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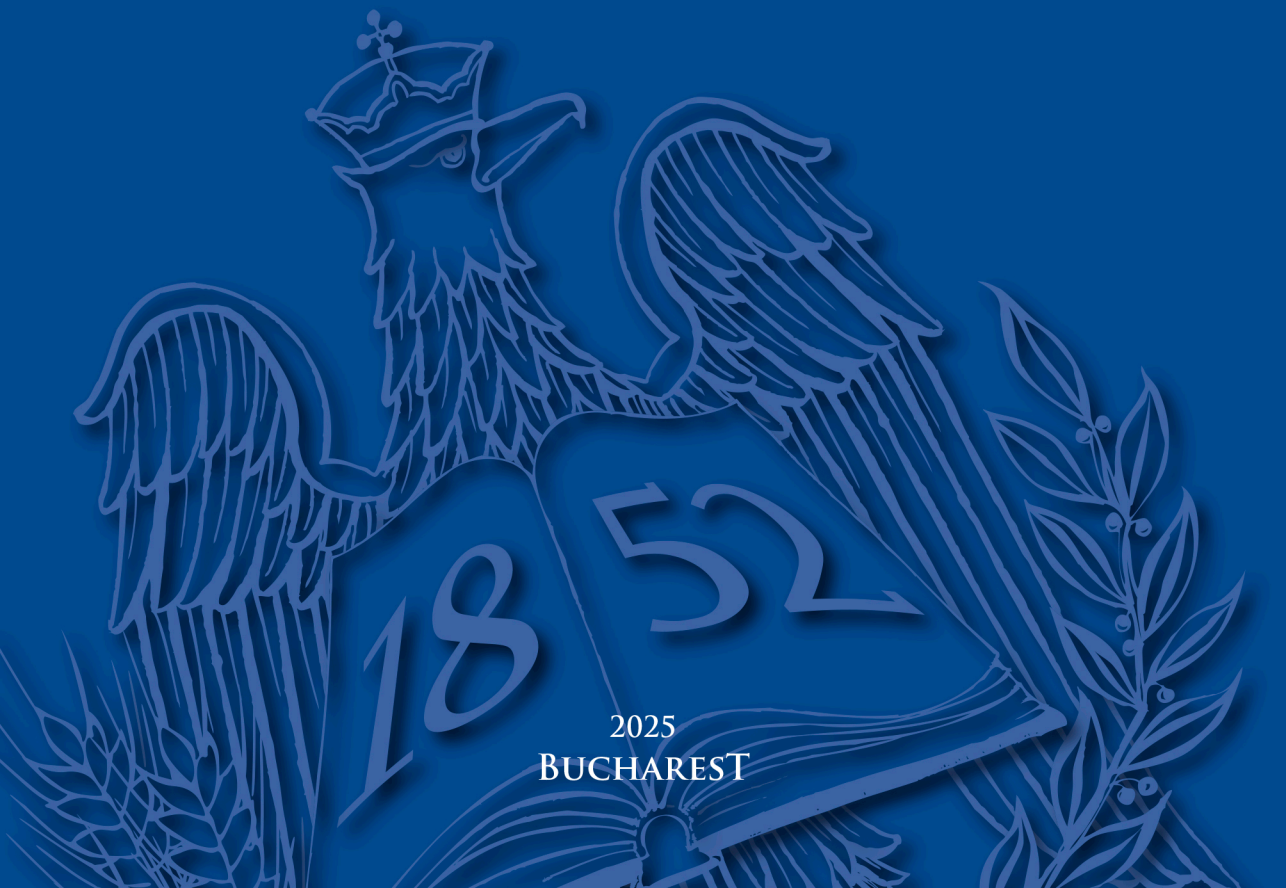

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SUMMARY

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THE COMPLEXITY OF GILL RAKERS EPITHELIA IN *GOBIO CARPATHICUS* VLADYKOV, 1925 – A MORPHOLOGICAL AND HISTOCHEMICAL DESCRIPTION

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Abstract

Gill rakers represent the inner surface of the gill arch, composed of rows of stiff strainers that serve to sort and position large food particles in the oesophagus. The histological structure reveals a central axe composed of cartilage, covered by an epithelium. However, due to their complex role combined with their anatomical disposition, the gill rakers epithelia displays a high histological complexity. Accordingly, the aim of the present study was to provide a throughout morphological and histochemical description of the gill rakers epithelia harvested from common omnivorous fish. Paired gills were harvested from Carpathian gudgeon fish Gobio carpathicus Vladykov, 1925 and fixed in 10% buffered formalin. The samples were processed with the paraffin embedding technique and subsequently stained with Goldner's trichrome method, PAS, Alcian Blue and combined PAS-AA techniques. The obtained results show that the gill rakers are covered by a stratified epithelium composed of several layers of cells, including basal cells and mucus-producing cells, together with taste buds. The histochemical assessment revealed mucous cells able to produce complex mucins, that are both PAS and AA- positive. However, even if the gill rakers are part of the gills, the covering epithelium reveals some features similar to the described structure of the anterior pharynx, but also the gill rakers epithelia display common features with the epidermal structure.

Key words*: fish epithelia, gills, histochemical reaction, mucus-producing cells.*

INTRODUCTION

Gobio carpaticus Vladykov, 1925, popularly called Carpathian gudgeon, from the Cypriniformes order, Gobionidae family is a common omnivorous fish found in the Danube drainage and in the Tisza catchment (Bănărescu, 1964). According to more recent molecular data, it seems that the specimens found in Mures and Tisza rivers may represent a distinct lineage (Kottelat and Freyhof, 2007), that may be at risk because of invasive species like the racer goby (*Babka gymnotrachelus* (Kessler, 1857)) or monkey goby (*Neogobius fluviatilis* (Pallas, 1814)) (Muntean et al., 2022).

The feeding mechanism of this common omnivorous fish implies several structures, the gills being one of the first that get in contact with the ingested food (Genten et al., 2009; Mokhtar, 2021). Gill rakers represent the inner surface of the gill arch, composed of rows of stiff strainers that serve to sort and position large food particles in the oesophagus and then in the stomach and intestines, being a good indicator for the fish diet (Genten et al., 2009).

The histological findings describe the gill raker as a dense core (cartilage or bone) which is covered by connective tissue and a stratified epithelium, similar to the one from the pharynx (Genten et al., 2009; Mokhtar, 2021).

This stratified epithelium found in the teleost gill raker is very different from the pharyngeal epithelium of mammals, which makes it almost unique and histologically diverse. It presents common features with the teleost skin epithelia due to their common capacity to secrete mucus (Genten et al., 2009). Its feature is extremely important, since it is involved in several functions like immune defence, lubrication of the ingested particles or the pre-gastric chemical digestion of foods (Genten et al., 2009; Tibbetts, 1997). Because the aquatic environment is rich in pathogenic organisms and substances, the capacity of the mucous cells to produce a secretion rich in lysozymes, antibacterial peptides, IgM, lectins or pentraxins is a great

way for a primary biodefence (Jung et al., 2002; Genten et al., 2009).
Accordingly, the histological complexity of the secretory epithelium from the rakers, corroborated with the rich function of the mucus shaped the aim of the present study that provide a thoroughly histological and histochemical description of the gill raker epithelia in Carpathian gudgeon.

MATERIALS AND METHODS

Paired gills were harvested from the Carpathian gudgeons (*Gobio carpathicus* Vladykov, 1925) caught in natural waters (Mureș reservoir, Romania), during recreational fishing. The samples were fixed with 10% buffered formalin for 5 days and subsequently demineralised with trichloroacetic acid 5% for 7 days. Following, the gills were processed with the usual paraffin embedding technique and sectioned at 5 µm thickness. The mounted sections were stained with Goldner's trichrome staining method (to underline the histological morphology of the gill rakers) and with PAS, Alcian Blue (pH 2.5) and combined PAS-AA techniques (to highlight the mucin-producing cells) (Suvarna et al., 2019). The microscopical assessment was achieved with an Olympus BX41 microscope equipped with a Olympus SC 180 digital camera (Olympus, Japan).

RESULTS AND DISCUSSIONS

The gill arch inner surface of the Carpathian gudgeon presented several comb-like structures, relatively short and thick, termed gill rakers (Figure 1). From a histological perspective, each raker presents a dense and hard core formed by hyaline cartilage, covered by a stratified epithelium and abundant connective tissue in between (*lamina propria*). However, the microscopic morphology of the epithelium displays differences at the level of the rakers when compared with the inter-raker region of the gill arch (Figure 1 A), the latter presenting a slightly lower density of mucus-producing cells and a slightly higher size in thickness. Despite the region addressed, the covering epithelium at the level of the gill arch was sustained by a lamina propria composed of both fibroblasts and fibrocytes, together with connective fibres. Therefore, the common aspect of the gill raker epithelium frames it in the buccopharyngeal epithelium type for the teleost fish (Walker et al., 1981; Tibbetts, 1997). Accordingly, several layers of cells, displayed on a very thick, mild wavy basal membrane can be observed. Moreover, taste buds are identified as intra-epithelial sensorial structures, with an onion-like aspect, usually on the apical part of the raker (Figure 1B).
From a histochemical perspective, both PAS reaction and Alcian blue staining highlighted mucus secreting cells. Few of them seem to be present in the intermediate part of the multi-layered epithelium but the cells appear to be displayed predominantly on the last epithelial layer, releasing their secretion product at the surface, forming a mucous coat at the apical part of the epithelium. However, on both histochemical stainings can be observed that the mucous coat is discontinuous, being absent (or very thin) at the level of the intra-epithelial taste-buds (Figure 1 C, D).
The histological architectonics of the epithelium in the case of Carpathian gudgeons was similar with the previously reported findings in fathead minnow (*Pimephales promelas*)(Walker et al., 1981) and other teleosts fish (Tibbetts, 1997; Vigliano et al., 2006; Bassuoni et al., 2021; Grau et al., 1992).
The high-magnification light microscopy revealed a few differences in terms of cytoplasmic details of the mucous cells. Accordingly, on all three histochemical stainings it was observed that at the level of the raker epithelium, the cells display a foamy aspect of the cytoplasm, due to the vacuoles with a small diameter (Figure 2 A, D, G). Nevertheless, in the inter-raker region, the mucous cells tend to be more spherical containing a cytoplasm with a vacuolated aspect (Figure 2 C, F, I). When it comes to mucous cells in teleost fish epithelia, an applied classification was made available. Therefore, the cells in the pharyngeal mucosa, together with the ones from the oral, oesophageal and rectal mucosae, are considered to be type-A saccular cells with rounded vacuoles and flattened nuclei disposed at the basal pole. The type-B cells represent the more known Goblet cells that are representative of the intestinal tract mucosa. They may display a PAS positive reaction, Alcian-blue positive

stain or both (Tibbetts, 1997). In the pharyngeal region, they are present in a stratified epithelium that displays a slightly similar morphology (to a certain extent) with the transitional stratified epithelium, being placed themselves in several layers (Tibbetts, 1997). The same pattern was observed also in the gill arch epithelia of the present study, where the mucus-producing cells were arranged in both apical and intermediate thirds of the epithelium.

In terms of tinctorial affinity, the mucous cells keep the same pattern in the two described regions. On the other hand, the mucus granules display a higher PAS positive reaction compared with the Alcian blue stain, suggesting that the biochemical componence may be predominantly composed of neutral glycoproteins, instead of acidic mucins (Tibbetts, 1997). It also should be mentioned that the decalcification step does not affect significantly the tinctorial affinity for those histochemical reactions (Gona, 1979). However, the positivity obtained using the combined staining method suggests a mixture of mucins in the cytoplasm (Figure 2 G, H, I).

It is known that the gill rakers may act like a sieve (Genten et al., 2009; Mokhtar, 2021). Considering this feature, they are in constant contact with water and particles that are coming from the environment, so the high amount of mucus here may have a multipurpose function. On the first hand, the mucus coat may act as a lubricant for food particles, facilitating an easy movement towards the pharyngeal cavity, but also in the pregastric digestion (Bassuoni et al., 2021; Tibbetts, 1997).

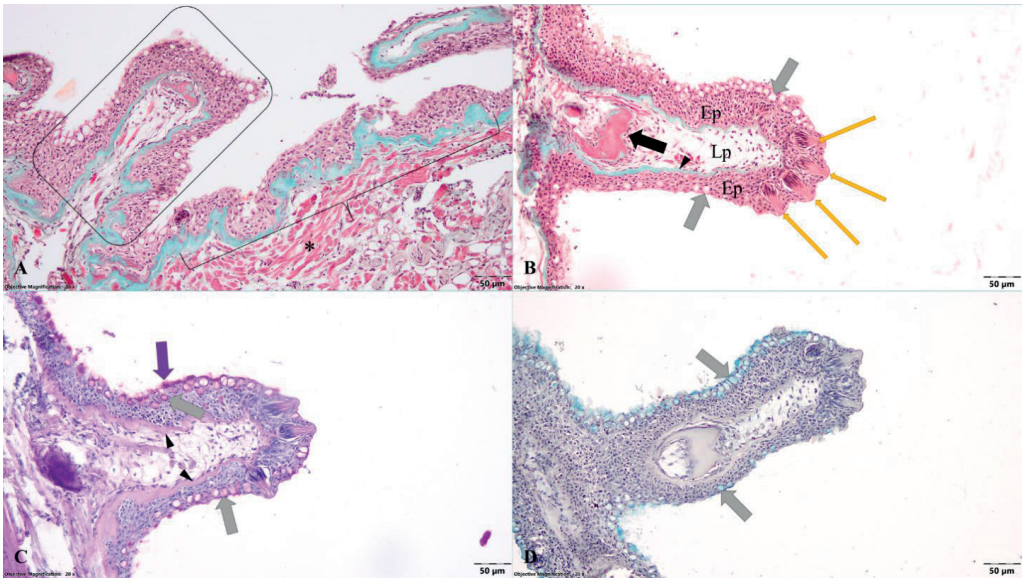

Figure 1. Gill arch of Carpathian gudgeon fish *Gobio carpathicus* Vladykov, 1925; A - Pharyngeal region of the gill arch presenting comb-like projections termed gill rakers (black square) and the inter-raker region (accolade), together with the underneath muscle tissue (*) - Goldner's trichrome stain; B - One gill raker formed by a mineralized tissue that serves as sustaining core (black arrow), covered by a stratified epithelium (Ep) with mucus-producing cells (grey arrows), intraepithelial sensorial structures - taste buds (long yellow arrows), a thick basement membrane (arrowhead) and lamina propria (Lp) - Goldner's trichrome stain; C - PAS positive mucus-secreting cells (grey arrows) in gill raker epithelium which release their secretion at the surface, forming a discontinuous mucous covering layer (purple arrow), PAS positive basement membrane with mild waves (arrowheads) - Periodic acid-Schiff reaction; D - mucous cells with a faintly positive reaction for Alcian Blue stain (grey arrows)

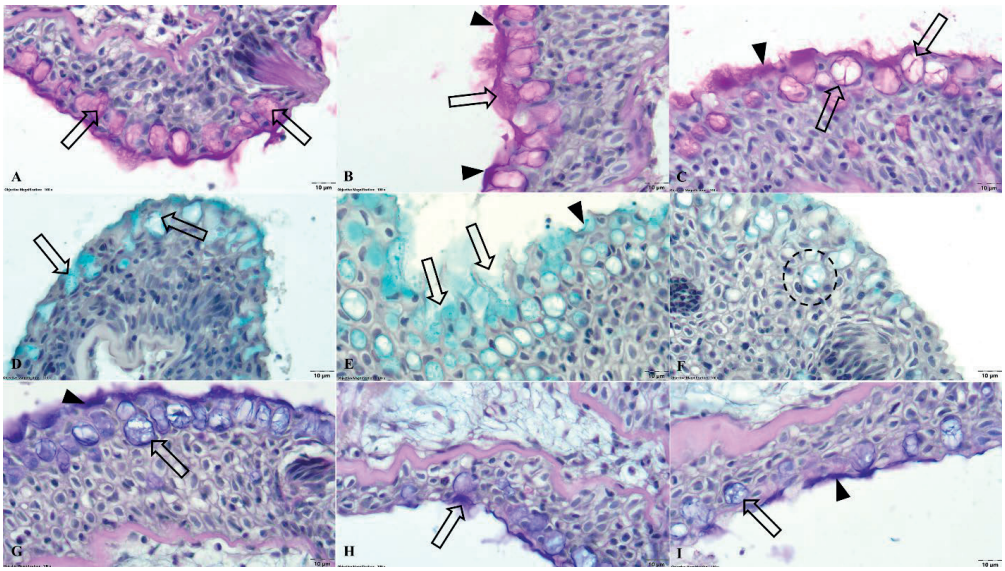

Figure 2 Histochemical details of mucus-producing cells from the gill arch of Carpathian gudgeon fish *Gobio carpathicus* Vladykov, 1925 (A, B, C - PAS stain; D, E, F - Alcian blue stain; G, H, I - PAS-Alcian combined stain; 100x magnification degree); A - Apical tip of the gill raker epithelium, displaying a high density of cells with saccular aspect, basal flattened picnotic nuclei and intracytoplasmic small and abundant vacuoles that give a foamy aspect; the vacuoles contain PAS positive mucus granules (empty arrows); B - PAS positive mucus coat at the surface of the gill raker epithelium (arrowheads) released through the apical pole of the cells (empty arrow); C - gill arch epithelium in the inter-rakers region displaying a slightly different feature of cells shape (more spherical - empty arrows) and composition (intracytoplamatic vacuoles with a bigger diameter that gives the cytoplasm a vacuolated aspect, with PAS positive mucous granules - arrowhead); D - mucus-producing cells faintly positive for Alcian blue stain, from the gill raker epithelium apical region, that present a granular cytoplasm (empty arrows); E - weakly positive mucus coat for Alcian blue stain highlighted at the surface of the gill raker epithelium (arrowhead) and the apparent releasing mechanism from the producing cells (empty arrows); F - Mucus secreting cells from the inter-raker region of the epithelium, presenting a basal picnotic and flattened nucleus and vacuolated cytoplasm faintly positive for Alcian blue stain (dashed circle); G, I - mucus producing cells positive for the double PAS and Alcian blue staining, maintaining the previously described morphology (G - raker epithelium; I - inter-raker region epithelium) (empty arrows) and the mucus coat at the surface of the epithelium (arrowheads); H - a mucous cell presumably intercepted during the mucus release phenomenon (empty arrow)

On the other hand, a thicker or less thicker mucus layer at the surface epithelium may protect it against mechanical abrasions (Bassuoni et al., 2021) and also acts like a chemical barrier, participating in innate or acquired immunity (Alexander et al., 1992; Jung et al., 2002).

