen fibers and the increase of bone resistance in the peri-implantar area (Munhoz et al., 2015)
Reverse torque test/ implant loosening test was the most commonly used method to measure the secondary stability of the implant. Implants showing mobility during this test should be considered for removal. This method is no longer used, as testing can lead to microcracks at the implant interface with the bone, which can lead to implant loss. (Sullivan D.Y. et al., 1996).

Non-invasive methods

Non-invasive methods are used in the clinical evaluation of human subjects (Gupta & Padmanabhan T.V., 2011; Meredith, 1998).
The implantologist's perception it is a simple method of assessing primary stability. It is based on the implantologist's perception when he inserts the implant. It has the disadvantage that it is a subjective method related to the surgeon's experience. The method cannot be reproduced or quantified (Swami et al., 2016).
Percussion test consists in evaluating the tonality of the sound emitted by touching a metal object by an inserted implant or by an abutment attached to the implant.
The method has proven to be ineffective as it is a subjective method that depends entirely on the human factor performing the test and does not have the ability to consistently discriminate sounds based on specific criteria. (Meredith, 1998; Al-Jetaily & AlFarraj Al-dosari, 2011).
Radiographic analysis is the most widely used non-invasive method. It can be used at any stage of post-implant healing and is useful to observe the lesions that may occur following implantation, but also to evaluate quantitatively and qualitatively the peri-implantar bone tissue. (Da Cunha, 2004).
The disadvantages of the method are represented by: visibility limitation in the case of conventional panoramic exposures, distortions of the radiographic image, bone loss at the interface with the implant is identified late (Da Cunha, 2004) and cannot accurately indicate the stability of the implant (Atsumi et al., 2007).
Another commonly used method is **computed tomography**. The method is used for planning the implantological treatment, determining the bone density, identifying the local pathological processes, but also for following the osseointegration (Ritter et al., 2014; Wang et al., 2013).
Regarding to **cutting torque resistance analysis** (CRA), the method was developed by de Johansson and Strid and later improved by Friberg. The amount of unit volume of bone removed by current fed electric motor and is measured by controlling the hand pressure during drilling at low speed. This energy is correlated with bone density and primary stability. It has the disadvantage that it cannot assess secondary stability or the potential for implant loss.
Periotest is a type of device developed to be able to quantitatively measure the movement of the tooth. Subsequently, the manufacturer recommends the use of this device to measure with high precision the mobility of the implant in the situation where no problems identifiable by radiological examination were detected (Drago, 2000).
The values recorded by the periotest depend directly on the characteristics of the peri-implantar tissue (bone, if the implantation was successful, or fibrous tissue, if the implant is compromised). The disadvantage of this method is given by the fact that the value

obtained is correlated with the direction and position of the excitation determined by the device (Tricio et al., 1995). The value of the read parameters sometimes differs from the real value of the existing biomechanical parameters (Caulier et al., 1997; Derhami et al., 1995).

Resonance frequency analysis (RFA) is one of the most commonly used methods in assessing the stability of the dental implant, being considered a non-destructive and non-invasive method (Meredith et al., 1996; Feng et al., 2015).

This method uses the principle of vibrations. The interpretation of the recorded response is based on the assumption that the resonant frequency is directly related to the rigidity of the bone-implant interface and the surrounding tissue (Zanetti et al., 2018).

Basically, the method targets 3 variables: implant rigidity/stability, implant/bone interface rigidity and peri-implantar bone tissue rigidity (Bavetta et al., 2019).

The higher the frequency resonance, the stronger the implant bone interface, indicating osseointegration (Satwalekar, 2015). Over time, obtaining low values may be associated with marginal bone loss and/or implant mobility, most often indicating the possibility of implant loss (Friberg et al., 1999; Barewal et al., 2003; Sjostrom et al., 2005; Lundgren et al., 2004).

Thus, by this method, the implants with risk of loss can be identified, but it can be considered as a method that indicates if the stability of the implant allows the subsequent prosthesis. (Gallucci et al., 2004; Glauser et al., 2004; Meredith et al. 1997; Kramer et al., 2005).

Resonance Frequency Analysis (RFA) is affected not only by bone tissue characteristics, but also by the effective implant length, diameter, and surface characteristics. This is the reason why no established normative base on RFA is available yet (Zanetti et al., 2018).

Insertion torque resistance measurement is the method that measures the torsional resistance that occurs during implantation. Torque resistance during implantation depends on dental implant characteristics (implant material, surface, architecture) and on bone tissue characteristics (resistance and density) (Ostman P., 2005; Boronat-Lopez A. et al., 2006; Konstantinovic, 2015).

A disadvantage of this method is that its result is influenced by the type of implant and the amount of fluid in the insertion pocket at the time of implantation. Also, the method does not take into account the force with which the implant is inserted（Ostman P. et al., 2005; Boronat-Lopez A. et al., 2006）.

Measurement of the lateral mobility applies to implants that may show a rotational movement, but which are stable to lateral movement (buccal-lingual or mesial-distal) and may have a favorable prognosis in terms of osseointegration (Konstantinovic et al., 2013; Sennerby et al., 2002).

Research and development methods

Further methods are being researched and constantly developed that can demonstrate, directly or indirectly, the presence of the osseointegration phenomenon. These methods include: *Implatest conventional impulse testing, Highly nonlinear solitary waves method, Electro-mechanical impedance method and Micro motion detecting device.*

CONCLUSIONS

The experimental animal model is indispensable in studies in the field of implantology because they allow in-depth cellular research that cannot be performed on human subjects due to the invasiveness of the research methods involved.

The newly discovered methods are initially tested on animal models and will be tested on human subjects after demonstrating their safety and efficiency.

So far, no universally accepted non-invasive method has been discovered to directly and objectively quantify the stability of the dental implant.

A non-invasive, fast, simple test to quantify implant osseointegration and stability is extremely necessary in current implantology.