Another interesting finding is related with the secretion mechanism of the mucus. In general, mucous cells are secreting their product through the merocrine secretion (Song et al., 2023; Specian et al., 1980). Exceptionally, the mucus can be released by the apocrine pathway, but only in some specialised glands, while the holocrine secretion is not common for mucus-secreting cells (Lodish et al., 2000; Kierszenbaum et al., 2015; Alberts et al., 2015). The latter mentioned mechanism implies that the whole cell undergoes apoptosis, releasing its entire content, this forming the secretion product (Kierszenbaum et al., 2015). In Carpathian gudgeon (but not limited to it), the unicellular glands disposed in the last layer of the epithelium seem to release their mucous product by a slightly different pattern than the common ones. If analysed thoroughly, it can be observed in several cells that the bulging apical pole of mucous cells presents a shattered appearance and the mucus release seems to be the consequence of an “explosion” (outburst) at the cellular surface (Figure 2 B, E). Moreover, those cells display also either karyopyknosis or karyolysis, suggesting features that are specific for the apoptotic cells (Häcker, 2000). However,

further research is needed in order to provide a thorough explanation about the detailed secretion mechanism.

CONCLUSIONS

The gill arch, including the rakers, displays a complex morphologic diversity. In *Gobio carpathicus*, a common omnivorous fish, a stratified epithelium with several secretory cells covers the whole gill arch, with a slightly different morphology in between the rakers and inter-rakers regions. The mucus secretion was released through the apical pole of the type A saccular cells, which are intensely PAS positive and faintly Alcian Blue positive. Thus, taking into consideration all the histochemical and morphological aspects described, the epithelium from the gill rakers is a stratified, mucus secreting structure, with an important involvement in several processes, vital for the fish.

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POLYMICROBIAL AETIOLOGY OF SEVERE RESPIRATORY DISTRESS IN SWINE: A CASE STUDY FROM WESTERN ROMANIA

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Abstract

The porcine respiratory disease complex represents a common polymicrobial condition that significantly impacts the worldwide swine industry. This study focused on identifying respiratory pathogens on a swine farm in Western Romania, where animals exhibited severe respiratory distress. A post-mortem examination was conducted on-site, followed by tissue sample and swab collection. Actinobacillus pleuropneumoniae, Pasteurella multocida, and Streptococcus suis were isolated. Real-time PCR analysis was conducted on five pooled samples, detecting Actinobacillus pleuropneumoniae in all of them. Alphainfluenzavirus influenzae (Swine influenza virus) was identified in two pools, while Circovirus porcine3 (Porcine circovirus 3) was present in one. All tested samples were negative for Betaarterivirus europensis (Porcine reproductive and respiratory syndrome virus), Mycoplasma hyopneumoniae, and Circovirus porcine2 (Porcine circovirus 2). Histopathology revealed necrotising haemorrhagic pneumonia or fibrinous suppurative bronchopneumonia. This study provides evidence of multiple pathogens in swine exhibiting severe respiratory distress, with Actinobacillus pleuropneumoniae as a predominant pathogen. The findings highlight the complex nature of porcine respiratory disease and underscore the need for targeted interventions to enhance swine health and production.

Key words*: swine, respiratory disease, Actinobacillus pleuropneumoniae.*

INTRODUCTION

Respiratory disease is one of the swine industry's biggest problems because it is linked to major production losses. The aetiology is typically polymicrobial and caused by the interplay of bacteria and viruses (Fablet et al., 2012; Opriessnig et al., 2011). The pathogens causing the porcine respiratory disease complex (PRDC) can create serious health issues when triggered by environmental factors or management deficiencies, such as overcrowding, mixing different sources of animals, temperature, continuous flow, ventilation, and sanitation (Assavacheep & Thanawongnuwech, 2022; Brogden & Guthmiller, 2002).

PRDC is usually characterised by anorexia, growth retardation, dyspnoea, cough, and fever (Thacker, 2001). The rate of morbidity may range from 30% to 70%, and mortality is considered between 4% and 6% (Opriessnig et al., 2011). Lesions are often encountered in the cranioventral area of the lung, where it fails to collapse; discoloration and consolidation may be found. Histopathological findings usually include bronchopneumonia along with interstitial pneumonia (Hansen et al., 2010). Depending on how the pathogens involved in coinfections or superinfections interact, the dynamic polymicrobial infections that are found in PRDC may result in varying clinical outcomes (Assavacheep & Thanawongnuwech, 2022). Implementing diagnostic and preventive strategies in impacted farms is challenging due to PRDC's complexity (Eddicks et al., 2021). It is often unclear who is the main pathogen and which one acts as a facilitating factor for secondary agents or further infections (Haimi-Hakala et al., 2017).

Both primary and opportunistic invasions can be caused by bacterial pathogens. Bacteria like *Bordetella bronchiseptica, Mycoplasma hyopneumoniae*, and *Actinobacillus pleuropneumoniae* are considered primary bacterial agents. The most common opportunistic agent is *Pasteurella multocida*, and the infection with other common

opportunistic agents like *Glaesserella parasuis*, *Trueperella pyogenes*, *Streptococcus suis, Actinobacillus suis*, and *Salmonella choleraesuis* may potentially result in respiratory conditions (Brogden & Guthmiller, 2002; Saade et al., 2020). The most common viral agents involved in the PRDC are *Betaarterivirus europensis* (Porcine reproductive and respiratory syndrome virus, PRRSV), *Alphainfluenzavirus influenzae* (Swine influenza virus, SIV), *Varicellovirus suidalpha1* (Aujeszky's disease virus, ADV), and *Circovirus porcine2* (Porcine circovirus 2, PCV2) (Assavacheep & Thanawongnuwech, 2022; Zimmerman et al., 2019). The range of infectious agents can intensify and prolong symptoms by interacting with one another in a complicated and perhaps synergistic way (Saade et al., 2020).

The purpose of this study was the investigation of an acute outbreak of respiratory disease and the identification of the pathogens causing it, in a Romanian swine farm.

MATERIALS AND METHODS

Sampling

A large-scale growing pig farm with an intensive system from Western Romania was the site of the study. The disease had a sudden onset, with animals showing signs of severe respiratory disease, dyspnoea, cough, severe apathy, anorexia, high fever, and sudden death as well as nervous signs. On-site post-mortem examination was performed, and representative tissue samples (lymph nodes, lung, and brain fragments) and swabs were collected and sent to the laboratory. Laboratory diagnosis was performed in Synevovet, Romania.

Molecular biological examination

Lung tissues and tracheobronchial lymph nodes were tested in five pools, each pool corresponding to a shed, for PRRSV, PCV2, PCV3, *A. pleuropneumoniae*, *M. hyopneumoniae*, and SIV by real-time PCR. Nucleic acid extraction was performed with the BioExtract Column kit (BioSellal, France). The following qPCR kits were used for pathogen detection: Bio-T kit PRRSV, with the differentiation of European and North American genotypes; Bio-T kit PCV2& PCV3 (BioSellal, France); EXOone *Actinobacillus pleuropneumoniae*; EXOone *Mycoplasma hyopneumoniae*; and EXOone Influenza A virus (Exopol, Spain), in compliance with the manufacturer's guidelines. The nucleic acids were amplified using the AriaMx instrument for real-time PCR (Agilent, United States).

Bacteriological examination

25 swabs were collected from affected tissues: lung, pericardial sac, lymph nodes, and brain, and they were cultivated on blood agar and chocolate agar growth mediums. The incubation was done in anaerobic conditions (thermostat with 5% CO_2) at 35-37°C for 20- 24 h. Then the colonies were selected based on their morphological characteristics, and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) technology was used to identify them.

Histopathology

During necropsy, lung samples were collected and then underwent a standard procedure involving 10% formalin fixation, paraffin embedding, sectioning, and H&E staining. Imaging and histological examination were performed using an Olympus CX23 microscope with an SC50 camera (Olympus, Tokyo, Japan).

RESULTS AND DISCUSSIONS

Reported gross findings include fibrinosuppurative, haemorrhagic, and necrotising pneumonia; interlobular oedema; and pleural adhesions (Figure 1, panels A to D). Laboratory diagnosis revealed the presence of several pathogens, viruses, and bacteria. By molecular biological examination, all the tested samples were positive for *A. pleuropneumoniae*, SIV was detected in two pools, and PCV3 was found in one pool. PRRSV-1, PRRSV-2, PCV2, and *M. hyopneumoniae* were not detected. From 16 isolates, five types of bacterial agents were identified by microbiological examination. *A. pleuropneumoniae*, *Escherichia coli* (*E. coli*), *Pasteurella multocida* (*P. multocida*), and *Streptococcus suis* (*S. suis*) were isolated from lung tissues; *Staphylococcus aureus* (*S. aureus*) and *E. coli* were present in the pericardium sac, and *E. coli* was also isolated from the brain tissue.

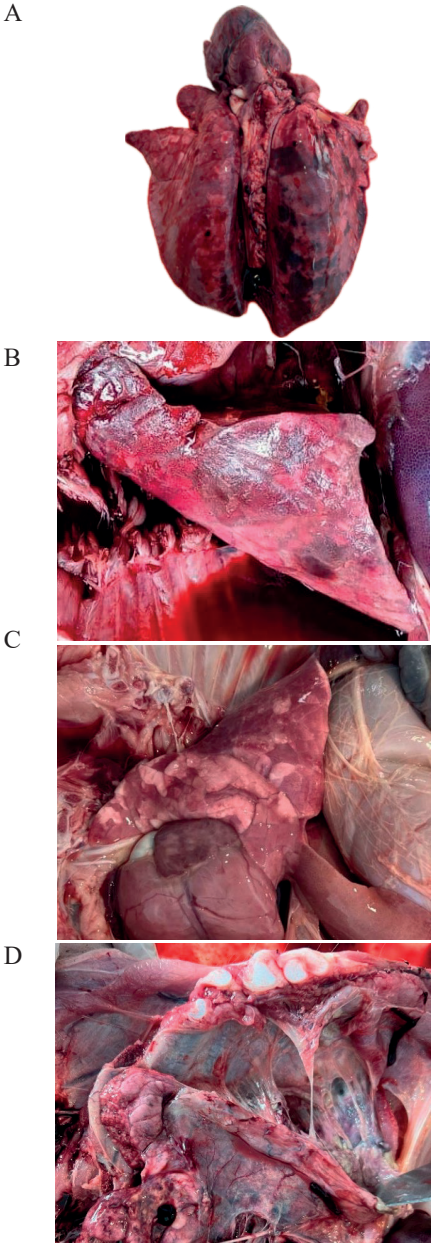

Figure 1. Gross pathological findings: (A) Deep red coagulative necrosis areas, sharply demarcated of fibrino-haemorrhagic and necrotising pneumonia; (B) Fibrinous exudate covering the pleural surface; (C) Cranioventral areas of consolidation; (D) Pleural adhesions

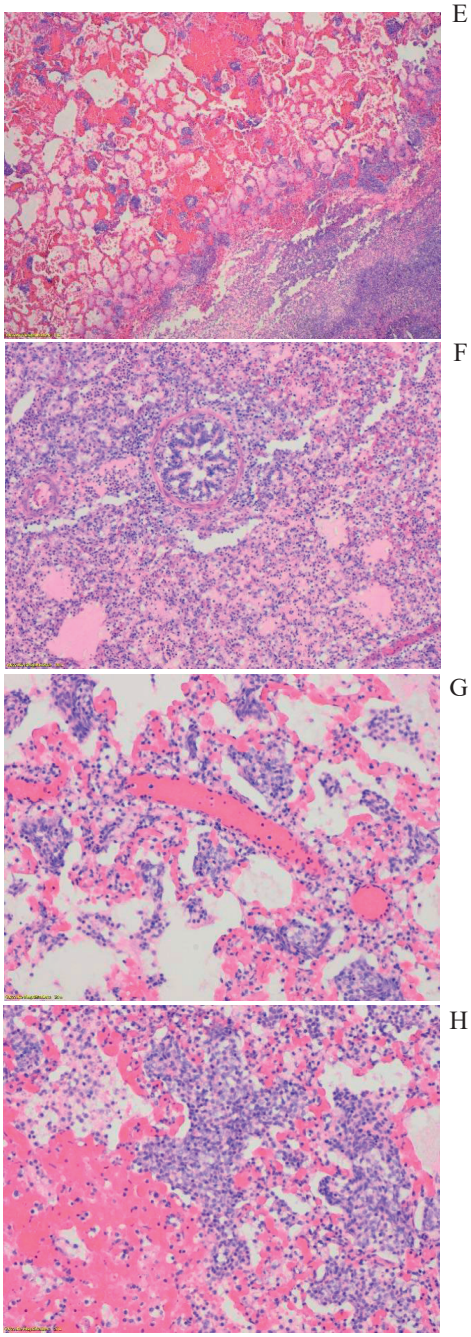

Figure 2. Microscopic appearance of lesions: (E) Lung, fibrinosuppurative bronchopneumonia and necrotising haemorrhagic pneumonia, H&E stain, 40x; (F) Lung, bronchiole infiltrated by inflammatory cells and filled with fibrinosuppurative exudate, H&E stain, 100x; (G) Lung, intra-luminal thrombosis of vessels (vasculitis), H&E stain, 200x; (H) Many degenerate neutrophils and macrophages admixed with abundant fibrin and necrotic cellular debris are filling and replacing alveoli and expanding interlobular septae. Alveoli contain oat cells, H&E stain, 200x

Histopathology revealed necrotizing haemorrhagic pneumonia fibrinous suppurative bronchopneumonia, with oat cell formation, fibrinonecrotizing vasculitis, and pleuritis (Figure 2, panels E to H). Table 1 summarises the pathogens detected in each shed, corresponding to qPCR detection and bacteriological examination.

Table 1. qPCR and bacteriological examination results corresponding to each shed

qPCR	*1*	*2*	*. 3*	*4*	*5*
PRRSV-1	Negative	Negative	Negative	Negative	Negative
PRRSV-2	Negative	Negative	Negative	Negative	Negative
PCV2	Negative	Negative	Negative	Negative	Negative
PCV3	Negative	Negative	Negative	**Positive (ct: 29)**	Negative
APP*	**Positive (ct: 31)**	**Positive (ct: 34)**	**Positive (ct: 24)**	**Positive (ct: 18)**	**Positive (ct: 29)**
*M.HYO**	Negative	Negative	Negative	Negative	Negative
SIV*	**Positive (ct: 39)**	Negative	Negative	**Positive (ct: 37)**	Negative
Bacteriological examination	*1*	*2*	*. 3*	*4*	*5*
APP*	Negative	Negative	**Positive**	**Positive**	**Positive**
P. multocida	Negative	**Positive**	**Positive**	**Positive**	Negative
S. suis	**Positive**	**Positive**	**Positive**	Negative	Negative
E. coli	**Positive**	**Positive**	Negative	Negative	**Positive**
S. aureus	Negative	Negative	Negative	**Positive**	Negative

**APP - Actinobacillus pleuropneumoniae; M.HYO - Mycoplasma hyopneumoniae; SIV - Alphainfluenzavirus influenzae (Swine Influenza Virus)*

The purpose of this investigation was to identify the pathogens causing an ongoing respiratory outbreak. One of the most important health issues that growing pigs face is respiratory infection (Hansen et al., 2010). Although most swine respiratory disease cases end in death due to bacterial pneumonia, the PRDC has a multifactorial causation, meaning that other infections and the interplay of pathogens, host background, and environmental variables play a significant role in its development. For effective control of the disease, all factors should be identified, including the underlying infectious cause, not only the actual cause of the deaths (Grant Maxie, 2016).

In the present study, many pathogens were present in the affected farm: two viruses, SIV and PCV3, and five bacteria, *A. pleuropneumoniae*, *S. suis*, *P. multocida*, *E. coli,* and *S. aureus*. In PRDC cases, four or more infectious agents are frequently found, single-pathogen positive rates being lower than those of multiple-pathogen combinations, leading to intricate and perhaps synergistic interactions that might worsen respiratory illness and lesions' severity and duration (Cheong et al., 2017; Zimmerman et al., 2019). In a retrospective study of the etiologic agents linked to respiratory illnesses in pigs in the United States, 88.2% of the cases examined included two or more infectious agents (Choi et al., 2003).

A. pleuropneumoniae was identified in all the sheds by qPCR, with cycle threshold (Ct) values between 18 and 34. In the shed with the highest *A. pleuropneumoniae* load, the bacteria were associated with SIV and PCV3; *S. aureus* was isolated from the same shed. It is considered that *A. pleuropneumoniae* can be a primary as well as a secondary pathogen (Brauer et al., 2012). Numerous factors can influence the *A. pleuropneumoniae* outbreaks, as well as the outcome of infection. The disease is greatly impacted by strain virulence and the presence of additional pathogens. For both acute and chronic (pleuritic) infections, a specific effect of the coinfection has been proposed (Fablet et al., 2012; Zimmerman et al., 2019).

In studies of coinfection between SIV and *A. pleuropneumoniae*, a considerably higher local cytokine response was reported (Czyżewska-Dors et al., 2017), and it has been demonstrated that the virus shedding and replication were higher in the coinfected pigs, with more severe symptomatology and lesions than the groups infected with one pathogen (Pomorska-Mól et al., 2017). An *in vitro* study of the coinfection between *A. pleuropneumoniae* and *S. suis* reported synergistic interactions between both pathogens and increased antibiotic resistance. When it was cultured along with *S. suis*, *A. pleuropneumoniae* developed without nicotinamide adenine dinucleotide and formed strong biofilms (Wang et al., 2020).

SIV was detected in two pools, with high Ct values (37 and 39). The virus is cleared in the lung tissue as fast as 72 hours postinfection (Grant Maxie, 2016), which may explain its inconsistent rates of detection. SIV is a key contributor to the PRDC, with an endemic course or rapidly spreading outbreaks of severe nonfatal disease (Grant Maxie, 2016). Other bacteria isolated in SIV-positive sheds included *S. suis*, *P. multocida*, *E. coli,* and *S. aureus*. Secondary bacterial infection is predisposed by the altered host response following SIV

infection due to complex mechanisms that would greatly raise the mortality rate (Lin et al., 2015). Coinfections between *S. suis* and the influenza virus are frequently encountered in clinical outbreaks (Lin et al., 2015). SIV infection can help *S. suis* colonise epithelial cells, being used as a vehicle, as bacteria adherence and cellular invasion were proven to improve when both infections evolve at the same time. SIV infection can also facilitate the entry of *S. suis* into the bloodstream through the respiratory tract. Additionally, during influenza infection, *S. suis* can enhance local inflammatory response in the respiratory system (Meng et al., 2015; Saade et al., 2020; Wang et al., 2013). In an *in vitro* study of the coinfection between SIV and *S. aureus*, the results showed a drastic enhancement in pathogeny, fatal disease, and extended lung lesions due to the virus haemagglutinin activation by the *S. aureus* protease (Tashiro et al., 1987). A diagnostic data study involving 2,872 pigs with respiratory disease identified SIV as the second most frequently detected pathogen, with 636 positive cases, of which SIV only was found in 89 (3.1%) samples, while the highest rate of coinfection was recorded with *P. multocida* in 148 (5.2%) (Choi et al., 2003).

PCV3 was identified in one pool, with a Ct value of 29. *A. pleuropneumoniae*, *P. multocida*, *S. suis,* and *S. aureus* were isolated from the same shed. PCV3 is present all over the world, in healthy and diseased herds (Opriessnig et al., 2020). A notable increase in PCV3 titres was observed in pigs with clinical disease development, compared to those without symptoms (Kedkovid et al., 2018). The connection between PCV3 infection and the occurrence of respiratory illness and lung damage has been documented in several studies (Kedkovid et al., 2018; Palinski et al., 2017; Phan et al., 2016; Savic et al., 2020; Shen et al., 2018; Zhai et al., 2017). Research, including those by Savic et al. (2020), Kedkovid et al. (2018), and Phan et al. (2016), has indicated that PCV3 co-infections with *A. pleuropneumoniae*, *P. multocida*, and *S. suis* are observed. PCV3 contributes to the disease development and the amplification of symptoms (Savic et al., 2020). Circoviruses interfere with the production of interferon and pro-inflammatory cytokines, thus affecting the immune response. Processes such as apoptosis, alteration of cell transport, and mitotic phase arrest also contribute to viral replication. Cytokine imbalance and lymphocyte depletion lead to weakened immunity, favouring the invasion of secondary or concurrent infections. This combination intensifies the severity of diseases associated with circoviruses (Fehér et al., 2023).

Pneumonia and septicaemia in pigs can be initiated by highly pathogenic *P. multocida* strains. *P. multocida* is regarded as a major contributor to respiratory issues and is considered the most frequently recovered bacteria in pigs suffering from pneumonia (Piva et al., 2023). *S. suis* coinfections are prevalent and can raise mortality rates by causing severe pneumonia (Lin et al., 2015). In general, animals with multiple bacterial infections, either co-occurring or following a primary infection, exhibit worse clinical symptoms, increased lung lesions, and decreased performance than those with single bacterial infections, alongside alterations in immune system responses (Saade et al., 2020). It is believed that bacterial development is favoured by viral infection of the respiratory system (Tashiro et al., 1987).

CONCLUSIONS

This study confirms the presence of multiple respiratory pathogens in swine exhibiting severe respiratory distress, with *A. pleuropneumoniae* as a predominant pathogen, SIV and its interactions with opportunistic bacteria, and PCV3, which might contribute to the severity of respiratory problems. Further research on lung diseases, as well as the impact of the interactions between the pathogens linked to these conditions on the severity of disease development, would lead to a better implementation of effective control measures. The findings highlight the complex nature of porcine respiratory disease and underscore the need for targeted interventions to enhance swine health and production.

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THE RELATIONSHIP BETWEEN MATING BEHAVIOUR, INCIDENCE OF TRAUMATIC INJURIES, AND PRODUCTIVITY IN A REPRODUCTIVE POULTRY POPULATION

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Abstract

This study was conducted in a poultry breeding farm, aiming to analyse the mating behaviour of birds from a population consisting of parental lines used for slow-growing coloured broiler production. The research focused on direct observations made in the growing hall, assessing the causal relationship between reproductive activity and the incidence of traumatic injuries due to sexual aggression, as well as their impact on final productivity. Following the analysis of reproductive behaviour, based on the examination of 1000 mating attempts, a detailed ethogram was developed. Data collected included details on courtship behaviour, the hen's response, and the outcome of each attempt. A total of 166 cases of aggressive trauma in hens were identified, with descriptions of each injury location and size. Productivity was evaluated based on the hatching rate obtained. The results revealed a significant correlation between mating behaviour, traumatic injury incidence, and productivity. The intensity of aggression peaked early in the laying period, within the first 5 weeks, when the population was still young and inexperienced.

Key words*: mating aggression, poultry behaviour, slow-growing coloured broiler, traumatic injuries.*

INTRODUCTION

Aggressive behaviour in chickens is not merely an expression of social hierarchy but also a response to factors such as stress and diet, serving as a key indicator of their welfare (Ndagimba et al., 2024). Physiological stress, reflected in elevated corticosterone levels and alterations in gut microbiota, has been associated with increased aggression, indicating that biological factors significantly influence social interactions in chickens (Gao et al., 2025). However, aggression levels vary between breeds, with some genetic lines displaying stronger dominance and conflict-driven behaviours, while others exhibit greater social tolerance (El-Shoukary et al., 2025).

In natural conditions, aggressive behaviour of roosters towards hens is uncommon, as the two genders maintain distinct social hierarchies, with male dominance typically exerted through passive means (Wood-Gush, 1958; Rushen, 1983; Kjaer & Mench, 2003). However, aggression of breeding roosters towards hens has been documented in the scientific literature, particularly in the context of industrial poultry farming (Jones & Prescott, 2000; Millman et al., 2000; Millman & Duncan, 2000a; 2000b; 2000c; Kjaer & Mench, 2003; De Jong, 2009). This type of behaviour, along with other deviant manifestations such as aggressive feather pecking, which can escalate to extreme forms of cannibalism, has a significant negative impact on the well-being and health of birds (Mitrănescu & Furnaris, 2012).

Previous studies suggest that behavioural manifestation has a genetic basis, potentially influenced by intensive selection aimed at improving productivity, often at the expense of optimizing social behaviour in poultry (Kjaer & Mench, 2003; Cheng & Jiang, 2020).

The present study provides a novel perspective by investigating this phenomenon in a population belonging to the parental line of slow-growing-coloured broilers. Unlike controlled experimental studies, this research was conducted in an industrial farming facility, allowing for an analysis of natural behavioural patterns in intensive rearing systems. The study establishes correlations between behavioural traits, lesions characteristic of sexual aggression, and productivity, offering an integrated understanding of this phenomenon.

MATERIALS AND METHODS

The present study was conducted on a poultry breeding farm equipped with its own hatchery. The research focused on direct observations carried out in the rearing hall, targeting a flock of breeding hens from parental lines used for producing slow-growing, coloured broilers.

The primary objective of the study was to collect data and observations directly from the rearing hall during observation sessions conducted according to a predefined schedule.

An essential aspect of the study was maintaining the specific conditions of industrial poultry farming technology, ensuring that observations did not interfere with the routine of the birds. No experimental conditions were introduced; the birds being engaged in activities characteristic of any intensive rearing hall.

Based on observations from previous rearing cycles, a hypothesis was formulated that the phenomenon of sexual aggression occurs during the early stages of sexual maturity in the studied birds (Lupu & Militaru, 2022). Consequently, the study period was defined as starting from the laying of the first egg, which marks the onset of sexual maturity (considering the sexual nature of aggression), and ending when no new cases exhibiting specific injuries were identified among the studied breeding hens.

Within this study, three distinct yet interdependent analyses were proposed to investigate the correlations between bird behaviour and final productivity, the latter being reflected in the hatching rate. The proposed studies are as follows:

- Study I: *"Observation, recording, and description of wounds identified in breeding hens as a result of sexual aggression."*
- Study II: *"Monitoring mating activities of roosters and breeding hens."*
- Study III: *"Calculation of the hatching rate corresponding to the period during studies I and II."*

The first two studies were conducted simultaneously, while the third study followed with three-week delay, corresponding to the incubation period of the collected eggs.

Study I: Observation, recording, and description of wounds identified in breeding hens as a result of sexual aggression

Five control visits were conducted daily to monitor and record any new cases of injuries observed in hens. During each visit, the entire rearing hall was thoroughly inspected to ensure the comprehensive identification of every affected bird.

Upon identification, each case was photographically documented, and the size of the wound was measured. Subsequently, the affected hens were marked with a coloured spray containing an antibiotic, providing also localized therapeutic effect. During subsequent visits, previously marked specimens were not recounted, allowing for the exclusive monitoring of newly emerged cases and avoiding duplication of records.

At the end of each day, the number of new cases was recorded in a database, new entry of information being consolidated weekly for each phase of the study. Additionally, wound measurements were recorded separately to facilitate the analysis of correlations between various variables.

To evaluate wound severity in a standardized manner, a scoring system based on wound size was applied. Each hen was assigned a single score corresponding to the largest or most visible lesion observed. The wounds were classified into three size-based categories:

- Score 1: wound smaller than 0.5 cm;
- Score 2: wound between 0.5 cm and 1 cm;
- Score 3: wound larger than 1 cm.

Only one wound per bird was recorded, based on its prominence, to ensure data consistency under field conditions.

Another point of interest was the localization of the wounds, which could be situated on the comb, the back of the head, the earlobes, as well as on the trunk and the lateral aspects of the thighs. The method used to assess wounds on the trunk and the lateral aspects of the thighs differed from the approach applied to identify wounds in the head region. Due to the dense feathering of the wings, which covers the lateral areas of the trunk, it was not feasible to consistently detect hens with trunk wounds during live observations.

As a result, an alternative strategy was implemented: during **manual depopulation**, all hens exhibiting visible trunk wounds were **identified and separated.** While post-mortem carcass examination may be considered a potential method for evaluating such wounds, it does **not allow for accurate quantification of live hens affected**. Therefore, **manual depopulation provided the most reliable opportunity** to record the presence of trunk wounds.

Study II: Monitoring mating activities of roosters and breeding hens

The mating behaviour was studied in the second part of the day, when its intensity is at its peak. It was proposed to observe 200 mating attempts each week of the study, analysing the behaviour of both roosters and hens, as well as the outcome of each mating attempt. A clear distinction is essential between the terms "mating attempt" and "mating". A "mating attempt" includes any behaviour reflecting the male's intention to initiate the act, whereas "mating" refers to the successful completion of the sexual act.

The observation of behaviour was conducted every Wednesday during each week of the study, as this day is approximately in the middle of the week. A behavioural analysis scheme was developed for this activity, which includes three main categories: ***Courtship behaviour***, ***Hen behaviour***, and ***Outcome of the mating attempt***.

Courtship behaviour includes:

"Absent courtship behaviour": the rooster does not display any courtship behaviour.

"Complete courtship behaviour present": the rooster displays the entire sequence of courtship behaviours as described in the scientific literature.

"Incomplete courtship behaviour present": the rooster displays partially or fully one or more elements of courtship. In this study, three basic courtship elements were selected, with the focus being on their expression frequency. These are: waltzing, vocalizations and food calling.

Based on these definitions, a three-level scoring system was applied to categorize the observed courtship behaviours: Score 0 (absent), Score 1 (incomplete), and Score 2 (complete).

The ***Hen behaviour*** category includes:

"Hen adopts the specific position voluntarily": this category refers to the female's behaviour. The hen voluntarily assumes the specific position (squatting) in response to the rooster's courtship behaviour or simply to his proximity (without the rooster displaying courtship behaviour). The hen does not need to be touched by the rooster to adopt this position.

"Hen adopts the specific position forcibly": This category refers to the female's behaviour. The hen adopts the specific position as a result of being forced by the rooster through his direct rush or approach.

"Hen flees": As a result of the rooster's rush towards the hen with the intent to mount, the hen does not adopt the specific position forcibly. Instead, she chooses to flee and move away from the rooster.

The mating behaviour can be concluded through four scenarios:

“Complete mating”: The hen willingly adopts the squatting position, and the rooster successfully achieves full cloacal contact. The presence of courtship behaviour (complete/incomplete) by the rooster is not mandatory. The essential elements of this category are the voluntary posture of the hen and the rooster's ability to establish cloacal contact.

"Forced complete mating": The hen adopts the squatting position forcibly, and the rooster successfully achieves full cloacal contact. The presence of courtship behaviour (complete/incomplete) by the rooster is not mandatory. The essential elements of this category are the forced posture of the hen and the rooster's ability to establish cloacal contact.

"Complete mounting without cloacal contact": In this case, the rooster successfully mounts the hen but does not achieve cloacal contact.

"No mounting occurred": This category includes cases where the hen flees or cases where the hen adopts the specific position (voluntarily/forcibly), but the rooster either deliberately stops attempting to mount or is driven away by a dominant individual.

Study III: Calculation of the hatching rate corresponding to the period during Studies I and II

Simultaneously, weekly hatchability percentages were recorded and compared with expected values provided by the commercial supplier.

Statistical analysis

To evaluate relationships between behavioural variables and experimental outcomes, two non-parametric statistical methods were applied: Spearman's rank correlation coefficient (ρ) and the Chi-square test of independence.

Spearman's correlation was used to examine monotonic associations between:

- The weekly percentages of rooster courtship behaviour and female responses during mating.
- The incidence of traumatic injuries and female behavioural responses.
- Female responses and mating outcomes.
- Female behaviour and hatchability, as well as the relationship between the total percentage of completed matings and hatch rate.

Analyses were performed on weekly percentage data collected over a five-week period. Spearman's method was chosen due to the ordinal nature of the data and the small sample size ($n = 5$). A p-value of < 0.05 was considered statistically significant.

To determine whether rooster courtship behaviour distribution changed significantly over time, a Chi-square test of independence was conducted using weekly observed frequencies for each courtship category.

RESULTS AND DISCUSSIONS

At the time of the first egg laying, the entire flock consisted of 5,078 females and 451 males (approximately 9 males per 100 females), raised together from day-old to depopulation in the same rearing hall. The study was conducted over a period of 5 weeks.

Study I: Observation, recording, and description of wounds identified in breeding hens as a result of sexual aggression

Study Period - Age of the birds - Number of cases with traumatic lesions

Over the course of the five-week study, a total of 166 cases of traumatic injuries specific to sexual aggression were observed and recorded. The first egg was laid when the hens were 21 weeks of age, this being considered as week one of the study. In week 1 of the study, only one case was recorded. In comparison to week 1, in week 2 of the study, when the birds were 22 weeks of age, 46 new cases were identified. There is a clear trend of increasing new cases, with a peak recorded in week 3 of the study, when the birds were 23 weeks of age. Starting from week 4 of the study, the number of cases decreases, reaching no new cases by week 6 (Figure 1).

These cases, in which injuries specific to sexual aggression are observed, occur within the first 5 weeks after the onset of egg laying, when the birds' age ranged between 21 and 25 weeks (Table 1).

Table 1. The number of new cases corresponding to each week of the study

Study week number	Age of the birds (weeks)	New cases-number/(%)
1	21	1 (0.6)
2	22	46 (28)
3	23	71 (43)
4	24	39 (23)
5	25	9 (5.4)
6	26	0 (0)
		TOTAL: 166 (100)

Percentage-wise, approximately half of the cases (43%) were recorded in week 3, when the birds were 23-week-old (Table 1).

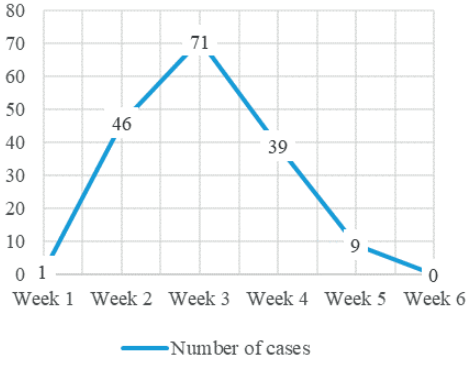

Figure 1. The curve of the number of cases with traumatic lesions recorded in the rearing hall

Injury size

Of the 166 cases identified during the 5 weeks of study, 58 cases involved injuries smaller than 0.5 cm (score 1), 64 cases had injuries ranging from 0.5 to 1 cm in length (score 2), and 44 cases involved injuries larger than 1 cm in length (score 3) (Table 2).

Thus, the majority of injuries throughout the observation period were classified as being between 0.5 and 1 cm in size (score 2). However, the highest value recorded in a single week (week 3) was 28 cases where the injury measured less than 0.5 cm in length (score 1).

Table 2. Number of cases per week according to the size of lesions

Study week no.	Total number of cases	< 0.5 cm	0.5 cm – 1 cm	> 1cm
1	1	1	0	0
2	46	18	17	11
3	71	28	26	17
4	39	8	17	14
5	9	3	4	2
TOTAL	166	**58**	**64**	**44**

Injury localization

In terms of localization, injuries were identified in 3 main areas: comb, head, and earlobes. Cases in which injuries were localized to the comb were recorded in weeks 1 and 2, while cases with injuries localized to the earlobes were only recorded in week 2. Cases in which injuries were localized to the head were recorded in all weeks except week 1. There is no significant difference regarding the location of wounds on comb and earlobes (Table 3);

The highest recorded value in the dataset is **149,** representing the total number of cases with lesions localized on the **head**. This constitutes the most frequent anatomical site observed during the five-week study period.

Table 3. Number of wounds according to localization

Study week no.	Total number of cases	Lesion localization		
		Comb	Head	Earlobes
1	1	1	0	0
2	46	8	30	8
3	71	0	71	0
4	39	0	39	0
5	9	0	9	0
TOTAL	166	9	149	8

Description of lesions

The lesions observed in hens, caused by the sexually aggressive behaviour of roosters, are located in two major regions: the head region and the trunk region. Lesions in the head region can be further classified into three specific zones: the comb, the earlobes, and the head (skin on the back of the head).

Lesions located in the trunk region can be further divided into two narrower areas: the dorsal area of the trunk and the lateral surfaces of the thighs.

A significant limitation of this necessary method is the inability to determine the time frame during which these injuries occurred, as well as the exact onset or cessation of their appearance. However, a description of these lesions can nevertheless be conducted. Lesions located on the trunk and the lateral surfaces of the thighs are characterized, in most cases, by the presence of skin flaps partial avulsion of the skin flaps and dry necrosis, affecting both the cutaneous edges of the wound and the exposed subcutaneous connective tissue and muscle masses.

Typically, there is no significant loss of tissue; however, wound edge approximation is impossible due to skin retraction and the presence of inflammation. In the perilesional area, fibrin deposition is frequently observed, indicating a chronic inflammatory process.

Figure 2. Hen, skin partial avulsion, dehydration and necrosis of the skin flap and subcutaneous connective tissue. Litter debris and clotted blood is noticed

Figure 3. Partial skin avulsion and skeletal muscle laceration and rupture

Figure 4. Massive skin retraction after avulsion, followed by dehydration and necrosis

Chronic lesions often display a surface contaminated with litter debris, suggesting prolonged exposure and a lack of adequate mechanical protection (Figures 2, 3, 4).