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A REVIEW OF CAUSES, DIAGNOSTICS AND EFFECTS OF PERIIMPLANTITIS

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Abstract

This paper aims to present a review of the causes, diagnostics, and effects of periimplantitis. To find relevant publications we used Google Scholar and PubMed as our databases and searched for articles from the years 2011-2021. Initial search results returned over 16000 articles in Google Scholar and 619 articles in PubMed, so a series of filters and criteria were used to select 28 papers used in this review. We found that periimplantitis represents an inflammation that affects both the hard and soft tissue around an implant. The most common cause of the disease is the accumulation of dental plaque around the dental implant. Periimplantitis is diagnosed by the following clinic signs and symptoms: swelling of the tissue around the implant, bleeding of the gingiva upon probing, the presence of harmful types of pathogen bacteria and bone crestal resorption (determined by radiologic evaluation). Many studies covered in this review were conducted on animals, using ligatures around the neck of the inserted implants to induce periimplantitis. Observations made while studying periimplantitis in animal trials can serve as a model for preventing, diagnosing, and treating periimplantitis in humans.

Key words*: dental plaque, diagnostic, ligatures, periimplantitis, prevention.*

INTRODUCTION

Dental implants have become the most widely used method to replace missing teeth. Although most dental implants are successful, there is a risk of further complications.

One such complication caused by dental implants is the disease called periimplantitis, which is an inflammation around the implant area caused by lack of proper hygiene and accumulation of bacteria. In advanced stages, it can lead to the loss of the affected implant. One study of 2,127 patients and 6,129 implants found that the prevalence of periimplantitis after a period of 2 years was 34% and 21% on the patient and implant levels respectively (Kordbacheh Changi et al., 2019).

With the number of dental implants increasing exponentially each year (Elani et al., 2018) it is becoming increasingly important to understand the causes, effects and treatment of periimplantitis.

This article will cover an overview from the literature from 2011-2021 on periimplantitis causes, diagnosis, and effects as well as other observations on the disease.

MATERIALS AND METHODS

Although there are several popular databases, such as IEEE Xplore and ScienceDirect, we decided to use Google Scholar as an initial search database, since it indexes scientific papers from all publishers. We also used PubMed as another database, due to its prestige in the medical field. Initially, we used the following keywords, to identify relevant publications: peri-implantitis OR periimplantitis OR peri implantitis AND diagnostic OR diagnosis OR prognosis OR prevention AND dogs OR humans. Since this search returned over 16000 articles, we decided to filter the results by searching for the following keywords, but only in the title: peri-implantitis OR periimplantitis OR peri implantitis. Also, to include only papers that discuss the diagnosis, prognosis, or prevention of peri-implantitis, we used the exclusion tool,

to eliminate articles that contain key words related to treatment (treat OR treatments OR treatment). This filtering step led to 1540 articles. We narrowed the search space even further, by including the following keywords: humans OR dogs or animals. The final filtering action led to 46 articles, which were examined (by reading the title and the abstract). Out of these articles, we selected 28 articles, based on the following criteria: English language, not before 2011, clinical trials, rather on animals than on humans, at least 3 patients for animal trials, patients with no other general risk factors, induced periimplantitis either by silk, cotton or metal ligatures in animals and periimplantitis by cementation in humans. As there is a lack of periimplantitis clinical trials on humans when compared to periimplantitis clinical trials on animals, the studies in this review will be focusing on studies conducted on animals, especially on canines. Table 1 summarizes the characteristics of the studied clinical trials, focusing on the procedures for inducing periimplantitis, the radiographic evaluation, number of subjects, type of implants, observations and results.

Table 1. Data about periimplantitis, extracted from reviewed clinical trials

Article	Type of subjects	No. of subject, no. & type of implants	Experimental procedures (to induce periimplantitis)	Observations on periimplantitis/periodontitis	Radiographic evaluation	Results
(Hall et al., 2011)	Humans	7 healthy patients and 7 patients that were having periimplantitis	No	The genetic markers have no significant difference between the healthy group and the perrimplantitis group	Yes	Patients with periimplantitis have the same gene expression as the healthy implant patients
(Slotte et al., 2012)	Humans	18 patients, 54 implants	No	Test qPCR as a non-invasive diagnostic tool	Yes	Further studies are needed
(Finne et al., 2012)	Humans	56 patients, 82 implants	No	Gingival recession can occur after the insertion of the implant	Yes	One piece implant has a high survival rate
(Degidi et al., 2012)	Humans	11 patients, 11 implants	No	Microbial adhesion to the implant or to the abutment of the implant is a risk factor for periimplantitits	Not mentioned in the paper	Implants that have the rougher abutment exposed to the oral cavity present a higher risk of periimplantitis
(Carcuac et al., 2013)	Animals	5 patients, 20 implants	Ligature to induce periimplantitis	Implant surface characteristics influence the inflammatory process in perrimplantitis	Not mentioned in the paper	More bone was lost at modified surface implants compared to turned surface implants.
(Charalampakis et al., 2014)	Animals	5 patients (dogs); 4 implants, 2 types (implant A: turned/implant B: iUnite, NobelBiocare AB)	Ligature to induce periimplantitis around implants and mandibular premolars. Ligatures were removed after 10 weeks	Microbial samples were taken with paper points after the removal of ligature (10 and 25 weeks after the removal)	Not mentioned in the paper	More bone was lost at modified surfaces in implants compared to teeth and turned surface implants
(Madi et al., 2014)	Animals	4 healthy female dogs, different implant types	Used cotton floss ligature to induce periimplantitis, removed after 4 months. The 5th open flap surgery was performed. Configuration of the periimplant bone defects were evaluated	Plaque accumulation on ligature caused periimplantitis. 20-30% of initial bone was lost around the implant	Yes	Periodontitis and induced periimplantitis in dogs are similar to periodontitis and periimplantitis disease in humans, respectively.
(Kütan et al., 2015)	Humans	56 patients, 56 implants	No	Platform switched placed 1 mm below the bone level will determine less resorption	Yes	The crestal bone level around implant is the success key factor