In addition to the primary necrosis, multiple scratches can be identified on the exposed muscle tissue, and the skin surrounding the lesions often appears frayed, most likely as a result of mating attempts. In severe cases, extensive destruction of the skin may be noted, potentially involving up to half of the thigh's surface. Generally, lesions are unilateral, affecting either the right or the left side of the body, with bilateral cases being rare. When tissue damage is extensive, lameness is observed, indicating functional impairment. Furthermore, in extreme cases, the occurrence of cannibalism may overlap with these lesions.

Study II: Monitoring mating activities of roosters and breeding hens

Throughout the entire observation period, a total of 1,000 mating attempts were monitored and described, with 200 attempts recorded per week. Among the 1000 mating attempts observed, no instance of complete courtship behaviour was recorded. In 49% of cases, incomplete courtship behaviour was observed, while in 51% of cases, courtship behaviour was entirely absent. From the perspective of the hen's behaviour, the specific mating posture was adopted voluntarily in 12.8% of cases, was forcibly induced in 54.5% of cases, and was avoided by fleeing in 32.7% of cases.

The proportion of attempts resulting in successful mating without coercion was 2.8%, while 14.2% of complete matings involved coercion. In 44.2% of cases, "mounting" occurred without cloacal contact, whereas in 38.8% of cases, the mating attempt did not result in mounting. These values vary depending on the week of the study and will be detailed for each individual week in the following sections.

The behaviour of the hen

A general trend of increased receptivity of the hen towards the rooster is observed, both directly through an increased number of cases where the female voluntarily adopts the specific mating posture, and indirectly through a decreased number of cases where the female attempts to escape. Even in the fifth week of observation, 53% of females adopt the position forcibly, with a slight decrease in this behaviour

observed in the third week of the study, likely due to an increase in the number of situations where the female chose to flee (Figure 5).

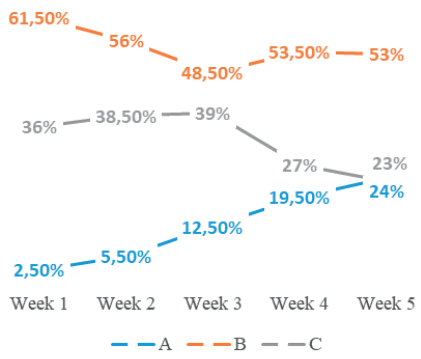

Figure 5. Behavioural development of the hen:
A. Percentage of mating attempts in which the female voluntarily adopted the specific position;
B. Percentage of mating attempts in which the female adopted the specific position by force;
C. Percentage of mating attempts in which the female fled

To explore potential associations between female behavioural responses during mating and the incidence of traumatic injuries, Spearman's rank correlation was applied using weekly percentages.

The analysis revealed no statistically significant correlations between the incidence of injuries and any of the three behavioural categories observed. A moderate negative correlation was found between the percentage of hens adopting the mating posture by force and injury incidence ($\rho = -0.60$, $p = 0.285$), while fleeing behaviour showed a strong positive trend ($\rho = 0.70$, $p = 0.188$). However, neither of these relationships reached statistical significance. Voluntary posture adoption showed no association with injury occurrence ($\rho = 0.10$, $p = 0.873$).**The courtship behaviour of the rooster**Throughout the five-week observation period, the courtship behaviour remained predominantly incomplete or absent. As shown in Figure 6, **no mating attempts included complete courtship behaviour (score 2), with this category consistently at 0%** each week. The proportion of **absent courtship behaviour (score 0)** ranged between 43.5% and 55%, peaking in **week 2.** Meanwhile, **incomplete courtship behaviour (score 1)** fluctuated between 45% and 56.5%, reaching its highest in **week 1** and lowest in week 2.

To assess whether the distribution of courtship behaviour changed significantly over time, a chi-square test of independence was applied. The analysis was based on numeric data derived from weekly mating attempt observations. Specifically, the number of mating attempts in which courtship behaviour was absent or incompletely present was calculated for each of the five observation weeks, using the reported percentages and a consistent total of 200 attempts per week.

The category of complete courtship behaviour was excluded from the analysis, as it was consistently absent (0%) across all five weeks, providing no variation for comparison.

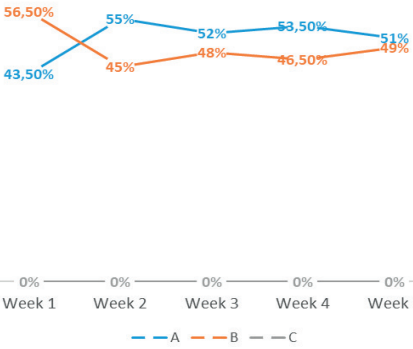

Figure 6. The courtship behaviour of the rooster:
A. Percentage of mating attempts in which courtship behaviour was absent;
B. Percentage of mating attempts in which courtship behaviour was partially present;
C. Percentage of mating attempts in which courtship behaviour was fully present

The observed frequencies were used to construct a contingency table, and the chi-square test revealed no statistically significant association between the week of observation and the type of courtship behaviour displayed ($\chi^2 = 6.36$, df = 4, $p = 0.174$).

To assess whether rooster courtship behaviour influenced female responses during mating, Spearman rank correlation was applied using weekly percentages of courtship categories (absent, incomplete) and hen behavioural outcomes (voluntary posture, forced posture, fleeing). The analysis revealed no statistically

significant correlations across any pair of variables. Correlation coefficients ranged from –0.30 to +0.30, with all *p*-values above 0.6. These results suggest that variation in courtship expression (limited to absent and incomplete behaviours) had no consistent or measurable effect on the way hens responded during mating attempts.

Outcome of the mating attempt

The outcome of the mating attempt is graphically detailed in Figure 7.

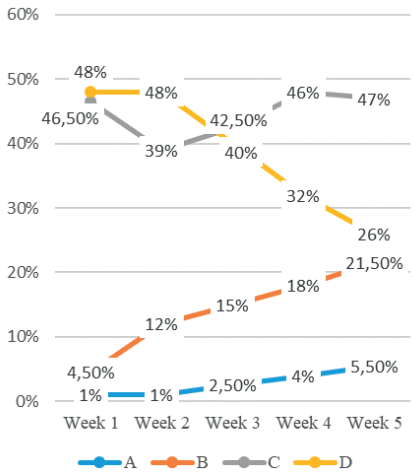

Figure 7. Outcome of the mating attempt:
A. Percentage of mating attempts that ended with a complete mating without force;
B. Percentage of mating attempts that ended with a forced complete mating;
C. Percentage of mating attempts in which mounting was complete but cloacal contact was not achieved;
D. Percentage of mating attempts that did not end with mounting

The number of mating attempts that did not result in mounting decreased throughout the study period, reaching 26% by week 5. For the number of mating attempts in which mounting occurred but no cloacal contact was made, variations were observed with both increases and decreases, with the values in weeks 1 and 5 being nearly identical (46.5% and 47%, respectively). The number of mating attempts that resulted in forced complete mating continuously increased from week 1 to week 5. The number of mating attempts that resulted in complete mating without coercion also increased throughout the study, although it remained much lower than the number of attempts that resulted in forced complete mating.

To assess the relationship between female behaviour, rooster courtship expression, and the outcome of mating attempts, Spearman's rank correlation was calculated based on weekly percentage data.

A strong and statistically significant positive correlation was found between the proportion of hens adopting a voluntary posture and the percentage of mating attempts that ended in complete mating without force ($\rho = 0.97$, $p = 0.005$). Similarly, voluntary behaviour showed a perfect positive correlation with the percentage of forced complete matings ($\rho = 1.00$, $p = 0.000$). These findings may reflect a general trend in which voluntary posture co-occurs with successful mating, regardless of coercion level.

Other associations were not statistically significant. Fleeing behaviour was positively but non-significantly correlated with the percentage of attempts where mounting did not occur ($\rho = 0.67$, $p = 0.219$), and forced posture was negatively associated with forced complete matings ($\rho = –0.70$, $p = 0.188$). No meaningful correlations were observed between courtship behaviour and any of the mating outcomes.

Study III: Calculation of the hatching rate corresponding to the period during which Studies I and II were conducted

The eggs collected over the course of the five weeks of the study were incubated, with the exception of those from week 1, due to the extremely limited number of eggs. In week 2 of the study, a total of 150 eggs were incubated for experimental purposes, with a very low hatching percentage anticipated. The obtained values are presented in Table 4. A clearer representation of this trend can be observed.

Table 4. The hatching percentage corresponding to each study week

The study week during which the eggs used for incubation were collected	Hatch rate percentage
Week 1	0% No eggs were set for incubation
Week 2	15%
Week 3	50%
Week 4	62%
Week 5	75%

The hatchability percentage is visibly affected in the initial weeks but shows a clear upward trend

throughout the study. In week 5, a 27% complete mating rate is correlated with a 75% hatchability percentage, a phenomenon explained by the hens' ability to store sperm and the fact that a single hen can mate with multiple roosters.

Although the hatching percentage is low at the beginning of the study, it increases steadily, reaching 83% after 6 weeks from the onset of egg laying.

The recorded hatchability rates increased progressively from 15% in week 2 to 75% in week 5. When compared to commercial expectations for the same genetic line, which range between 70% and 80% for weeks 22 to 25, the observed values were considerably lower in the early part of the laying period, with convergence occurring only by the fifth week.

The results revealed a **perfect positive correlation** between the percentage of voluntary posture and hatch rate ($\rho = 1.00$, $p = 0.000$), suggesting that cooperative female behaviour during mating may strongly influence reproductive success.

A Spearman correlation was performed to determine the relationship between the total percentage of completed matings (both with and without force) and hatch rate. The analysis revealed a **perfect positive correlation** between the overall completion rate of mating attempts and hatchability ($\rho = 1.00$, $p = 0.000$), indicating that successful copulation, regardless of its nature, is a strong predictor of reproductive output.

The relationship between negative female behavioural responses during mating and hatch rate was also explored using Spearman correlation. A strong negative trend was found between fleeing behaviour and hatch rate ($\rho = –0.80$, $p = 0.200$), suggesting that increased avoidance behaviour by hens may be associated with reduced reproductive success. However, this correlation did not reach statistical significance. Forced posture showed a weaker, non-significant negative correlation with hatch rate ($\rho = –0.40$, $p = 0.600$).

Overview of the phenomenon of sexual aggressiveness in the present study

Wounds specific to sexual aggression

The manifestation of sexual aggression is closely correlated with mating behaviour. Sexual aggression occurs when the natural stages of mating behaviour are partially or entirely absent, leading to the emergence of behavioural abnormalities. Roosters, in their desire to mate, no longer perceive the avoidance signals emitted by hens, and they end up forcing matings. As a result of forced matings or even just forced mating attempts, males inflict specific injuries on females.

These injuries, primarily affecting the skin in the head and torso regions, are localized precisely where the male grips the hen during mating. The injuries occur through tearing of the skin with the claws or beak during the mating attempt. Injuries caused by sexual aggression differ from those resulting from dominance-based fights and are categorically distinct from the injuries associated with cannibalism due to their location and morphological characteristics (Lupu & Militaru, 2022).

The study and understanding of animal behaviour, a field known as ethology, are essential for assessing species' well-being and adaptability. In birds, ethology helps identify factors influencing stress, aggression, and social interactions, which are relevant for both research and management in controlled environments (Bălăceanu, 2021).

In the present study, the phenomenon of aggressiveness is characteristic of the onset of the laying period and extends over a time span of 21 to 25 weeks, as confirmed by previous studies. De Jong et al. (2009) observed that males exhibit aggressive behaviour immediately after being introduced into female groups, with forced sexual interactions occurring during the initial encounters between males and females, starting at 20 weeks of age.

The presence of these localizations (comb and earlobes) exclusively in weeks 1 and 2 suggests the idea of sexual immaturity on the part of the roosters, as they were unsure how to grip the hen during mating.

The head was the most frequently affected region, likely due to sexual aggression during forced mating attempts, where males may peck or restrain females by targeting the cranial area. Most injuries were classified as score 2, corresponding to wounds between 0.5 and 1 cm, indicating a moderate but repeated level of trauma. The peak incidence of score 1 injuries in week 3 coincided with the highest number of new cases overall, occurring when the birds

were 23 weeks old and still within the early post-laying period. This suggests that the initial weeks after the onset of egg production represent a critical window in which the frequency and intensity of sexually aggressive interactions escalate rapidly before stabilizing.

Despite not reaching statistical significance, the strong positive correlation between fleeing behaviour and injury incidence ($\rho = 0.70$) highlights a potential biological link between avoidance responses and the risk of trauma. This suggests that hens subjected to aggressive mating attempts may experience both behavioural stress and physical injury, especially during the early reproductive period.

Trunk and thighs lesions exhibited characteristics repeated trauma without healing tendency, likely linked to repeated forced mating. The presence of necrosis, inflammation, and contamination suggests prolonged exposure without healing. Such injuries may impair mobility and, in severe cases, provoke cannibalism, highlighting the welfare risks associated with unregulated sexual aggression.

When examining courtship behaviour in roosters, it was either absent or weakly expressed. Throughout the study, courtship behaviour was absent in 51% of cases, while in 49% it was incomplete. The analysis of courtship behaviour revealed no statistically significant variation in its distribution across the five-week study period. Although weekly fluctuations in the proportion of absent and incomplete behaviours were observed, the chi-square test indicated that these differences were not meaningful at a statistical level. This suggests a general consistency in courtship expression over time. Numerous previous studies support and confirm this observation, indicating that in many cases recorded under commercial breeding conditions, mating is not preceded by courtship behaviour, or when it does occur, it is expressed with low intensity (Millman et al, 2000; Jones et al, 2001; De Jong et al, 2009). These deficiencies appear to be correlated with selective breeding for production traits. Kjaer and Mench (2003) suggest that genetic selection for high production efficiency may have inadvertently influenced behavioural patterns, including courtship expression.

Furthermore, it remains unclear whether the deficiency in courtship behaviour and the occurrence of hyper-aggressiveness are independent issues or interconnected phenomena. There is a possibility that these traits are genetically linked to a production characteristic, such as the development of a prominent breast, a trait that has been a key focus of selection in breeding programs. Duncan (2009) highlights the potential genetic correlation between physical traits selected for production efficiency and alterations in mating behaviour, raising important questions about the unintended consequences of modern poultry breeding practices.

Hens are generally expected to respond to the rooster's courtship behaviour; however, under certain circumstances, they may exhibit avoidance strategies or even rejection behaviours (Willis & Ludlow, 2015).

Contrary to expectations, our study found no clear relationship between female receptivity and the rooster's courtship behaviour. These results suggest that variation in courtship expression had no consistent or measurable effect on the way hens responded during mating attempts.

Instead, female receptivity showed a gradual upward trend throughout the study. This increase could suggest a progressive adaptation of the females. Another possible explanation is that the initial stress, combined with male aggressiveness, gradually leads to a reduction in defensive behaviour.

The role of females in the manifestation of sexual aggressiveness remains insufficiently understood. It is not yet clear whether hen behaviour is a direct consequence of male aggression during mating interactions or if females fail to display a typical mating response, which in turn leads to male frustration (Millman et al., 2000). Finally, no strong preference for a particular rooster lineage was observed. Researchers hypothesize that female avoidance of males results from learned experiences rather than an innate aversion (Millman & Duncan, 2000a).

When examining the relationship between mating behaviours and outcomes, the data revealed that voluntary female posture was strongly associated with the successful completion of mating, both with and without the

use of force. This suggests that female cooperation may play a key role in mating efficiency. In contrast, courtship behaviour - limited in this study to absent or incomplete forms - showed no clear association with mating success. Although fleeing behaviour tended to correlate with failed mounting attempts, this relationship was not statistically significant, pointing to the multifactorial nature of mating dynamics.

The male-to-female ratio plays a crucial role in ensuring optimal fertility rates (Duncan, 2009). With the introduction of genetic lines selected for increased breast yield, concerns have grown regarding male aggressiveness and its negative effects on mating behaviour and fertility. Currently, a 1:12 mating ratio is more commonly used (Leeson & Summers, 2010). However, in practice, this ratio often approaches 1:20, as a considerable number of roosters fail to successfully fertilize hens (Duncan, 2009).

In the context of reproductive performance, several behavioural variables were examined for their potential association with hatch rate. The strong positive correlation observed between voluntary female posture and hatchability suggests that cooperative behaviour during mating may directly contribute to successful fertilization. Likewise, the perfect correlation between the total number of completed matings (whether forced or not) and hatch rate highlights the importance of copulation success in determining reproductive output. While fleeing behaviour showed a notable negative trend, and forced posture also correlated negatively with hatch rate, these associations were not statistically significant. Even so, the consistent direction of these patterns supports the idea that female responsiveness and mating outcome are key elements influencing reproductive efficiency in the early laying period.

According to commercial performance expectations for this **genetic line,** hatchability between weeks 22 and 25 typically ranges between 70% and 80%. In the present study, observed hatch rates during weeks 2 and 3 were considerably lower than these standards, suggesting that, beyond physiological maturation, additional factors such as mating-related stress or trauma may have interfered with reproductive success. However, as the study progresses, a clear upward trend in hatching rates is observed, suggesting a possible adaptation within the flock. This improvement may be influenced by a stabilization of mating interactions, a reduction in excessive male aggression, or an increase in successful copulation attempts over time.

The cause of the lesions observed in the study is multifactorial, involving both male and female behavioural patterns. While roosters reach physical maturity, their behavioural development remains incomplete, leading to excessive aggression and uncoordinated mating attempts. Simultaneously, the lack of receptivity in females exacerbates this dynamic, as their avoidance or passive resistance contributes to unsuccessful and potentially injurious mating interactions.

These findings suggest that the temporal mismatch between physical and behavioural maturity in roosters, combined with female non-receptivity, creates conditions that heighten the risk of lesions. The intensity of this phenomenon decreases after the five-week study period, suggesting a potential behavioural adaptation over time. This decline may be attributed to progressive social stabilization, a reduction in male hyper-aggressiveness, or an increase in female receptivity as the flock dynamics adjust. Future research should explore whether this decline is a consistent pattern across different genetic lines and housing conditions, as well as investigate possible interventions that could accelerate this stabilization process, thereby minimize the risk of lesions and improve overall reproductive success.

This study addresses a significant issue that remains unexplored at the national level and has received limited attention internationally regarding the impact of sexual aggression in roosters on the welfare, productivity, and fertility of breeding flocks. International research has predominantly focused on parent stock of conventional fast-growing broilers, while little attention has been given to parent stock of coloured broilers, despite their growing economic importance. The findings highlight that the absence of proper courtship behaviour and aggressive mating attempts not only compromise the physical integrity of the hens but also negatively affect reproductive success, ultimately reducing overall farm performance. In an industry where optimizing productivity is

essential for economic sustainability, identifying and mitigating factors that contribute to this behaviour becomes a priority. Given the lack of national research on this topic and the narrow scope of international studies, the present study serves as a necessary starting point for a deeper understanding and improved management of behavioural dynamics in coloured broiler parent flocks

CONCLUSIONS

The phenomenon of sexual aggression emerges with the onset of sexual maturity, which coincides with the initiation of egg-laying and occurs within the age range of 21 to 25 weeks.
Specific lesions associated with the phenomenon of sexual aggressiveness are observed only in the first 5 weeks after egg-laying begins.
Head area lesions were recorded in 3.26% of the hen population.
Lesions were observed in 89.8% of cases on the back of the head, 5.42% on the comb, and 4.81% on the earlobes. The localization of lesions on the comb and earlobes was specific only to the first two weeks of the phenomenon's manifestation.
In terms of size, 35% of lesions were smaller than 0.5 cm, 38.5% measured between 0.5–1 cm, and 26.5% were larger than 1 cm.
No cases of complete courtship behaviour were recorded. Throughout the study, in 51% of cases, courtship behaviour was entirely absent, while in 49% of cases, it was incomplete, with no significant differences observed over the course of the study.
The cause of the lesions is complex and involves both male and female behaviour. Roosters exhibited behavioural immaturity despite reaching physical maturity, while the lack of receptivity in females exacerbated the mechanism leading to the occurrence of lesions.
The hatching percentage is visibly affected in the first weeks but shows a clear upward trend throughout the study.

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STRUCTURAL PECULIARITIES OF THE VENOUS WALL BEFORE AND AFTER VALVES IN THE FEMORAL VEIN IN BROILER CHICKENS

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Abstract

The veins transport the blood from the capillaries to the heart and to prevent blood reflux, the veins dispose valves. This study aims to describe the microscopic structure of the femoral vein in broiler chickens, before and after the valves. The right femoral vein was collected from a 10-day-old broiler chicken that was histologically processed by embedding in paraffin and the sections were stained by the Verhoeff-trichrom method. In the case of the femoral vein in broiler chickens, before the valves, the tunica media is made up of smooth muscle cells without being compactly arranged, while the tunica adventitia is predominantly fibrous. In the segment after the valves, the tunica media is more compact than in the segment before the valves and the tunica adventitia is fibro-elastic. At the borderline between the media and the adventitia, there are 3-4 elastic lamellae similar to those in the external elastic lamina of the arterial wall. At the level of the agger, the tunica media is twice as thick as in the segment after the valves. These structural differences of the venous wall are directly related to ensuring changes in the hemodynamic parameters in the phases of the valvular cycle (opening phase, equilibrium phase, closing phase and closed phase).

Key words*: femoral vein, microscopic structure, broiler chickens, valves, Verhoeff-trichrome.*

INTRODUCTION

The venous system transports unidirectionally the blood from the capillary network to the heart (Miclăuș et al., 2017). Veins have a wall consisting of 3 tunics: intima, media and adventitia (Bacha and Bacha, 2000).

The approximate proportion between the three tunics is 5 - 15 - 80%. From a structural point of view, the tunica media of the veins can be muscular, fibrous, musculo-fibrous or musculo-elastic. Generally, tunica adventitia is made of irregular dens connective tissue (Rus, 2021).

Blood reflux in the venous system is prevented due to the existence of valves (Renaudin et al., 1999; Lurieet et al., 2003). The venous valvular system is represented by semilunar folds of the tunica intima.

The valves have two faces: luminalis and parietalis. The area below the valve insertion base on the venous wall is called agger (Caggiati, 2013). The valves present a connective axis covered by the endothelium. The lumen of the veins, in the portion after the valves, is more dilated and is called the sinus (Safir and Lev, 1952). Blood circulation at the level of the valves is divided into 4 phases: opening, equilibrium, closing and the closed phase (Lurie et al., 2006).

The detailed knowledge of the structure of the venous wall represents numerous advantages, especially since in human medicine numerous surgeons use fresh chickens as non-living animal models to practice vascular microanastomosis techniques (Galeano and Zarabini, 2001; Couceiro et al., 2013; Kang et al., 2017). Some authors state that the chickens are preferred to other non-living animal models because the chickens have some great advantages such as: easy storage, convenient disposal, low purchase price, less preparation time and absence of ethics issues (Kim et al., 2013; Kang et al., 2017).

This study aim is to describe the microscopic features of the femoral vein in broiler chickens, before and after the valves.

MATERIALS AND METHODS

The right femoral vein was collected from a 10-day-old broiler chicken during the necropsy. It was fixed in 10% formalin for 5 days. Later, the harvested fragment was included in paraffin and 5 µm thick sections were made. The obtained

sections were stained by the Verhoeff-trichrome method to appreciate both the elastic and collagen components of the venous wall structure on the same section. The microscopic slides were examined with an optical microscope (Olympus BX 41) and photographs were taken (Olympus SC 180). The obtained images were processed with Olympus cell Sens software.

The study was approved by the Bioethics Committee of the University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, no. 453, from 29.05.2024.

RESULTS AND DISCUSSIONS

The right femoral vein, in 10-day-old broiler chickens, is a muscular vein, and the approximate proportion between the 3 tunics is similar to that described in specialized literature. Valves were intercepted on the section (Figure 1A). At the level of the tunica intima, no particular aspect was observed, displaying the typical structure, made of endothelium.

The tunica media consists of circularly arranged smooth muscle cells. Into the tunica media, it can be seen that there are small differences in the segment before (Figure 1B) and after (Figure 1C) the valves, in the sense that the muscle cells are somewhat better represented in the segment after the valves and have a more compact arrangement.

At the level of the tunica adventitia, structural differences were also found. Thus, in the segment before the valves, the tunica adventitia is formed by dense irregular connective tissue in which collagen fibres are the majority, but from place to place, rare elastic fibres can be observed (Figure 1B). In the segment after the valves, the amount of elastin is much higher so that we can affirm the fact that the tunica adventitia is fibro-elastic (Figure 1C).

In the inner third of the tunica adventitia, the amount of elastin is greater than that of collagen. Thus, at the limit between the tunica media and the tunica adventitia, there are 3-4 elastic lamellas similar to those described in the external elastic lamina in the arterial wall. In the middle and outer third of the tunica adventitia, the amount of elastic fibres is slightly higher than in the segment before the valves, but the collagen fibres are the majority.

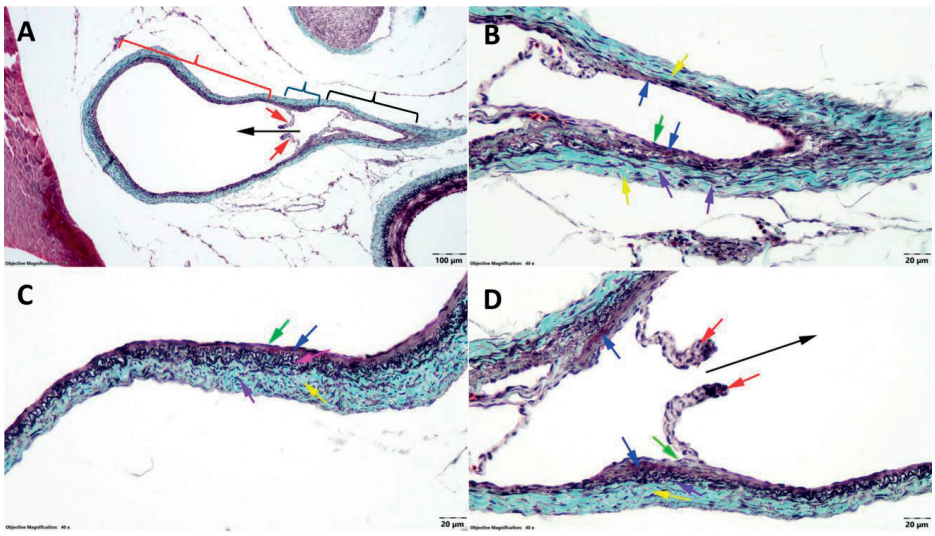

Figure 1. The microscopical appearance of the right femoral vein in 10 days broiler chicken, Verhoeff-trichrome stain; A - general features of the venous wall, black brace - the segment before the valves; blue brace - the agger area; red brace - the segment after the valves; B - details of the venous wall before the valves; C - details of the venous wall after the valves; D - details of the venous wall at the agger area; A, B, C, D - black arrow - the direction of blood flow; red arrow - valves; green arrow - endothelium; blue arrow - tunica media; pink arrow - elastic lamella; yellow arrow - collagen fibres into the tunica adventitia; purple arrow - elastic fibres into the tunica adventitia

In the area of insertion of the valves on the venous wall, which is called agger, the tunica media is significantly thicker (Figure 1D), being almost twice as thick as in the segment after the valves. Due to the fact that in the agger area tunica media it is much thicker, this has the role of preventing the opening of the vein in this region during the closed phase of the valves cycle. The same aspects are also described by Caggiati in humans (2013).

These structural differences of the venous wall before and after the valves seem to be related to the hemodynamic parameters in the 2 segments. Thus, in the segment before the valves, the predominantly fibrous wall prevents the excessive dilation of the veins, so that the occurrence of valvular insufficiency is prevented, respectively the blood reflux during the closed phase of the valves.

Renaudin et al. (1999) claim that valvular deficiency and blood reflux is a consequence of vascular wall distension. Instead, in the segment after the valves, the much more elastic structure of the venous wall allows dilation of the vein at the level of the sinus during the opening and equilibrium phase.

The differences regarding the media in the two segments would be explained by the fact that in the segment after the valves, where the tunica media is more compact and more developed, it would participate in the good functioning of the closing phase, where the contraction of the smooth muscle cells of the tunica media would increase the blood pressure on the mural side of the valves, and valve closure is facilitated. The increase in blood pressure in the mural part of the valves that exceeds the one in the luminal part leads to their closure is also reported by other authors (Lurie et al., 2006).

CONCLUSIONS

In the case of the femoral vein in broiler chickens, before the valves, the tunica media is made up of smooth muscle cells without being compactly arranged, while the tunica adventitia is predominantly fibrous. In the segment after the valves, the tunica media is more compact than in the segment before the valves and the tunica adventitia is fibro-elastic. At the borderline between the tunica media and the tunica adventitia, there are 3-4 elastic lamellae similar to those described into the external elastic lamina of the arterial wall. At the level of the agger, the tunica media it is twice as thick as in the segment after the valves.

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PARTICULARITIES OF THE THYROID CONTROL OF LAYING CYCLE IN EGG-LAYING CHICKEN HYBRIDS DIFFERENTLY SELECTED FOR EGG PRODUCTION

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Abstract

The present work investigates the changes in thyroid control of the laying cycle in Lohmann and Leghorn egg-laying selected hybrids versus unselected Sussex hens. Blood thyroxine (T4) concentration showed an upward trend from 20 to 32 weeks of age (woa), then T4 concentration decreased until 100 wk. The blood T4 evolution curve in Sussex hens was consistently below hybrid chickens (P<0.05). Blood triiodothyronine (T3) also showed an upward trend from 20 to 32 woa, followed by a plateau until 36 woa, after which, the T3 showed a slow downward trend until 100 woa. The increase in T4 and T3 levels during the period of laying was related to the increase in photoperiod. Both hybrid and Sussex hens showed a decrease of T4 to T3 conversion after 32 woa. The conversion capacity decrease was higher in hybrids versus Sussex. Blood T3 evolution levels correlated negatively and weakly with egg-laying intensity (r=-0.81 in Sussex, r=+0.05 in Lohmann and r=+0.17 in Leghorn) but positively with oviduct weight/body weight ratio (r=0.81 in Sussex, r=0.66 in Leghorn and r=0.49 in Lohmann hens).

Key words*: tetraiodothyronine, triiodothyronine, egg-laying cycle control, egg-laying intensity, egg-laying chicken hybrid.*

INTRODUCTION

In birds, as in mammals, there are two forms of iodinated thyroid hormones (ITH) circulating in the blood: the prohormone T4 and the active hormone T3. T3 is the biologically active form because it binds to thyroid hormone receptors with higher affinity than T4. In birds, peripheral tissues can convert T4 to T3 by the action of 5'-deiodinase (Harr, 2002; Hudelson and Hudelson, 2009; Kaneko, 1997, cited by Sönmez, 2021). However, the conversion of T4 to rT3 (reverse T3) is faster than its conversion to T3. Therefore, low levels of T4 may be present in the serum of birds when increasing amounts of rT3 are produced (Decuypere et al., 2005). Iodinated thyroid hormones get involved directly, through systemic actions, or indirectly, through metabolic actions, in the control of reproductive function in the hen. It is well known, for example, that in hypothyroidism, ovulation is inhibited, leading to infertility. Treatment with ITH restores normal ovarian activity. Sechman (2013) reproted that hyper-thyroid state evoked by administration of T3 for a few days diminishes LH, estrogen and progesterone levels, reduces the weight of the ovary, induces atresia of preovulatory follicles and eventually causes stoppage of egg laying. ITH are also involved in moulting of the birds (Dascălu et al., 2000). Cole (1965) described hereditary hypothyroidism in White Leghorn fowls with obesity, delayed maturity, poor laying rate, reduced egg size, decreased fertility and hatchability. Kowalik and Sechman (2017) published a study on the role of ITH in birds underlaying the genomic and non-genomic actions, and their effects on egg laying intensity. Sechman (2013) demonstrated that the ovary expresses mRNAs for membrane receptors for ITH in laying hens, revealing the direct intervention of the thyroid in the steroidogenesis processes of this organ. The process of genetic selection of inbred strains of egg-laying hybrids, however, led to changes in the response of the reproductive system to the control exerted by the pituitary-thyroid axis (Hanlon et al., 2021). Our study summarizes the extent to which the implications of the hypothalamic-pituitary-thyroid axis in the regulation of reproductive function differ in Lohmann and Leghorn hybrid birds compared to Sussex hens.

MATERIALS AND METHODS

The research was carried out on three groups of hens: two groups of egg-laying hybrids (Lohmann brown classic and White Leghorn hybrid) compared to a group of Sussex hens, as a control, raised in industrial system. The birds were monitored from the age of one day. Feeding and watering of the birds were done *ad libitum*. The determinations began at the age of 18 weeks of age (woa) on a number of 48 capita in each group. Also, at the age of 18 woa, photostimulation began with the progressive increase of the light period from 8 hours, by 1:30 hour per week, until a duration of 14 h of light was reached (at 22 woa). This photoperiod was maintained until 61 woa, when an additional photostimulation of another 1 h was added and maintained until the end of the experiment (100 woa). The number of eggs laid was recorded daily. Blood samples were taken at different set time intervals, up to 100 woa, to determine serum T4 and T3 concentrations in all the three groups. Body weight was monitored at the same time intervals. At the ages of 22, 32, 61, 65 and 100 woa, 5 animals from each group were slaughtered to determine the weight of the oviduct. Serum T4 and T3 concentrations were determined by ELISA using dedicated kits provided by DBC (Diagnostics Biochem Canada Inc) for "research only" purposes. A standard curve was drawn for T4 determination, relating the colour intensity to the T4 concentration (Figure 1).

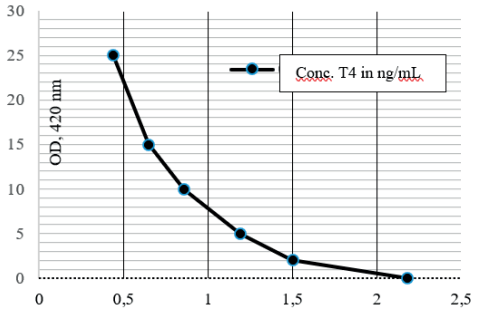

Figure 1. Standard curve for T4 concentration

Absorbance (OD) was read at 420 nm. T3 concentration (in ng/mL) was determined similarly using dedicated reagents.

The data were statistically processed. SAS v9.4 Software (SAS Institute Inc. 2015. SAS/STAT® 14.1 User's Guide. Cary, NC: SAS Institute Inc) was used for the statistical analysis and interpretation of the data. The data regarding the production and growth para-meters were analysed with ANOVA (PROC MIXED). Pearson correlation coefficient (r) was also calculated and expressed as strong, moderate, weak or none. Differences between groups were considered significant for the probability of the null hypothesis $P < 0.05$.

RESULTS AND DISCUSSIONS

Particularities of ITH secretion during the laying cycle

The overall analysis of the T4 evolution graph reveals a general ascending path for all three groups (Figure 2). The slope became more pronounced after the 32nd week of life of the hens, which suggests a forcing of the negative feedback mechanism regulating hypothalamic-pituitary T4 secretion after this age, decreasing in turn its degree of conversion to T3, the active hormone, as shown in Figure 4. The T4 concentration presents higher average values in hybrids compared to Sussex ($P = 0.029$). It is also noted that the two hybrid groups showed higher T3 levels than the Sussex hens, with the differences over the entire period from 18 to 100 woa being 2.31 ng/mL in the Lohmann group and 2.85 ng/mL in the Leghorn group ($P = 0.011$). The standard deviation values were also higher in the hybrid groups. The differences between the Sussex hens and the hybrid groups were significant ($P < 0.05$) at the ages at which they were calculated: 21, 32, 61 and, respectively, 100 woa for T3.

The T3 evolution curves of the hybrid groups show wider variations from one determination to another, generating a "sawtooth" appearance, compared to Sussex hens (Figure 3). We can speculate that hybrid hens are more sensitive to stress, reacting more intensely to the action of different factors: environmental, nutritional or other, compared to Sussex hens. After reaching the peak of laying, the overall trend of the T3 evolution curves was a downward one in all groups ($P < 0.01$), which may be associated with a decrease in oxygen consumption and the decrease in thyroid activity or a decrease in the intensity of metabolic processes as they age.