Article	Type of subjects	No. of subject, no. & type of implants	Experimental procedures (to induce periimplantitis)	Observations on periimplantitis/periodontitis	Radiographic evaluation	Results
(Neilands et al., 2015)	Humans	25 healthy patients, 25 patients with periimplantitis	No	A higher rate of Porphyromonas/ Prevotella and anaerobic Gram positive cocci was present in patients who were having periimplantitis than in healthy patients	Yes	More longitudinal studies required
(Ioannidis et al., 2015)	Humans	40 patients, 40 implants	No	Narrow implants prevent perrimplantitis	Yes	Ti-Zr 3,3 mm implants have the same success rate as the Ti implants with 4.1 mm diameter.
(Ishii et al., 2016)	Animals	3 patients, 12 pure titanium implants	Used dental floss over a period of 90 days to induce inflammation	UV radiation slows down periimplantitis. Rx shows less bone loss in UV-irradiated periimplantitis	Yes	If the implant is exposed to ultraviolet radiation, the bone resorption will be less pronounced when compared to the areas that weren't exposed to the radiation.
(Derks et al., 2016)	Humans	62 patients, 596 implants	No	The progression of the periimplantitis is not linear	No	The onset of periimplantitis usually appears 3 years after the use of implant
(Gamper et al., 2017)	Humans	60 patients, 151 implants	No	Both implant systems show a very high survival rate	Yes	No significant differences between the one piece and two piece dental implants
(Lin et al., 2017)	Animals	6 dogs, 36 Straumann dental implants	3 months after the implant stainless stell ligatures and silk ligatures were placed to produce periimplantitis	After ligature removal, the inflammation partially recovers. Bone loss was observed	Yes	To induce periimplantitis, SSL ligatures are much more effective than silk or cotton ligatures
(Wu et al., 2017)	Animals	6 patients (Labrador dogs)	Used cotton floss ligature to induce periimplantitis, removed after 2 months	Connectivity tissue loss, increase in inflammation in B and T cells, neutrophils, and macrophages.	Yes	Periimplantitis induced by ligatures in dogs is similar to human periimplantitis.
(Mencio et al., 2017)	Humans	20 patients, 20 implants	No	The patients were divided in two groups: group A - screwed implant- abutment connection and group B: cemented implant-abutment connection	No	Implants with screwed connection have a higher risk of developing periimplantitis than cemented implants.
(Christiaens et al., 2017)	Humans	23 patients, 50 implants	No	The best predictor for evaluating the periimplantitis is the bone sounding	Yes	Intraoral Rx may underdiagnose the periimplantitis. Therefore, clinical observation is very important for diagnostic
(Abi-Aad et al., 2018)	Humans	24 patients, 173 implants	No	No difference was found between the immediate implant and the conventional implant	Yes	Immediate implant loaded have similar results as one stage implant
(Huang et al., 2018)	Animals	6 dogs	Cotton ligature, oral hygiene was neglected to encourage bacteria formation for a 12-week period	Marginal bone loss	Yes	Implants located at the bone crestal position showed less infra-osseous problems than those implants placed at the subcrestal position in a periimplantitis.

Article	Type of subjects	No. of subject, no. & type of implants	Experimental procedures (to induce periimplantitis)	Observations on periimplantitis/periodontitis	Radiographic evaluation	Results
(Morelli et al., 2019)	Animals	6 patients	After 6weeks old ligatures were replaced, and they were removed completely after 3 months	Bleeding on probing	Yes	Bone resorption was more accentuated in narrower implants. Narrow implants show a tendency of faster progression of the induced periimplantitis compared to standard ones.
(Wu et al., 2019)	Animals	5 dogs, 20 Straumann implants	Used cotton floss ligature to induce periimplantitis	Inflammation, bleeding on probing suppuration. Bone loss was observed on RX	Yes	Bacteria proliferates around the implant and affects the stability of the implant
(Galarraga et al., 2019)	Humans	4 patients, 5 implants	No	All implants were lost in the subjects due to severe bone loss	Yes	Periimplantitis affects bone to implant contact. Radiographic evaluation does not show to full extent of periimplantitis
(Bolle et al., 2019)	Animals	6 dogs, 57 implants, 4 types (Straumann, Nobel, Global, Twinkon)	Submarginal ligatures	Implant type may influence bone loss and healing during implantitis.	Yes	Implant type may influence bone loss and healing during implantitis. Parameters of implant type may present risk factors for the periimplantitis. Further research must be conducted.
(Guarnieri et al., 2020)	Humans	56 older humans	No	Bone loss, chronic inflammatory infiltrate	Yes	Extremely careful cementation process to avoid further complications
(Yoon et al., 2020)	Animals	6 dogs, 24 implants	Silk ligature to induce periimplantitis	Mechanical debridement may impede periimplantitis progression of periimplantitis. Bone loss was observed on RX.	Yes	Mechanical debridement may impede periimplantitis progression of periimplantitis
(Galárraga-Vinueza et al., 2020)	Humans	4 patients	No	Biopsies reveal that cytokines, macrophages, and neutrophils are present at periimplantitis sites	Yes	Results are uncertain
(Pamato et al., 2020)	Humans	21 patients, 52 implants	No	A comparison between the titanium base abutments and the cement-retained abutment has shown no significant difference	Yes	Titanium base abutments have no negative impact in soft and hard tissue that surround the implant
(Scarano et al., 2021)	Humans	-	-	Cemented retained restoration on implants ensure a better fit and a better distribution of loading during function	Yes	Periimplantitis is associated with the excess of cementum around the implant tissue

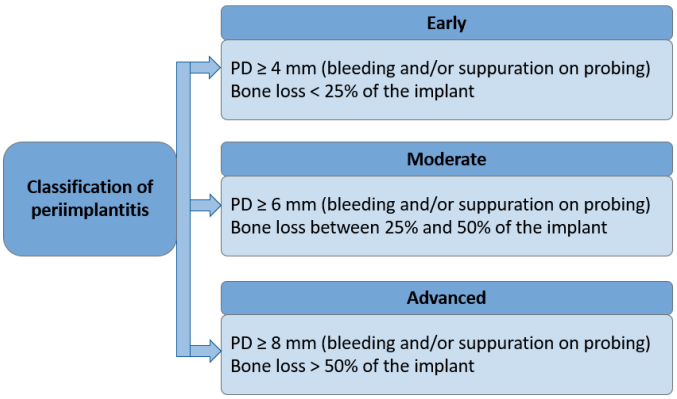

Figure 1. Classification of periimplantitis (according to Froum & Rosen, 2012)

RESULTS AND DISCUSSIONS

Periimplantitis is defined as an inflammatory response of the tissue surrounding the dental implant. The main factor that causes periimplantitis is dental plaque accumulation around the neck of the implant. Early stages begin with mucositis, when the biofilm causes inflammation at the gingival level surrounding the implant. If the dental plaque is not properly removed, the inflammation of the gingiva will continue to progress until mucositis becomes periimplantitis. Mucositis is limited to affecting the soft tissue only. It becomes periimplantitis when the gingival inflammation starts affecting the bone. Therefore, periimplantitis includes the inflammation of the soft tissue as well as bone resorption. Some clinical parameters that are used for diagnosis of the periimplantitis disease are: dental plaque, periimplant mucosa health, bleeding on probing, keratinized mucosa around the implant, suppuration and resorption of the crestal bone. Insufficiency of keratinized mucosa around the dental implants has been associated with an increase of dental plaque accumulation, mucosal recession and peri-implantitis (Kungsadalpipob et al., 2020) A careful evaluation of all the factors stated above helps determine the presence and severity of periimplantitis. Figure 1 shows the different stages of progression in the periimplantitis disease.

In clinical trials conducted on animals, to study periimplantitis, there is a series of steps that must be followed. The first step is the extraction of the teeth that will be replaced. After approximately 3 months, a dental implant procedure will take place through a surgical intervention. The purpose of the surgical intervention is the replacement of the natural tooth root with an artificial one. After the surgery is complete, a period of three months is necessary for healing of the surgical area. During the three months, controlled hygiene with mouth wash is applied to prevent the premature formation of inflammation. After the healing process and osteointegration of the implant into the bone is complete, one of three different ligature types are applied around the neck of the implant to induce periimplantitis. The three main types of ligatures are: metal, silk and cotton.

For studies conducted on humans, the periimplantitis was caused by leftover cementum in the sulcus of the gingiva around the implant and dental plaque.

The dental plaque includes the microbiota that coats the implant. Certain strains of microflora can cause dental implant failure. Some of these strains include: bacteria which is black pigmented such as Prevotella nigrescens, Prevotella intermedia, and Phorphyromonas gingivalis as well as bacteria that are gram negative such as Spirochetes, Bacteroides, Forsythusfusobacteria, and Aggregatibacter actinonomycetemcomitans (Heydenrijk et al., 2002). Radiographic imagistic is important for detecting and determining the stage of the periimplantitis. If the implant is found to present mobility, then it is completely compromised and must be removed. A clinical diagnosis characterized by bleeding upon

probing with or without suppuration, an increased peri-implant pocket depth ≥5 mm or a radiograph showing marginal bone loss ≥2 mm is an indication of periimplant disease. (Nguyen-Hieu et al., 2012).

The best way to avoid periimplantitis, implant failure and removal is detecting the early stage of periimplant mucositis and managing it. (Jepsen et al., 2015).

Currently the diagnosis of implants is based mostly on clinical and radiological signs. However, molecular tests, as well as biomarkers in peri-implant crevicular fluid (PICF) show promise and may help detect and prevent early periimplantitis (Alassy et al., 2019; Carinci et al., 2019).

Implant narrowness, shape and coating material are some of the factors which can affect periimplantitis risk. However, most studies can agree that if the implant is surrounded by dental plaque then there is a high likelihood of periimplantitis developing. To avoid the formation of the dental plaque that causes periimplantitis a good dental hygiene is required. Excellent dental hygiene usually results in the prevention of periimplantitis and the complications associated with this disease, which determines the successful rate of the implant.

CONCLUSIONS

Based on current literature, our review on periimplantitis revealed that the most common cause of the disease is lack of proper hygiene and accumulation of dental plaque around the dental implant. Periimplantitis is associated and diagnosed with swelling of the affected area, bleeding of the gingiva, the presence of certain types of pathogen bacteria and bone resorption. All studies covered in this review on animal trials used ligatures to induce periimplantitis so it can be observed. Preventing, diagnosing, and treating periimplantitis in its early stages maximizes the success rate of the implant.

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MISCELLANEOUS

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STRATEGIC MANAGEMENT OF THE AVIAN INFLUENTZA (AI) / HIGHLY PATHOGENIC AVIAN INFLUENZA (HPAI)

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Abstract

Avian Influenza (AI) is a contagious disease, first described in Italy, in 1878, by Peroncito, as a disease with high mortality in poultry, next documented as a type A influenza virus, in 1955, by Schäfer W., and then recognized as a transboundary disease spreading across international borders, occurring worldwide (consistent evidence support the transboundary character of the disease, particularly from 2003 to our days, in Asia, USA, Latin America, Europe, Oceania). Data available shows that AI has significantly affected the world economy and society in the last decades, causing disruption of poultry industry and global trade, changes in poultry industry and markets policies, affecting livelihood of vulnerable people, producing considerable control costs, and starting with 1997 it is also highly recognised as a public health threat that can cause illness or death in humans. Despite the new tools used by the modern management (advanced science technologies, statistics tools, etc.) the disease still leave behind losses into the global poultry industry and sometimes human deaths (Hong Kong, Cambodia, Djibouti, Egypt, Iraq, Turkey etc). The paper aims to review the knowledge on the management of AI (HPAI) in the world, to identify gaps or weaknesses in the management of the AI and to try to provide guidelines on how countries can be better organized to react to an outbreak of AI/HPAI and/or to identify better ways to diminish the devastating impact of the disease upon societies, consumer, trade between the countries, economy.

Key words*: animal health, crisis management, risk factors, economy trade JEL classifications: H12, Q18.*

INTRODUCTION

The article reviews the evidence available in relation to crisis and emergency management practices in respect of Avian Influence (AI) outbreaks in the world. Documented evidence show that it is a disease with history, first described in 1878, in Italy, by Peroncito [1,2], then confirmed in 1955 that is a Influence virus by *Schäfer* W. [1,2] and in 2003 recognized as a transboundary disease, spreading across international borders, occurring worldwide (consistent evidence support the transboundary character of the disease, in Asia, USA, Latin America, Europe, Oceania). Today evidence show that AI is still a problem with global impact which requires attention from all parties involved (government, scientists, industry, population).

In this context, the paper is reviewing the crisis management process implemented in case of AI outbreaks (the epidemiology of AI, the monitored data, the shortcomings and the gaps identified by different bodies or risk assessor – auditors of EC, EFSA researchers, OIE, FAO, WTO – international organisations etc) and the progress registered by different techniques to support crisis management (modelling, risk management, genotyping, mapping technique comparisons studies, etc) in the world, and different factors that influence the management of the AI virus (gaps in scientific information, shortcomings identified in the official animal health controls applied by the competent authorities, statistical approaches rather than biological evidence etc.).

MATERIALS AND METHODS

Retrospective method was used. Data collected, registered and notified to OIE, EC by countries, audit reports, reports issued by EC, FAO, OIE, official presentations, data published in various scientific articles concerning the AI were reviewed.