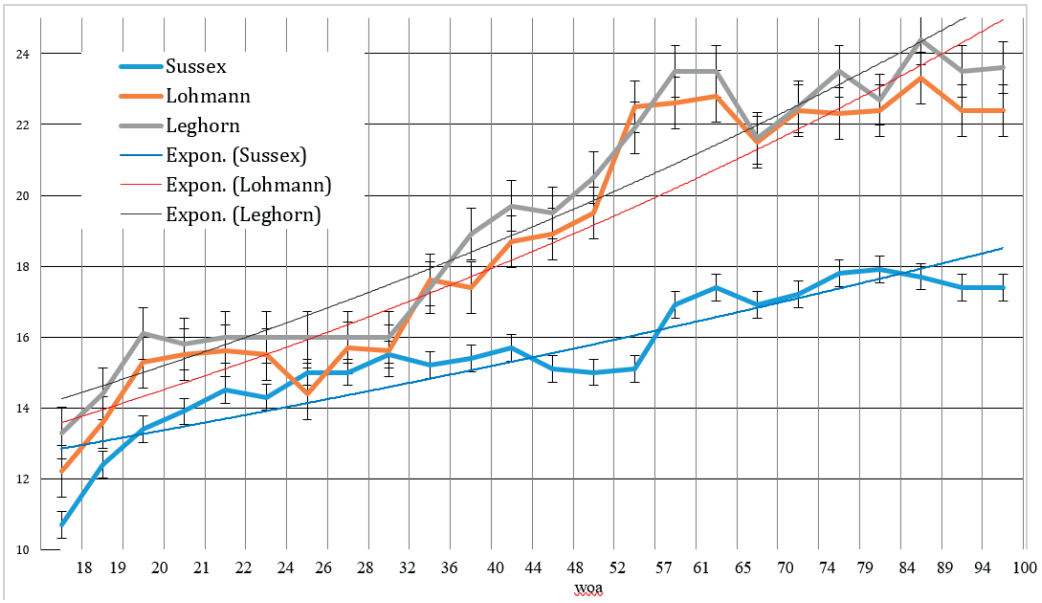

Figure 2. Evolution of the tetraiodothyronine concentration (as ng/mL) in blood serum of the three breed/hybrids of egg-laying hens from 18 to 100 weeks of age. From 61st week of age, the photoperiod was supplemented by 1 hour of light

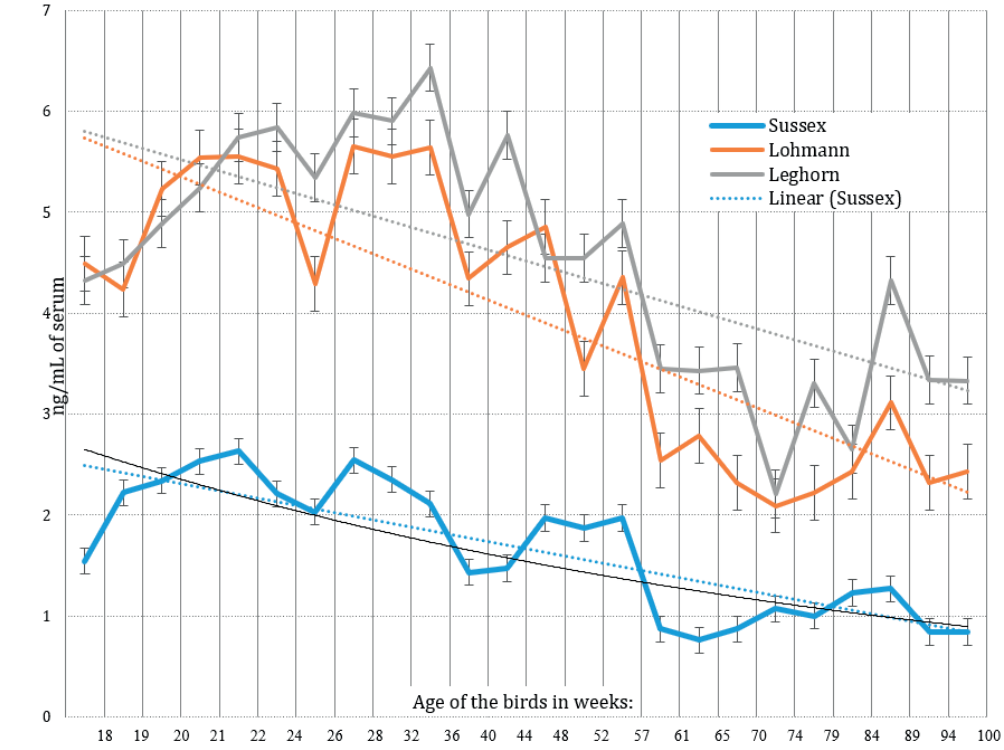

Figure 3. Evolution of triiodothyronine concentration (as ng/mL) in blood serum of the three breed/hybrids of egg-laying hens from 18 to 100 weeks of age. From 61st week of age, the photoperiod was supplemented by 1 hour of light

The decrease was more pronounced in Sussex compared to hybrid groups. The rate decrease conversion of T4 to T3 is revealed by the increase in the T4/T4 ratio values (Figure 4).

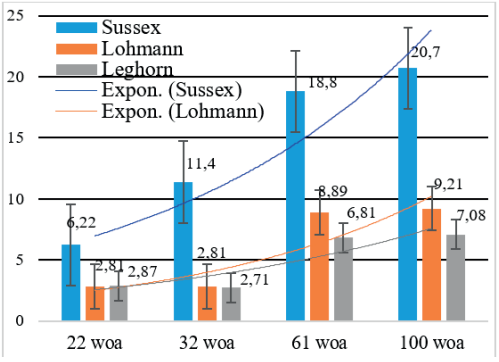

Figure 4. T4/T3 ratio in Sussex hens and laying hybrids, at four key times of the laying cycle: 21 woa (entry into laying), 32 woa (peak of laying), 61 woa (change in photoperiod) and 100 woa (end of technology exploitation period); woa = weeks of age

As a mechanism, the decrease trend in T3 levels as seen in Figure 3 may be the result of a conversion rate of T4 to T3 (Stojević et al., 2000).

Particularities of ITH secretion under the effect of photoperiod

In our research, the three groups of birds benefited from a directed light program. The effect of photoperiod on the blood concentration of thyroid hormones can be analyzed during the interval from 18 to 22 woa, when the birds of the three groups were applied a photostimulatory light program (14 hours lighting), and from the age of 61 woa, when a photostimulation hour was added (15 hours of lighting). According to data from the literature (Bédécarrats et al., 2009; Bunaciu et al., 2009; Zhao et al., 2023) the upward evolution of the T3 concentration from 18 to 22 woa in all three groups of birds has a double explanation: on one hand it can be attributed to the increase in photoperiod. On the other hand, it is considered that only the reaching of the maturity weight would be sufficient to determine these hormonal evolutions, the consequence of which being the onset of egg laying. The increase in the duration of the light period at the age of 61 woa did not generate significant changes in T3 or T4. However, the specialized literature has proven in recent years that, in birds, an adequate level of ITH is of paramount importance for the normal functioning of the female reproductive system.

ITH/egg-intensity ratio and ITH/oviduct weight ratio

Pearson correlation between the evolution of T3 and T4 concentration as independent variables and the laying frequency as dependent variable at the ages of 22, 32, 61, 65 and 100 woa allowed the observation that, in Sussex hens, the laying intensity correlates more strongly with the blood level of T4 and T3 than in hybrid hens (Table 1). In the Lohmann hybrid, the values of the correlation coefficient r were 0. This fact illustrates a higher sensitivity of Sussex hens to the action of ITH compared to Lohmann or Leghorn hens. In other words, in Lohmann and Leghorn hens, the laying frequency does not correlate with the secretion of ITH. On the other hand, according to Stojević et al. (2000) the variations in the intensity of production (of meat) largely follow the variations in the blood T3 concentration of young broiler hens. In contrast, the slight increase in T4 concentration during the experimental period is most likely related to the higher rate of iodinated thyroid hormone production.

It is worth noting that blood plasma in birds does not contain a specific protein for binding and transporting ITH as in mammals (Farer et al., 1961), which is why T3 and T4 bind to plasma albumin whose affinity and transport capacity are lower for T3 than for T4 (Sturkie, 1986).

Table 1. Pearson correlation coefficient (r) of serum T4 and T3 and the laying frequency of different-age hen groups during egg-laying cycle

	Age (in weeks):	22	32	61	65	100	r values
Sussex	Laying fr*	1.4	5.18	4.62	4.76	3.64	-
	T4	13.9	15.5	16.4	17.4	17	0.68
	T3	2.53	1.35	0.87	0.76	0.84	-0.81
Lohmann	Egg fr.	3.85	6.3	5.6	5.6	4.2	-
	T4	15.5	15.6	22.6	22.8	22	0.00
	T3	5.54	5.55	2.54	2.78	2.43	0.00
Leghorn	Egg fr	3.71	6.15	5.46	5.46	3.85	-
	T4	15.8	16	23.5	23.5	23	-0.04
	T3	5.24	5.9	3.45	3.43	3.33	0.17

*Laying fr = laying frequency, expressed as number of egg/week/bird.

To eliminate possible errors determined by the influence of breed size, the analysis of the correlation between the evolution of ITH blood level and oviduct weight was performed using the oviduct weight/body weight ratio values. The results of this monitoring are presented in Table 2.

Table 2. Pearson correlation coefficient (r) of serum T4 and T3 (as ng/mL) and the oviduct weight (ovw)/body weight (bw) ratio in different-age hen groups during egg-laying cycle

Age in weeks:		22	32	61	65	100	r values
Sussex	T4	13.4	15.5	16.4	17.4	17.4	-0.84
	T3	2.53	1.35	0.87	0.76	0.84	0.81
	Ovw/bw	2.78	2.54	2.53	2.56	2.33	-
Lohmann	T4	15.3	15.6	22.6	22.8	22.4	-0.60
	T3	5.54	5.55	2.54	2.78	2.43	0.65
	Ovw/bw	3.41	3.13	3.14	3.12	2.73	-
Leghorn	T4	15.8	16	23.5	23.5	23.6	-0.54
	T3	5.54	5.9	3.45	3.43	3.33	0.49
	Ovw/bw	4.34	3.83	3.96	3.92	3.43	-

From the analysis of the data presented in Table 2, it is found that the Sussex breed presented the highest correlation values, both for T4 and T3. In all of hens used in the experiment, T4 correlated negatively with the age of the birds, while T3 correlated positively with the age of the birds, and this asynchronicity is difficult to explain. Analysis of the period from 61 woa (the time of application of the one-hour photoperiod supplement) to 65 woa (five weeks of photostimulation) illustrates slight decreases in the oviduct weight/body weight ratio ($P > 0.05$) in hybrids, with a slight increase being present in Sussex, which could be interpreted as a higher sensitivity of the Sussex breed to supplemented photoperiod. Data in the literature regarding the influence of ITH on oviduct weight in laying hens are scarce. Mimura (1937) reports a weight value of 45.5 g for the oviduct in adult Leghorn hens, with a correlation coefficient (r) value with body weight calculated at $r = +0.59$, while Mahajan & Joshi (2020) report a weight of 57 g for the same structure in 25-year-old Leghorn. According to Dawson (2001), treatment of birds with thyroxine can mimic the effects of long photoperiods, which confirms our results where an increase in photoperiod from 8 hours to 14 hours in 18-wk-old chicken was accompanied by an increase in ITH concentration.

CONCLUSIONS

The research of particularities of thyroid control of the laying cycle in hens selected for egg-laying (Lohmann and Leghorn) allowed to identify some particularities of ITH secretion compared to an unselected breed of hens (Sussex). Over an evolution period from 18 to 100 woa, the curve of blood T4 evolution in Sussex chickens was constantly below that of hybrid chickens. The increase in iodinated thyroid hormones, both T4 and T3, during the period of entry into laying was related to the increase in photoperiod. Supplementation of photoperiod in the middle of the laying cycle did not significantly modify the evolution of the concentration of the two ITHs in either hybrids or Sussex. The evolution values of T4/T3 ratio were constantly higher in Sussex versus hybrid chickens. The blood concentration of T4 and T3 does not correlate with either the frequency of laying or the weight of the oviduct in hybrids versus Sussex chickens.

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MORPHOLOGICAL CHARACTERISTICS OF THE LONG BONES OF THE PELVIC LIMB IN THE RED-NECKED WALLABY (*MACROPUS RUFOGRISEUS*)

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Abstract

Red-necked wallaby (Macropus rufogriseus) belongs to the Diprotodontia order, Marsupialia infraclass, Macropodidae family, and the genus Macropus, and is spread particularly on the Australian continental. The Macropodidae family encloses all the marsupial herbivores, from which the kangaroos and the wallabies are the best known. This case study provides a complete description of the long pelvic limb bones in the red-necked wallaby. The morphological features of the skeleton provide valuable data in practice, allowing accurate species identification in case of disputes. The following conclusions emerged: the gluteal line is very high, the ischial tuberosity is rounded and drawn lateral, the iliopubic eminence is very developed, the greater trochanter is large, rectangular and undivided, the tibial intercondylar eminence is very high and elongated, and the articular surfaces relatively equal, the epipubic bones are L-shaped and the cranial extremity is the longer half.

Key words*: wallaby, pelvic girdle, femur, tibia.*

INTRODUCTION

The red-necked wallabies *(Macropus rufogriseus)* are members of the *Macropodidae* family, closely related to kangaroos. These species are fascinating marsupials with diverse environmental adaptations, regarding the body shape and unusual hooping locomotion, as well as their ability to be energy efficient (Thornton et al., 2022). Their behaviour is also particular, as in other eutherian species, they may use conflicts and reconciliation to maximise the after-benefits (Cordoni & Norscia, 2014). The skeletal system of marsupials, namely macropodoids, exhibits unique adaptations for characteristic locomotion: the hind limbs are about twice as long as the forelimbs and the tail is strong and sturdy for balance. In this species, the most developed hind limb bone is the tibia. This type of locomotion most likely evolved to enhance predator avoidance (McGowan & Collins, 2018). Some authors have introduced the term 'pentapedal' locomotion, thus describing the tail as a fifth limb to support weight while the hind limbs are balanced forward (Windsor & Dagg, 1971; Dawson et al., 2015). This pentapedal locomotion does not characterise all members of the family *Macropodidae*, but it is a habit preference. Most of the species that engage in this type of locomotion inhibit open spaces, like forests, grasslands or woodlands. (Dawson et al., 2015). Another preference of these hoppers is forelimb use; many individuals display lateralisation in limb usage. (Spiezio et al., 2016).

The literature includes studies on locomotion, osteology and musculature in other related species, such as tail anatomy in the western grey kangaroo (*Macropus fuliginosus*) (Dawson et al., 2014), pelvic limb musculature and locomotor apparatus of the curs and leg in the eastern grey kangaroo (*Macropus giganteus*) (Hopwood & Butterfield, 1976; Hopwood & Butterfield, 1990). Research on wallabies, particularly their skeletal structures, offers valuable insights into their evolutionary history, biomechanics, and ecological roles.

MATERIALS AND METHODS

The pelvic limb bones from an adult red-necked wallaby were used for morphological description. The bones belong to the collection of the Anatomy discipline at the Faculty of Veterinary Medicine in Bucharest.
The most captivating aspects were documented and photographed. The description and identification comply with the Nomina Anatomica Veterinaria (N.A.V. 2017).

RESULTS AND DISCUSSIONS

The coxal bone

The ilium presents a very high gluteal line on the lateral surface, which divides this surface into two relatively equal fossae. The iliac crest is convex and tuberous, and both sacral and coxal angles are divided into two spines. Before the acetabular cavity, a preacetabular tubercle is observed at the distal extremity of the gluteal line (Figure 1). The greater ischiatic notch and ischiatic spine are reduced, and the lesser ischiatic notch is very wide.
On the medial surface of the wing, a small groove marks the site of the detachment of the gluteal crest (Figure 3).
The contribution of each pelvic bone is observed on the margin of the acetabular cavity. The acetabular notch is located caudoventrally, the second marginal notch is located cranioventrally, and the last one is dorsal.
The ischiatic tuberosity is rounded and drawn lateral. The ischiatic arch presents, centrally, a tubercle with a ventrocranial orientation.

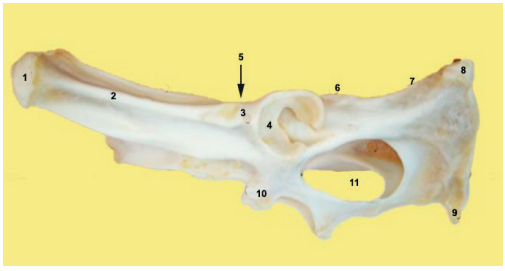

Figure 1. Hip bone of the red-necked wallaby (*Macropus rufogriseus*) - lateroventral view (original):
1. Iliac crest; 2. Gluteal line; 3. Preacetabular tubercle; 4. Acetabular cavity; 5. Greater ischiatic notch; 6. Ischiatic spine; 7. Lesser ischiatic notch; 8. Ischiatic tuberosity; 9. Ischial tubercle; 10. Iliopubic eminence; 11. Obturator foramen

There is an obvious iliopubic eminence on the lateral side of the cranial border of the pubis. At the symphysis level, the bone shows a highly developed pubic tubercle (Figure 1). The obturator foramen has an oval shape (Figure 2). A rough surface for muscle insertion is on the ventral surface of the ischiatic plate.

Figure 2. Pelvic girlde of the red-necked wallaby (*Macropus rufogriseus*) - dorsal surface (original):
1. Greater ischiatic notch; 2. Ischiatic tuberosity; 3. Lesser ischiatic notch; 4. Ischiatic spine; 5. Iliopubic eminence; 6. Ischiopubic symphysis; 7. Dorsocranial iliac spine; 8. Dorsocaudal iliac spine; 9. Iliac crest; 10. Obturator foramen

Figure 3. Right coxal bone of the red-necked wallaby (*Macropus rufogriseus*) - dorsomedial side (original):
1. Auricular surface; 2. Iliopubic eminence; 3. Obturator foramen; 4. Ischiatic tuberosity; 5. Ischiatic arch

The femur

The spherical articular head is supported by a very high neck at the femur's proximal end.
The femoral head has a more cranial position, and the fovea is centrally placed. The greater trochanter, relatively rectangular in appearance and undivided, exceeds by far the articular surface of the femoral head (Figure 4).
On the medial face, at the proximal end, the elongated and high second trochanter, with a ridge appearance, is observed. A tubercle for muscular insertion is observed on the caudal face in the central portion of the femoral body.