RESULTS AND DISCUSSIONS

During the last century the entire world experienced a large number of AI health crisis

with politic, economic and social impact [1, 2, 8, 9, 11, 16, 17, 21].

Despite the negative impact of the crisis, markets changed, restrictive measures on trade, human deaths, they also left us to the world a measurable print, or legacy, respectively we learned from them around the clock.

In 1878, the AI was first time differentiated from the other diseases that cause high mortality rates in birds, by Perroncito E. [1]. 81 years later, over a period of 36 years (from 1959 to 1995), sources mention that 15 outbreaks of HPAI viruses were recorded in poultry (1) *They also, point out that at that time the AI was causing high mortality but the outbreaks were patchy and limited to small regions.*

However according to the data available, until 1997 (119 years later), when the *first human case was detected and reported in Hong Kong* [1,2] the poultry outbreaks were reported mostly globally.

In 1996, the H5N1 virus was found in China in a commercial geese and it was believed to originate from H5 viruses in wild migratory birds [12]. However, this outbreak gave rise in 1997 to the next outbreaks of H5N1 in the farms of Hong Kong and led to human infections and deaths. Surveillance revealed that H5N1 was widespread in poultry and the birds were culled.

Only after that moment the disease was propelled in the centre of attention of the whole world (scientists, officials, public), recognised as a public health concern and a transboundary animal disease and *monitored more carefully and systematically diagnosticated in the world. After that moment and based on the reported data it can be noticed that the AI outbreaks were rather frequently diagnosed and reported* (Figures 1, 2, 3).

During the next 10 years (from 1997 to 2007), literature recorded further 11 HPAI outbreaks in poultry. This time it was point out that some of them affected millions of birds and spreaded across Asia [10], Europe and Africa, in over 60 countries.

Europe experienced six major HPAI episodes, three of which occurred in the last years (2016-2017 [2, 9], 2017–2018 and 2019-2020 [6].

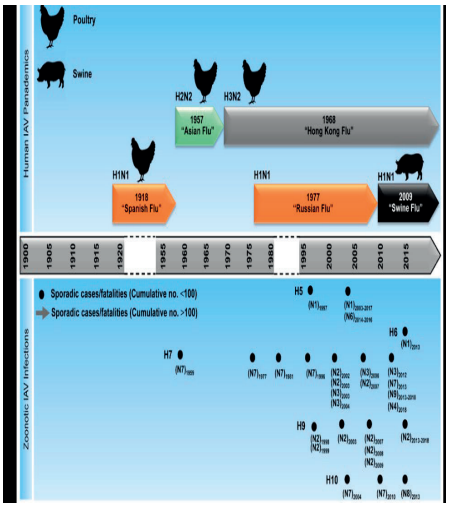


Figure 1. The timeline (1900-2015) of flu pandemics and epidemics caused by influenza A virus (authors Ahmed Mostafa, Elsayed M. Abdelwhab, Thomas C. Mettenleiter, and Stephan Pleschka)

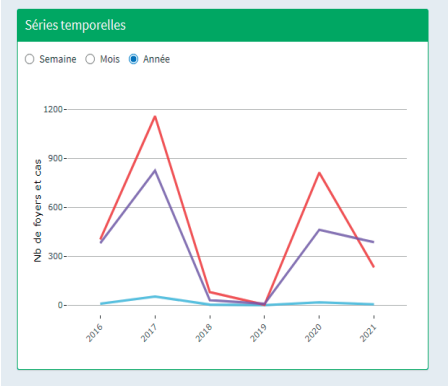


Figure 2. The flu pandemics and epidemics caused by influenza A virus between 2016-2021 in Europe (serotypes identified H5N1, H5N3, H5N5, H5N6, H5N8, H5Nx) (Source: ADNS/DGAL/FAO/OIE)

Documented evidence [8] show that the avian influenza type A viruses appear *to be adapted very well in aquatic wild birds, in waterfall, gulls, shorebirds etc and also specify that the complete host range*[19] *for AI in wild birds is not completely know.*

We remind here also about the past pandemics when the non-human Influenza A viruses changed and infected people: in 1918 (when H1N1 – determined as an avian origin gene, however there is no universal consensus, and 50 million deaths worldwide were registered), in 1957-1958 (H2-N2 avian origin gene, when another 1.1 million deaths worldwide were

registered),in 1968 (H3-N2 avian origin gene as well, when 1.1 million deaths were registred worldwide,) and 2009 (H1-N1 virus – half a million death world wide),
Since then, studies have been conducted in the entire world, and revealed new scientific information, but also new gaps [Verhagen et al., 2021]. Partially the studies addressed problems and provided valuable knowledge to the world, but also recommended further research (for example the inclusion of new methodes such as whole genome sequencing, continuing on host identification and avian ecology and introducing the developments in the risk based surveillance of Avian Influenza [Verhagen et al.,2021].
In Europe in 2005, the European Commission and the World Bank hosted an international conference on AI and pledged million of euro to fight against AI with the experts of the Republic of China.
In 2015, EFSA organised, a workshop where it was analysed the state of knowledge, etiology and epidemiology of the IA virus in animals, the threats and the gaps:

I. targeting four major subjects:
- interaction host – pathogen (to understand host range restriction [18], to identify mechanisms by which viruses adapt to new host species),
- methods of diagnostics (improving the diagnostics, respectively developing more rapid molecular tests and early detection),
- surveillance and risk management (risk analyse of introduction the virus into EU, new risks tools),
- prevention (developing efficient vaccine and vaccination programme, to validate bio security measures) and control

II. mapping the AI EU projects funded by EU in last 10 years

III. Analysing and discussing:
- the status of knowledge on IA surveillance, monitoring and control,
- the transmission evidence for AI from animal to human and how was identified by the surveillance measures,
- the viral characteristics associated with animals AI virus and human infection,
- the epidemiological risk factors/and drivers connected with IA transmission and spreading between species, and within animal populations,
- the scientific gaps need it to be identify and address through research,
- how to rank a pandemic risk posed by a given AI virus.

At the end, they realised that at that time the understanding of the transmission of the virus intra and between animal species and from animals to humans, on host-range, drivers of virulence **was in fact basic**. They admitted that the standardisation of the diagnostic tools the integration of the databases and networks, as well as international cooperation are the keys to success. Also, they realised that there is a need to involve scientists from many sciences in order to achieve greater impact in AI management [13, 14].

They identified the needs, and set up the long and short goals. the objectives, and
- proposed a revised animal health framework based only on scientific science,
- prioritised the interventions related to prevention, preparedness in case of a crises, research and innovations programmes,

In this context, other new studies were initiated at molecular level [8,9], studies in the unexplored areas of immunology – in order to improve the vaccine responses, to create vaccines that can be mass-delivered and studies in epidemiology in order to clearly understand the modes and routes of transmission within and between animal species/ to humans, and studies of predictive biology, and mathematical modelling in order to predict and improve the overall management of the disease [13, 14], studies concerning the sampling methodology /test results of the surveillance programme carried out by Member States [1-21].
On the other hand, epidemiological and genetic comparison studies [13] were carried out by scientists in different countries between *the* 2016-2017 *episode* and the two previous episodes that took place in Europe (2005-2006 and 2014-2015) (source Comparison of 2016-2017 and Previous Epizootics of Highly Pathogenic Avian Influenza H5 Guangdong

Lineage in Europe). The study concluded that a record in Magnitude was registered in 2016-2017 and they underlined that they noticed significant variations between the episodes (temporal, spatial, epidemic curve, seasonality etc) concluding that is difficult to predict future HPAI epizootic. They also recommended global surveillance of the virus changes in order to provide valuable information for preparation, detection and control of HPAI.

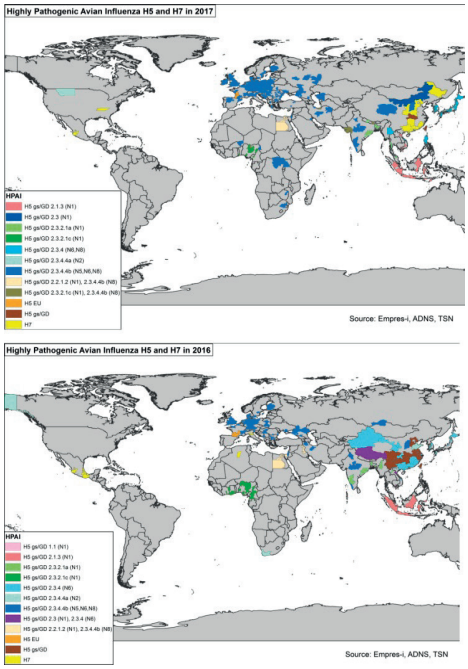


Figure 3. EFSA Journal, Volume: 15, Issue: 10, First published: 16 October 2017, DOI: (10.2903/j.efsa.2017.4991

Consecutive crisis, the CE/the other affected countries **drafted legislation**, carried out evaluation of the **entire/partial management control system** [3], **audits** [7], promoted collaboration, organised workshops, training reunited their forces in focused research etc.

One point of legislation everywhere require to draw up Contingency plan [5] which represent a management tool to help them to control operations (before, during and after unforeseen event) and return to its daily operations as quickly as possible after an outbreak /pandemic episodes.

The legislation foreseen a minimum set of requirements:

- cooperation between authorities & stakeholders /a chain of command and a coordinator unit called National Disease Control Centre,
- setting a Local Disease Control Center,
- detailed instructions concerning the controlling actions for addressing the outbreak, to protect the public and animal health and to reduce the negative effects,
- clear details on responsibilities staff established (who does what and when!),
- real time alert exercise,
- equipment & materials available any time,
- diagnostic labs facilities & capacity for rapid diagnosis,
- legal powers to ensure the implementation
- holding registration and identification of the high density areas that presents risks,
- emergency vaccination,
- a permanent operational expert,
- training,
- communication with the general public who directly /indirectly/ voluntary /involuntary participate in one way /another to the crisis management plan influencing the favourable /unfavourable implementation of the plan,
- access to financial resources.

The EU co-financed the Member States Plans and veterinary emergency actions.

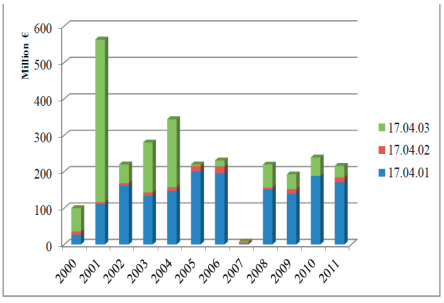
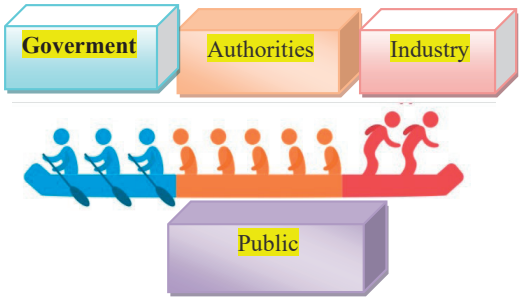


Figure 4. EU Funding for Animal Health 2000-2011 (source - Evaluation of the EU rapid response network, crisis management and communication capacity regarding certain transmissible animal diseases, 01.08.2012)

However, based on documents reviewed and analysed (EC audit reports [7], FAO reports on AI) we can emphasize that cooperation

between the key relevant stakeholders is a critical condition during the control/management of a disease, particularly in pandemic occurrences. They should act as one [13, 14].

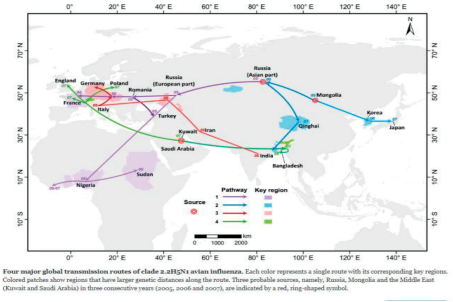


One more frequent problem identified during audits [7] on the implementation of a crisis management plan is poor coordination of all the stakeholders listed above, reflected in the shortcomings identified during the implementation of the actions planned, non effective and efficient use of resources, inconsistent protection of the public and animal health, and huge negative impact on economy, society, environment etc on long time.

An additional difficulty met in reality is that the decision makers have to overcome political, mass media, industry, and people pressures and to keep everybody happy, so to speak, to motivate them, to involve them in the process, to make them to understand and accept the unpleasant, unpopular and costly measures. Sometimes, the pressures influence their decision-making process and operations, affecting in the end the achievement of the targeted objectives. Pressures encourage ongoing events that can cause general uncertainty and weaken the country control system put in place to defend the human and animal health.