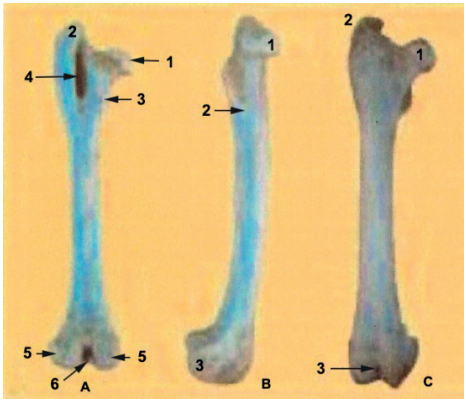

Figure 4. Femur of the red-necked wallaby (*Macropus rufogriseus*) (original):
A. Caudal surface of the left bone: 1. Femoral head; 2. Greater trochanter; 3. Lesser trochanter; 4. Trochanteric fossa; 5. Femoral condyles; 6. Intercondylar fossa; B. Medial surface of the left bone: 1. Femoral head; 2. Lesser trochanter; 3. Medial condyle C. Cranial surface of the right bone: 1. Femoral head; 2. Greater trochanter; 3. Femoral trochlea

The distal articular surface is represented by two condyles caudally, separated by an intercondylar fossa, and cranially by a trochlea, with unequal ridges, the lateral one being more developed. Above the lateral condyle is a reduced supracondylar fossa.

The epipubic (marsupial bones) are relatively "L"-shaped, at a wide-open angle, and longer at the cranial end. They are articulated through the caudal end to the ventral face of the pubic bones, almost on the cranial border, immediately near the pubic symphysis (Figure 5).

The cranial margin of the tibia is high, elongated, with a sharp edge, and slightly inclined towards the lateral side (Figure 6).

Figure 5. Epipubic or marsupial bone of the red-necked wallaby (*Macropus rufogriseus*) (original):
1. Caudal extremity; 2. Cranial extremity

The thin, sharp lateral border is ridge-like and reaches the body's middle third.

The distal third of the body of the tibia is cylindroid. Reduced muscle insertion lines are observed on the caudal surface, and a first-order vascular foramen is observed in the upper third of this face.

The tibia has an elongated, high intercondylar eminence and relatively equal condylar surfaces.

Figure 6. Right tibia and fibula of the red-necked wallaby (*Macropus rufogriseus*) (original):
A. Tibia: 1. Tibial crest; 2. Lateral border; 3. Medial malleolus; B. Fibula: 1. Proximal extremity; 2. Distal extremity; 3. Vascular groove

Distally, the tarsal articular surface is divided by a low median relief into two unequal parts, the medial one elongated and deep, the lateral one wide and relatively shallow. The medial malleolus is evident. Laterally, there is an articular surface for the fibula.

The slightly flexible fibula has a flattened body with a prominent groove on the medial side, which starts from its middle third and ends at the distal end. The distal end of the fibula completes the distal articular surface of the tibia on the lateral side (Figure 6).

CONCLUSIONS

The gluteal crest is very high and divides the iliac wing into two relatively equal fossae. On the medial surface of the wing, a small groove marks the site of the detachment of the gluteal crest. The contribution of each pelvic bone is observed on the margin of the acetabular cavity.

The ischial tuberosity is rounded and drawn laterally. The iliopubic eminence is significantly developed.

The femur presents the relatively rectangular undivided greater trochanter far exceeding the femoral head's articular surface. On the medial surface is the elongated, high, ridge-like second trochanter.

The epipubic or marsupial bones are relatively "L"-shaped and wide open, and the cranial extremity is the longer half.

The tibia has an elongated, high intercondylar eminence and relatively equal condylar surfaces. A first-order vascular foramen is observed on the caudal surface of the body's upper third. Distally, the tarsal articulation surface is divided by a low median relief into two unequal parts, the medial one elongated and deep, the lateral one wide and relatively wide.

The slightly flexible fibula has a flattened body, with an obvious groove on the medial side, which starts from its middle third and ends at the distal extremity.

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THERAPEUTIC POTENTIAL OF PLATELET-RICH PLASMA (PRP) IN DOG OSTEOARTICULAR DISORDERS

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Abstract

Platelet-Rich Plasma (PRP) therapy, derived from autologous canine blood, enhances tissue repair and modulates inflammation by concentrating platelets and growth factors. This study involved 20 dogs of various ages, breeds and gender, with joint traumatic injuries, such as coxofemoral luxation, patellar luxation, cruciate ligament rupture and degenerative joint disease (DJD), diagnosed through radiological exams. PRP was prepared by extracting blood into tubes containing separation gel and anticoagulant, followed by high-speed centrifugation. The PRP was injected into affected joints under general anaesthesia and administered according to each patient's specific treatment protocol. Periodic evaluations at 15, 30, 60 and 90 days, as well as at 6 months and 1 year have demonstrated excellent recovery without complications, highlighting the healing potency of PRP for both traumatic and degenerative conditions. The improvement was observed in 60% of cases after one administration, with 50% (n=10) of non-surgical and 10% (n=2) of surgical patients. Additionally, 10% of non-surgical and 30% (n=6) of surgical patients required two PRP administrations. The study demonstrated the safeness of treatment, even for unresponsive patients to conventional anti-inflammatory therapies.

Key words*: Platelet-Rich Plasma, degenerative joint disease, inflammation, healing.*

INTRODUCTION

Degenerative conditions of synovial joints encompass multiple conditions, which involve an interaction between biological and mechanical factors affecting the articular cartilage, subchondral bone, and synovium, being either monoarticular or polyarticular (Da Costa, 2010). Primary degenerative joint disease of the synovial joint is diagnosed when no predisposing factor exists, being more often observed in the category of geriatric dogs. In such cases, aging process of joint may be accelerated. Postmortem examination reveals the aging and fibrosis of cartilage as incidental findings as a result of mild degenerative changes of the articular surface (Smith et al., 2012).

Secondary degenerative joint disease of the synovial joints is associated with abnormalities in the primary joint or in supporting structures, leading to premature degeneration of the cartilage. Any condition that directly causes the degeneration of the articular cartilage creates instability or results in abnormal directional force that may predispose to degeneration of articular cartilage (Osland et al., 2011).

Osteoarthritis is characterized by an imbalance between the synthesis and degradation of the components of articular cartilage, resulting in the breakdown of the articular cartilage (Liu et al., 2023).

Osteoarthritis has historically been classified based on the analysis of synovial fluid, comparing the levels of inflammatory proteins across various types of arthritis. Some studies have demonstrated that the synovial fluid in osteoarthritis (considered non-inflammatory) contains significantly fewer inflammatory proteins compared to the synovial fluid found in rheumatoid arthritis or septic arthritis (Enomoto et al., 2019).

Among the various conditions affecting the joints, gout is a well-known pathology in humans, but it can also be seen in dogs, though much more rarely. Gout is characterized by the deposition of urate crystals in the joints and periarticular tissues, leading to inflammation and severe pain (Soare et al., 2022).

Articular cartilage is avascular and lacks nerve endings, with limited capacity for self-regeneration when articular conditions develop (Zaragoza et al., 2015).

Platelet- rich plasma (PRP) is an emerging treatment that offers a safe solution by stimulating a natural repair process, containing a high concentration of platelets and growth factors such as IGF-1 (Insuline- Like Growth Factor), TGF-Beta (Transforming Growth Factor Beta), VEGF (Vascular Endothelial Growth Factor), EGF (Epidermal Growth Factor) and PDGF (Platelet-Derived Growth Factor) (Cook et al., 2015). PRP is an autologous blood product that provides critical haemostatic effect when tissue injury occurs. Thus, platelets are among the first blood cells to migrate to the affected area, releasing beta growth factor, which initiates the healing process (Farghali et al., 2021).

These growth factors promote the formation of new blood vessels, trigger mitosis, activate macrophages and stimulate collagen production from fibroblasts (Iacopetti et al., 2020). Direct administration of PRP can support tissue healing with improved quality and speed, without the risk of disease transmission or autoimmune reactions (Lopez et al., 2019).

The aim of this research is to present a relatively new, safe and natural approach to treating joint disorders of various types. The advantages of PRP therapy include its ability to be administered as often as needed for optimal results, the increased concentration of growth factors and the acceleration of the healing process. The treatment is performed under anaesthesia to ensure accurate and precise administration, with studies reporting no side effects (Da Costa, 2010).

MATERIALS AND METHODS

The study was conducted on 20 dogs, including both genders, ranging in age from 1 to 16 years and representing a variety of breeds: Golden Retriever, Pekingese (n=2), Pomeranian, German Shepherd, Beagle, Romanian Raven Shepherd, Yorkshire Terrier (n=2), small-sized mixed breed, American Staffordshire Terrier, Staffordshire Bull Terrier, Labrador Retriever (n=2), Maltese (n=2), Rottweiler, medium-sized mixed breed, and West Highland White Terrie (n=2). Of the 20 dogs, 85% (n=17) had joint conditions of both traumatic and degenerative origin, while only 15% (n=3) dogs presented solely with degenerative osteoarticular conditions.

During the study, 60% (n=12) received a single PRP administration, of which 50% (n=10) were non-surgical and 10% (n=2) were surgical patients, while 40% (n=8) underwent two administrations, with 10% being non-surgical patients and 30% (n=6) being surgical patients. For an accurate diagnosis, canine gait analysis, which involved visual and subjective observation of the dogs from multiple angles at both the walk and trot on a flat surface to detect lameness. Additionally, the Canine Osteoarthritis Staging Tool (COAST) was utilized as standardized system for assessing the severity of osteoarthritis. Radiographic imaging and clinical examination were also conducted to provide a complete assessment of joint health.

Periodic evaluations were conducted at 15, 30, 60 and 90 days, as well as at 6 and 12 months, using the canine gait analysis and the COAST system to assess for signs of lameness or pain. These assessments aimed to determine whether a single PRP dose was sufficient or if a second dose was necessary for optimal therapeutic outcome.

PRP preparation

Blood collection has been performed with a G21 green butterfly needle with adapter and a kit with a 10 ml PRP New Life ACDA tube. The PRP tubes are pre-labelled, red-capped tubes. Each tube has a pre-calibrated vacuum for the collection of 10 ml of blood. Inside each tube is an inert polymer-based separating gel and an anticoagulant essential for the separation of platelet-enriched plasma (injectable fluid) from the red blood cell concentrate, which is achieved through centrifugation.

For the preparation phase, two stages are followed:

Stage 1 - Preparation of collected blood for centrifugation: After collecting the blood, the tubes are gently inverted 3-4 times to prevent microclot formation. The tube is then placed into the centrifuge with an angle rotor, set to 4000 rpm for 7 minutes.

Stage 2 - Preparing the patient for intra-articular administration of Platelet-Rich Plasma under anaesthesia. Sedation of the patient was achieved using Medetomidine and Butorphanol, with Propofol for maintaining the anaesthesia.

All the collected samples undergo the same preparation protocol for obtaining PRP. The preparation of PRP involves collecting autologous venous blood using the Buffy Coat Method (consists of blood extraction, collection and high-speed centrifugation until it separates into three layers: platelet-poor plasma (PPP), platelet-rich plasma (PRP) and red blood cells (Wang et al., 2024). To obtain a high concentration of platelets, the excess platelet-poor plasma (PPP) is gently aspirated from top to bottom, avoiding contact with the platelet at the bottom of the tube. The aspirated volume represents approximately 50% of the total plasma obtained after centrifugation. The tube containing the remaining liquid and the platelet pellet in the leukoplastic layer is gently rotated until the platelet-rich plasma (PRP) is obtained. For each dog a 5ml syringe is used to extract the PRP. The difference between PPP and PRP is visibly noticeable, with PRP appearing darker due to the higher concentration of platelets.

Once the patient is anesthetized, they are connected to a pulse oximeter. The affected limb is positioned to allow for the precise insertion of a needle attached to an empty syringe into the joint capsule. After the needle is inserted, gentle aspiration is performed to confirm correct placement. The empty syringe is then detached and replaced with one containing PRP for administration.

The final volume of PRP was determined by the amount recovered after centrifugation, which varied for each dog. The administered dose ranged between 1 and 3 ml, depending on individual differences. Several factors influenced the PRP yield, including haematocrit levels, baseline platelet concentration, physiological state, hydration status and technical variations during processing. These variables explain why some patients produced a higher PRP volume while others yielded less, despite the same initial blood collection volume.

RESULTS AND DISCUSSIONS

After the first administration, 60% (n=12) of the dogs showed significant improvement, with progressive pain reduction, increased use of the affected limb, and improved joint flexibility during palpation, flexion, and extension. However, 40% (n=8) required a second PRP injection due to suboptimal recovery observed during follow-up evaluations. Among them, 30% (n=6) were post-operative cases, while 10% (n=2) were non-operated.

Among the operated dogs (n=8), six of them required a second PRP administration, indicating a bigger need for additional treatment due to persistent pain and lameness. Conversely, in the non-operated group (n=12), 10 dogs responded well to a single administration, while only two required a second administration.

Dogs diagnosed with purely degenerative joint conditions 15% (n=3) required only a single PRP administration. In contrast, dogs presenting with both traumatic and degenerative conditions were more variable in their response: 45% (n=9) required one injection, while 40% (n=8) needed two administrations for optimal recovery.

At 15 days post-treatment, 60% (n=12) of the dogs that received a single PRP injection showed complete clinical improvement, displaying no pain, lameness, or gait abnormalities. Their condition remained stable at 30, 60, and 90 days, as well as at 6 and 12 months, with no recurrence of symptoms.

Conversely, the 40% (n=8) that required a second PRP dose still exhibited pain and lameness at the 15-day evaluation. By 30 days, their symptoms remained unchanged, leading to the decision to administer a booster PRP injection. Following the repeated dose, all 8 dogs demonstrated significant improvement, with no clinical signs of lameness, pain, or discomfort at their 15-day follow-up. The radiographs taken before and after the PRP therapy show healing post-surgery (Figure 1).

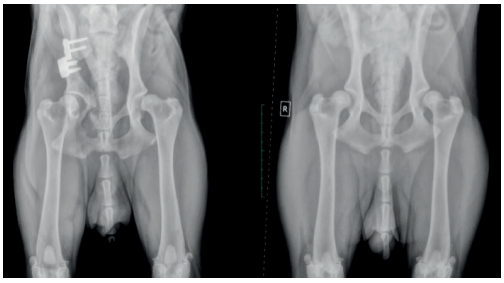

Figure 1. Internal plate fixation in iliac fractures with concurrent mild coxo-femural subluxation after surgery. 60 days follow-up radiograph imaging demonstrates complete healing after the second PRP administration, with the joint showing no further signs of damage or abnormality

Additionally, in a separate case where no surgical intervention was performed, comparative radiographs (Figure 2) reveal stabilization of degenerative changes such as periarticular osteophyte formation, joint space narrowing and remodelling of the femoral condyles and tibial plateaus.

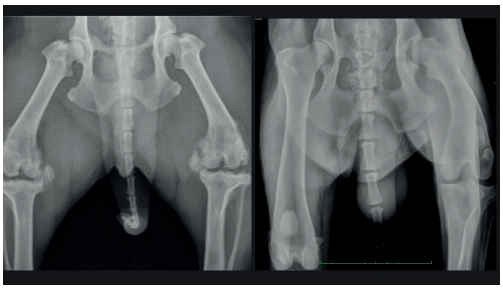

Figure 2. 90 days follow-up radiograph imaging demonstrates notable improvements including preservation of joint space width, absence of further osteophyte development and reduction in periarticular soft tissue density, suggesting decreased inflammation and healing after one PRP administration

Continued evaluations at 30, 60, 90 days, 6 months, and 1 year confirmed full recovery, with no further recurrence.

All 20 dogs responded positively to PRP therapy, with no cases of treatment failure or recurrence of symptoms during the 1-year follow-up period.

Dogs that received two PRP administrations were predominantly post-surgical cases (30%), while 10% of post-operative dogs required only one injection due to faster recovery.

PRP therapy was administered to 15% of dogs with degenerative conditions only and 85% of dogs with both degenerative and traumatic joint diseases. The results suggest that PRP is effective in both groups, with a higher need for a subsequent administration in post-surgical and trauma-related cases.

No adverse reactions were observed following PRP injection, neither after the first nor the last administration.

Temporary discomfort, lasting from a few hours up to a maximum of 24 hours, was observed only in post-surgical dogs (n=8), while no cases of local inflammation or pain were reported after PRP administration in any of the patients.

The 60% success rate after a single administration aligns with previous reports in veterinary medicine, confirming PRP effectiveness as a non-pharmacological alternative to long-term NSAID therapy. However, most existing studies focus on experimental models or human applications, making this investigation an important step toward validating PRP in clinical veterinary practice (Anderson et al., 2021).

A key observation was that 30% of post-surgical dogs required two PRP injections to achieve full recovery. This suggests that a single PRP administration may be not sufficient to counteract the post-surgical inflammatory response, necessitating booster administrations to optimize tissue regeneration.

The severity of the inflammatory process, the complexity of the intervention and its extent can influence the number of PRP administration. However, in cases involving trauma-associated osteoarthritis, a significant proportion (40%) required two injections to reach full functional recovery.

This may indicate that traumatic joint injuries induce a more complex inflammatory response, requiring more aggressive regenerative stimulation compared to chronic degenerative conditions.

One of the most significant findings was the absence of adverse effects, reinforcing PRP safety profile as a regenerative treatment. Compared to NSAIDs or corticosteroids, which can have systemic side effects, PRP offers a biological, low-risk alternative.

Post-injection discomfort was observed only in surgical cases, most likely due to underlying tissue trauma rather than PRP-related inflammation. This supports previous evidence that PRP is well tolerated and does not induce significant inflammatory reactions.

Small sample size (n=20) limits general applicability and the absence of a control group (e.g., untreated dogs or NSAID-treated cases) prevents direct comparison of PRP efficacy against conventional therapies.

Variability in breeds, sizes, and ages may introduce response differences that were not fully controlled.

The results of this study suggest that PRP could become a adequate therapeutic option for managing canine osteoarthritis and joint trauma, reducing the reliance on chronic NSAID use and minimizing drug-related side effects.

PRP has been previously used by various authors in combination with other therapies, demonstrating positive results in the management of osteoarticular conditions. Studies integrating PRP with anti-inflammatory drugs, physical therapy, or other regenerative treatments have reported improved pain relief and functional recovery, suggesting that multimodal approaches can enhance therapeutic outcomes.

However, our study took a different approach by assessing PRP's effects as a standalone treatment, without the influence of additional therapies. Sergio López et al. administered Carprofen as a rescue analgesic at least once during the first week post-treatment. The inclusion of an NSAID in their study may have influenced the evaluation of PRP's true efficacy, as the analgesic effect of Carprofen could have masked or altered the natural progression of recovery.

In our study, the absence of adjunctive medication allowed for a more direct assessment of PRP regenerative potential. The results indicated a clear and progressive improvement in joint function and pain relief, with some patients requiring an additional dose for optimal recovery. Importantly, no adverse effects were observed, and no additional pain management was necessary during the evaluation period.