In summary, in reality these goals listed above (collaboration, coordination, lack of pressure) are not so easy to achieve [7], and additionally there are also other many factors that can interfere in the implementation of the plan, such political instability, other interests, lack of government support and commitment to unpopular measures, illegal movements and trade of the domestic and wild birds, people culture (lack of trust, lack of education, poverty etc), climate and wild bird migration changes, competency of the veterinary authority etc

Phylogenetics analysis of the viruses brought information on the main paths in the world (see Figures 5, 6).

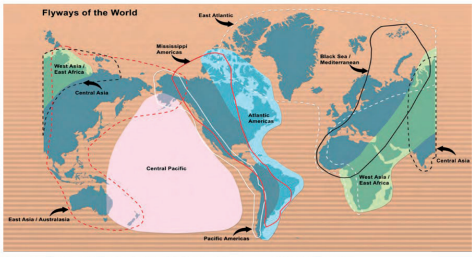


Four major global transmission routes of clade 2.2H5N1 avian influenza. Each color represents a single route with its corresponding key regions. Colored patches show regions that have larger genetic distances along the route. Three probable sources, namely, Russia, Mongolia and the Middle East (Kuwait and Saudi Arabia) in three consecutive years (2005, 2006 and 2007), are indicated by a red, ring-shaped symbol.

Figure 5. Major global transmission routes of H5N1 avian influenza. (source global spatiotemporal and genetic footprint of the H5N1 avian influenza virus, Ruiyun Li, Zhiben Jiang & Bing Xu)

Additionally, the phylogeographic analyses confirmed the role of migratory wild birds in the circulation of H7N3 strains from North America into Mexico in 2012–2013.

Equally, same method have been used to reveal the HPAI H5N1 transportation by different bird species across Asia [11] and that the spread of HPAI H9N2 strains in Asia was a mixture of long-range distribution by wild birds attached with more restricted spread via the domestic bird trade.



Migration flyways of the world. Note that Alaska is a major crossroads for several flyways.

Figure 6. Flyways of migratory water fowl. Flyways run approximately north–south, and also overlap in northern regions, including in Siberia, Greenland, Alaska and across the Bering straits, which allows occasional transmission of influenza viruses between North America and Eurasia.
Flyways from http://wpe.wetlands.orgIwhatfly

In case of AI, the virus can be transmitted from bird to bird in birds the AI virus is shedded by feace and/or through contaminated material and usually the problems start when the domestic production systems are coming in close contact with wild birds [12].

The **poultry production system** and the trade of poultry, hatching eggs, one day chicks, etc are associated with risks [5, 16].

In general all countries in the world have developed measures to regulate risks production, risk trade [5, 6, 16], such as: legislation, bio security guidelines, financial incentives, compensations schemes etc however from documented evidence and current epidemiological data (audit reports [7], scientific studies) it is clear that there are not enough (Table 1).

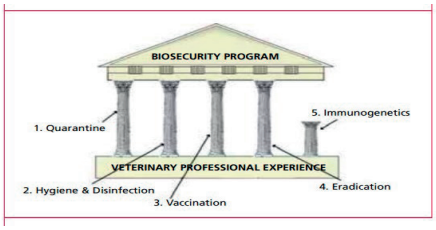


Figure 7. Biosecurity and supporting strategies for disease control and prevention (T. J. Bagust)

Different systems are exposed to different risks and every poultry farm have its own risk profile [5, 7, 16, Table 1] and capacity to spread the virus to the next farm, therefore measures and strategies developed and applied by the operators should be tailored to each farm (system), after a risk management is completed, in order to address their proper risks, otherwise it won't work. In other words, one size doesn't fit all and this feature must be seriously taken in consideration by each farmer, competent authority, government etc respectively each participant to the poultry production/trade system.

Table 1. Poultry system in five South east Asian countries affected by HPAI in 2003-2005

Country	Industrial	Large commercial	Small commercial	**Backyards**
Cambodia		<1%	<1%	**99,9%**
Indonezia	3,5 export and consumption	21,2	11,8	**63.4**
Lao		small	10	**90**
Thailand	70 % production export	10 % production	**10% production, 98% producers**	
Vietnam	small	20-25% production. Few producers	10-15% production, few producers	**65 % production**

Based on the data in the table and based on the impact of AI in these countries is very clear that the production system and the bio security measure have a critical role in the control of the waves of AI.

The world's **poultry population and the trade** have been rapidly grown in the last decades (Figure 8), driven both by demand and supply, according to FAOSTAT data, generating huge density of poultry populations and frequent movements.

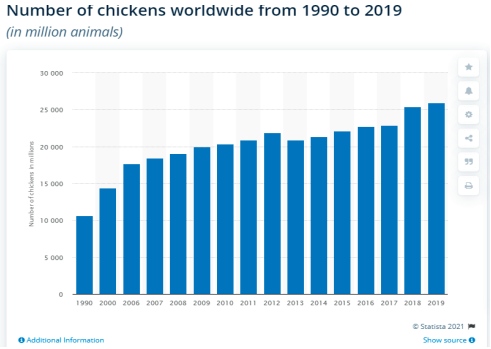


Figure 8. Number of chickens worldwide from 1990-2019

Statistics data shows that United States (18.262 MT) and Brazil (12910 MT), respectively the largest producers of poultry in the world, were/are rarely affected by AI. In contrast, the Republic of China (12300 MT) and the EU-27 (11560 MT) which are smaller producers then USA and Brazil, were/are frequently affected by the AI.

Analysing data related to biggest exporters of poultry in Brasil (3889 MT) and USA (3014 the AI is rare. On the other hand in EU-27 (1276 MT) and in Thailand (690 MT), both being big smaller exporters compared with Brasil and USA, both are frequently affected by the AI (Thailand was affected before 2004, then after they reviewed the production system, no new cases were reported).

Therefore we can conclude only for the cases described above that the size of production /trade can be factors that can trigger AI waves (EU-27, China, Thailand etc) in certain circumstances. The data are not comparable because many factors influence the final result

Particularly, documented evidences shows that Thailand before 2004 [6, 16, 15, 17, 19] was among the world major poultry exporters which produced almost 1 billion chickens per year. The production at that time was formed from commercial farms (some 9000 farms) but also

from backyards (around 2.6 million BY) where poultry were raised for food in all the villages. On the other hand Thailand is one of the nine major migratory water birds flyways in the world and home of 50 millions migratory water birds (the flyway include 22 countries such as Russia, China, South East Asia [11], New Zealand and Australia).

Thailand experienced in 2004 [16] an outbreak of H5N1 avian influenza in poultry and in humans. More than 62 million birds died or culled and out of 17 human cases, 12 died. The epidemiological investigation revealed that most people were infected via direct contact with ill or deceased poultry, or when living in households with abnormal poultry deaths. The Government endorsed a three year plan. However, even epidemiologically speaking Thailand seems to be one of the countries regularly affected by HPAI, Thailand has not notified any HPAI since 2009.

Thailand suffered the greatest impact of the disease in 2004 (75% reduction in exports) followed by China, (63% reduction,) Hong Kong (55%) and 27% in the USA, whereas Brazil was the only country that increased (6%) exports (Taha, 2007) [2].

In USA in 2007, more than 190,000 wild birds were tested for AI and have not been detected in wild birds anywhere in North America even potential pathways (Figure 9) of avian influenza introduction from Asia [11] to North America is very clear exist.

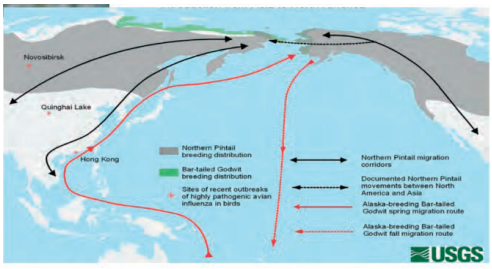


Figure 9. potential pathways of avian influenza introduction from Asia to North America

Outbreaks in domestic poultry caused by AI in USA, occurred, from time to time. For example, HPAI H5 viruses (H5N1, H5N2 and H5N8) were identified in 21 U.S. states from 2014 to 2015. According to CDC data in the past, there have only been a small number (fewer than 10 in 15 years) of reported human infections with North American avian influenza A H7 viruses. Most were associated with poultry exposure and have resulted in mild respiratory illness and/or conjunctivitis.

Some studies emphasise there is insufficient knowledge about the relation of avian influenza virus (AIV) to migratory birds in South America. According to some articles no occurrence of HPAI was reported in domestic or wild birds in Brazil. However, a few studies published data on LPAIV in Brazilian native fauna and exotic resident avian species. It seems that AI is considered an exotic disease in Brazil.

In 2011, in poultry, the United Nations Food and Agriculture Organization [9] considered six **countries to be endemic for HPAI H5N1 virus**:

- → Bangladesh,
- → China,
- → Egypt,
- → India,
- → Indonesia,
- → Vietnam.

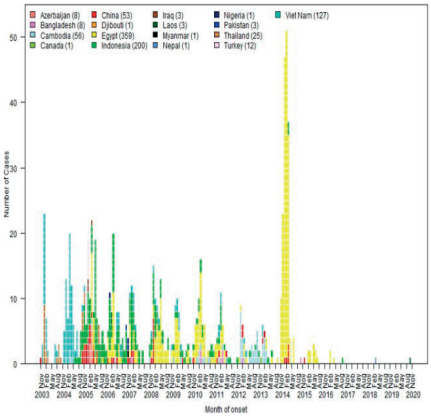


Figure 10. Epidemiological curve of AI (H5N1) cases in humans 2003-2020 (source, WHO)

In 2011, a FAO report concludes that countries listed above become endemically infected because they had **high proportion of poultry reared in very large 'backyard'**, which were sold under conditions of very poor bio security. According to them the poultry production in place plays a critical role in the maintenance and spread of H5N1 HPAI [20], backyard poultry being part of the cycle of infection, especially when the sale and movement of backyard poultry was done through long and

complex market chains in conditions of poor bio security, with unreported outbreaks.

It seems that, measures have been developed everywhere in the world and that are similar covering prophylactic, and control and measures (minimum requirements) and they are also applied almost the same into entire world, even there are huge differences between the poultry production systems in countries (respectively huge technological differences between high income counties vs low income countries) and also in the same country (commercial farms vs family mixed own flocks, sustaining livelihoods) and environmentally. These observations are also backup by the data monitored by FAO who highlight in different reports how in developing countries small producers hardly ever take bio security measures or vaccinate their birds because they are familiar to lose part of flocks because of diseases. It seems that these kinds of farms are the most plausible mechanism to spread the virus between places which are not connected by the flyways of the migratory birds.

CONCLUSIONS

Today even all stakeholders (government, legislators, researchers, industry, public etc.) acknowledge the globalization, the emergence of transboundary diseases (e.g., zoonotic influence, emerging Coronaviruses - MERS, SARS, Covid etc) the threat related to the increase of worldwide recognized diseases (e.g., ASF, Salmonella, West Nile Virus, Bluetongue, AMR etc) and despite the huge amount of studies carried out we have also to admit that new scientific gaps have been identified and need to be addressed through further research and surveillance using new technologies. Biology is not simple, is not easily predictable (especially when we are referring to mutation, gene flow, genetic drift, or natural selection).In this context there is a need of good epidemiologists and scientists from different sciences. Is it critical, that in order to identify ways to manage better the AI pandemics we have to read firstly *the gaps in scientific information* (*ecology of influenza viruses, the adaptability of the virus to new host species, drivers of virulence, transmission mechanism etc).*

As we saw from the review that are too many factors to manage and most of the time the data for different countries cannot be compared. Therefore, the future is the One Health approach. Countries must work together as partners and create a global network that can be used as a big management tool in building the strengths of the already existing programs and to broaden the beneficial effects of the critical capacity-building efforts, to promote epidemiological research in order to share methodology and scientific experience worldwide and to encourage all the stakeholders to work together (government – legislators – farmers – industry – mass media- people).

Another lesson learned from crises is that one size doesn't fit all and the profile risk of each farm, each country must be seriously taken in consideration by each farmer, competent authority, government etc respectively each participant to the poultry production / trade system. The poultry production in place plays a critical role in the maintenance and spread of H5N1 HPAI [14] , backyard poultry being part of the cycle of infection, especially when the sale and movement of backyard poultry is done through long and complex market chains in conditions of poor bio security, with unreported outbreaks.

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