The findings indicate that PRP alone can be an effective treatment for osteoarticular conditions, reducing the need for supplementary analgesia. However, further research directly comparing PRP with and without NSAID co-administration is needed to determine the most effective clinical protocol.

Additionally, some researchers have opted for repeated PRP administrations at fixed intervals regardless of clinical response. For example, Xue et al. applied PRP in three consecutive doses over a set period, whereas in our study, a second administration was only performed if the initial response was insufficient. Despite these differing protocols, both approaches led to similar long-term improvements in joint function, indicating that while multiple doses may be beneficial in some cases, they are not always necessary.

In our study, we used the buffy coat method for preparing platelet-rich plasma (PRP), a technique that isolates the platelets from the blood by separating the buffy coat layer after centrifugation. This method is known for its simplicity and efficiency in obtaining a high concentration of platelets. Other studies, however, have used different PRP preparation methods, such as the single-spin method (Goodale et al., 2023), double-spin method (Shin et al., 2017), and gel separation techniques (Dalğın et al., 2020), which involve more complex processes but are aimed at achieving a higher platelet yield or different plasma characteristics.

For instance, Shin et al., used a double-spin technique to separate the platelets from plasma, which resulted in a higher concentration of growth factors compared to the buffy coat method. However, their findings indicated that both methods were effective in promoting healing and reducing pain in musculoskeletal conditions, although they noted slight variations in platelet concentrations.

Similarly, Dalğın et al., utilized the gel separation method to prepare PRP, achieving a higher volume of plasma enriched with platelets. Despite these differences in preparation, the clinical outcomes for both methods were comparable, with improvements in pain reduction and functional recovery in patients.

Although the methods used by others differ from the buffy coat method in terms of platelet concentration and preparation complexity, our study shows that the buffy coat method is equally effective in producing satisfactory clinical results for musculoskeletal conditions. This suggests that even simpler techniques can be a viable and cost-effective option for PRP preparation without compromising the therapeutic efficacy, as seen in the positive outcomes reported in both our study and in studies employing more advanced PRP preparation methods.

CONCLUSIONS

This study demonstrates that PRP is an effective and reliable treatment for osteoarticular conditions in dogs. The results showed significant improvements in pain reduction and mobility, with no adverse effects observed. Despite the simplicity of the buffy coat method compared to other more complex PRP preparation techniques, the clinical outcomes

were similar, indicating its potential as a cost-effective alternative. Further studies are needed to confirm the long-term benefits and refine treatment protocols for osteoarticular pathologies.

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ADVANCED IMAGING AND SURGICAL INTERVENTION IN THE MANAGEMENT OF A CANINE ADRENAL PHEOCHROMOCYTOMA – A CASE REPORT

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Abstract

Pheochromocytomas are rare adrenal medullary tumours in dogs, often associated with severe systemic effects due to catecholamine secretion. This report details a six-year-old spayed mixed-breed dog presented for a corneal melting ulcer, where polyuria-polydipsia prompted further investigations. Biochemical tests showed low serum cortisol (1.5 µg/dL) and cTSH (<2.5 ng/dL), with no other abnormalities. Abdominal ultrasound revealed a left adrenal mass adherent to the kidney and caudal vena cava, further confirmed by CT to involve significant vascular structures. Surgical intervention included total nephrectomy, adrenal mass excision, and vena cava reconstruction. Despite the technically successful surgery, the patient succumbed to postoperative cardiovascular complications (severe arrhythmia and hypertensive crisis) within hours after surgery. Histopathology confirmed pheochromocytoma without renal infiltration. This case highlights the essential role of advanced imaging in diagnosis and surgical planning and underscores the perioperative risks of managing such aggressive tumours.

Key words*: abdominal ultrasound, adrenal neoplasm, adrenalectomy, computed tomography (CT), pheochromocytoma.*

INTRODUCTION

Pheochromocytoma is a rare neuroendocrine tumour that affects the adrenal gland in dogs, originating from the chromaffin cells of the adrenal medulla. This condition can have severe systemic implications due to excessive catecholamine secretion, leading to various clinical manifestations, including episodic hypertension, polyuria, polydipsia, tachycardia, weakness, and collapse (van den Berg & Galac, 2024).

The diagnosis of pheochromocytoma is challenging due to its nonspecific symptomatology and variable clinical presentation. Biochemical examination, including plasma metanephrine measurement, is a promising method but is not always available in veterinary practice (van den Berg & Galac, 2024). For this reason, imaging plays a crucial role in the diagnostic and treatment planning of these tumours.

Abdominal ultrasound is often used as an initial method for identifying adrenal masses. Although it provides useful information about the tumour's size and structure, it cannot accurately assess vascular invasion or metastases (Bargellini et al., 2016). The use of contrast-enhanced ultrasound (CEUS) has proven to be a promising tool in differentiating pheochromocytomas from other types of adrenal tumours due to its ability to highlight tumour perfusion (Nagumo et al., 2020).

Computed tomography (CT) is considered the method of choice for diagnosis and staging, offering superior details regarding tumour size, its relationship with adjacent structures, and the presence of vascular invasion (Gregori et al., 2015). A comparative study between CT and histopathological examination showed that computed tomography has high sensitivity in detecting vascular invasion and tumour extension (Davidson, 2022).

Surgical intervention remains the preferred approach for pheochromocytoma, with adrenalectomy representing the gold standard. However, due to the high risk of intraoperative

complications, such as severe haemorrhage and hemodynamic instability, adequate perioperative preparation is essential (Yoshida et al., 2016). Modern monitoring techniques and anaesthetic management, including the preoperative use of alpha-adrenergic blockers such as phenoxybenzamine or prazosin, are crucial for minimizing intraoperative hemodynamic fluctuations and improving postoperative prognosis (Yoshida et al., 2016; Hayes, 2022). However, some studies have shown that short-term outcomes may still be acceptable even in the absence of preoperative medical management, depending on individual case factors (Appelgrein et al., 2020).

For inoperable patients or those with high surgical risk, alternative therapies such as stereotactic radiotherapy have been investigated, although data on long-term efficacy remain limited (Linder et al., 2023).

This study aims to contribute to the specialized literature by presenting a case of canine pheochromocytoma diagnosed through advanced imaging and treated surgically, with a focus on perioperative challenges and prognostic implications.

MATERIALS AND METHODS

The patient was a spayed female mixed-breed, approximately six years old, adopted from a shelter four years prior to presentation. The initial reason for presentation was a comprehensive ophthalmologic examination, which led to a diagnosis of corneal melting ulcer and secondary uveitis in the right eye. During hospitalization, the dog was observed to have a polyuria-polydipsia syndrome, which prompted further investigation to identify the underlying cause.

Haematological and biochemical tests, including complete blood count, serum biochemistry panel, and hormonal assays, were conducted using IDEXX ProCyte Dx and Catalyst Dx analysers (IDEXX Laboratories, Westbrook, Maine, USA). Based on the results, advanced imaging was deemed necessary to investigate a suspected adrenal ethology.

An abdominal ultrasound was performed using an Esaote MyLab 7 ultrasound system (Esaote, Genova, Italy) equipped with an SC3123 convex transducer operating at a frequency range of 3-10 MHz. Based on this scan, an intra-abdominal mass was identified, prompting further imaging evaluation.

To achieve a more accurate assessment of the mass and to exclude the presence of pulmonary metastases, a contrast-enhanced computed tomography (CT) study of the thorax and abdomen was performed with the patient under sedation. The scan was carried out using a Philips Access 16-slice helical CT scanner (Philips, Suzhou, China), and included both pre-contrast and post-contrast phases. The contrast agent (Iohexol, Omnipaque 350 mg I/mL) was administered intravenously at a dose of 2 mL/kg. Image acquisition was performed with a slice thickness of 2.5 mm and a reconstruction increment of 1.25 mm. High-resolution multiplanar reconstructions were generated at 1 mm thickness with a 0.5 mm increment. Additional scan parameters included 180 mAs per slice, tube rotation time of 0.75 seconds, and a pitch of 1.0625. Multiplanar reconstructions were obtained using soft tissue, bone, and lung windows, providing detailed visualization of abdominal and thoracic structures, including vascular anatomy and potential metastatic sites.

Immediately after the completion of the CT scan (which indicated an invasive adrenal mass with vascular involvement), and while still under the same general anaesthesia, the patient was transferred directly to the operating room for surgical management.

A ventral midline laparotomy was performed with the patient in dorsal recumbency to access the abdominal cavity. Intraoperative exploration revealed a large adrenal mass firmly adherent to the cranial pole of the left kidney (Figure 1). Dissection was technically demanding due to the tumour's extensive involvement of the surrounding tissues, including the caudal vena cava.

Given the strong adhesion between the adrenal mass and the left kidney, an *en bloc* resection of the tumoural complex was performed, involving simultaneous removal of the affected adrenal gland and ipsilateral kidney. One of the most significant intraoperative challenges was the tumour's invasion into the wall of the caudal vena cava, which required partial venous resection and meticulous primary venorrhaphy to restore vascular continuity (Figure 2). Despite the complexity of the procedure, the mass was successfully excised in its entirety.

Figure 1. Intraoperative view - adrenal-kidney tumoural block being carefully dissected and isolated

Figure 2. Intraoperative image showing the caudal vena cava (CVC) post-tumour resection.

The adrenal-kidney tumoural block was submitted for histopathological examination. Macroscopically, the mass appeared cavernous and friable, with alternating regions of necrosis and haemorrhage. Tissue sections were processed routinely and stained with Hematoxylin and Eosin (H & E) for microscopic evaluation. Histologically, the neoplasm consisted of a dense proliferation of polygonal cells.

In the immediate postoperative period, the patient received intensive postoperative monitoring. Initially, recovery from anaesthesia was uneventful, with stable vital parameters. However, approximately two hours after surgery, the patient developed acute gastrointestinal haemorrhage, manifested by bradycardia and severe bloody diarrhoea. Despite prompt initiation of intravenous fluid therapy for hemodynamic stabilization and intensive supportive care, the dog progressed to cardiopulmonary arrest and failed to respond to advanced resuscitation measures, ultimately resulting in death.

RESULTS AND DISCUSSIONS

The initial clinical presentation of the patient, a six-year-old spayed mixed-breed dog, was unrelated to adrenal pathology, with referral for ophthalmological examination and diagnosis of a corneal melting ulcer with secondary uveitis. However, the incidental observation of polyuria–polydipsia during hospitalization prompted further diagnostic work-up. Haematological and biochemical analyses revealed a low serum cortisol level (1.5 µg/dL) and decreased cTSH (<2.5 ng/dL), findings that raised suspicion of adrenal dysfunction and supported the need for advanced imaging.

Ultrasound examination identified at the cranial pole of the left kidney, a large and heterogeneous intra-abdominal mass measuring approximately 62.6 mm in diameter, poorly defined margins, and contained areas of reduced echogenicity suggestive of internal necrosis. The mass was also noted in close proximity to the caudal vena cava, suggesting potential vascular involvement (Figure 3).

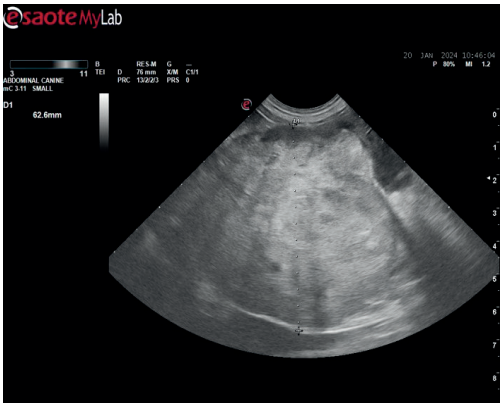

Figure 3. Ultrasound image showing a large, irregular and heterogeneous mass, appearing adherent to the cranial pole of the left kidney and in close contact to the caudal vena cava

These sonographic features are consistent with those described in the literature for adrenal pheochromocytomas, which are often large, heterogeneous, and capable of compressing or infiltrating surrounding structures (Bargellini et al., 2016; Nagumo et al., 2020).

Figure 4. Post-contrast CT image in a dorsal oblique plane, reconstructed in a soft tissue window. The image highlights a large, heterogeneous adrenal mass measuring approximately 66.4 × 93.8 × 89.7 mm, located cranial to the left kidney and displacing it caudomedially

Figure 5. Post-contrast CT image in transverse plane at the level of the mid-abdomen, in a soft tissue window. The heterogeneous mass appears enlarged, with mixed attenuation consistent with necrotic and viable tumour regions

To further characterize the lesion and evaluate for potential metastases, a contrast-enhanced CT scan of the thorax and abdomen was performed. The tumour was identified as a large left adrenal mass measuring 66.4 × 93.8 × 89.7 mm, with hypoattenuating regions indicative of necrosis. It formed a contiguous mass with the left kidney, causing caudomedial displacement and encasement of the renal artery and vein. Notably, the tumour invaded the lumen of the caudal vena cava over a length of approximately 20 mm. These findings reinforced the diagnosis of an invasive adrenal neoplasm with a high potential for intraoperative complications. In comparison with literature reports, this case demonstrates imaging characteristics similar to those described by Gregori et al. (2015), where CT was shown to have high sensitivity in detecting vascular invasion. The use of multiplanar reconstructions further enhanced surgical planning, allowing precise visualization of the extent of vascular involvement (Figures 4 and 5), and such tools can be further enhanced by the use of 3D-printed anatomical models for surgical planning, as previously reported (Almeida et al., 2019).

Additional CT findings included left ventricular hypertrophy, bilateral renal calculi, and splenomegaly. While these may not be directly related to the adrenal pathology, they are important when assessing overall patient stability prior to surgery. In human and veterinary literature alike, the presence of caudal vena cava invasion is considered a poor prognostic indicator and significantly increases surgical risk (Mayhew et al., 2019).

Surgical excision was performed through *en bloc* resection of the adrenal mass and the ipsilateral kidney due to strong adhesion between the two structures. Intraoperatively, the tumour was found to infiltrate the wall of the caudal vena cava, necessitating partial resection and venorrhaphy. Despite the complexity of the procedure, complete tumour removal was achieved, with clean macroscopic margins.

Histopathological analysis confirmed the diagnosis of adrenal pheochromocytoma. Macroscopically, the mass was cavernous and friable, with extensive areas of haemorrhage and necrosis. Microscopically, the tumour consisted of polygonal neoplastic cells arranged in lobules and cords, separated by a fine fibrovascular

stroma. The cells exhibited mildly acidophilic cytoplasm, pleomorphic hyperchromatic nuclei, and occasional mitotic figures. Multifocal vascular thrombosis, necrosis, and neutrophilic infiltrates were observed. Capsular invasion by nests of neoplastic cells was noted in multiple areas, suggesting a high malignant potential. No evidence of neoplastic infiltration was found in the renal parenchyma, supporting the hypothesis of displacement rather than direct invasion (Figure 6).

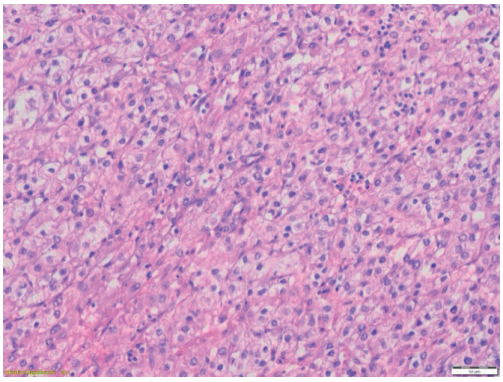

Figure 6. Histopathological image of the excised adrenal mass, stained with Haematoxylin and Eosin (H & E), at 20× magnification

These histological features are in agreement with those described in the literature for canine pheochromocytomas, which are characterized by a high degree of local invasiveness and metastatic potential (van den Berg & Galac, 2024; Zini et al., 2019). Capsular penetration, cellular pleomorphism, and vascular thrombosis are key features associated with poor prognosis. In the immediate postoperative period, the patient initially recovered without incident. However, within two hours of surgery, the dog developed acute gastrointestinal haemorrhage, bradycardia, and haemorrhagic diarrhoea. Despite intensive supportive care and intravenous fluid therapy aimed at hemodynamic stabilization, the patient deteriorated rapidly and progressed to cardiopulmonary arrest, ultimately unresponsive to resuscitation efforts. Such rapid postoperative decompensation is consistent with literature describing catecholamine surge and vascular instability following pheochromocytoma excision (Davidson, 2022). Removal of the tumour source may trigger abrupt hemodynamic collapse due to sudden catecholamine withdrawal.

This case illustrates the diagnostic and surgical challenges associated with canine pheochromocytomas. The use of advanced imaging modalities, particularly contrast-enhanced CT with high-resolution multiplanar reconstruction, proved essential for tumour characterization and preoperative planning. Histopathological findings confirmed the aggressive nature of the neoplasm, consistent with previously reported cases.

While adrenalectomy remains the gold standard treatment, the prognosis is strongly influenced by the extent of vascular invasion and perioperative stability. By contrast, dogs with small, non-invasive adrenal tumours generally have more favourable surgical outcomes and a reduced risk of complications (Cavalcanti et al., 2020).

In this case, adrenalectomy combined with total nephrectomy was technically feasible, but extensive invasion of the caudal vena cava posed a major challenge, requiring partial resection and difficult suturing of the vascular wall. The unfavourable postoperative evolution, with rapid deterioration and hemodynamic collapse, confirms the significant risks associated with these interventions - risks that are well-documented in the literature (Davidson, 2022).

Advancements in imaging diagnostics and perioperative therapy could improve long-term success rates. Recent reports have also explored the use of intraoperative Indocyanine Green (ICG) fluorescence imaging to aid in tumour localization and resection, offering promising outcomes (Yu & Lee, 2024). In some cases, stereotactic radiotherapy or palliative therapeutic approaches could serve as viable alternatives, but further studies are needed to evaluate the effectiveness of these methods (Linder et al., 2023). Laparoscopic adrenalectomy has also been successfully applied in selected cases with non-invasive adrenal tumours, providing favourable outcomes with reduced surgical trauma (Pitt et al., 2016). Additionally, favourable outcomes have been described even in bilateral adrenalectomies, provided that intensive perioperative management is applied (Oblak et al., 2016).

CONCLUSIONS

This case of invasive adrenal pheochromocytoma in a dog illustrates the diagnostic and surgical complexity of managing such rare but aggressive tumours.

A multimodal diagnostic approach - combining clinical evaluation, laboratory analysis, ultrasound, and CT - was essential for detecting adrenal involvement and vascular invasion.

While abdominal ultrasound can raise initial suspicion of adrenal involvement, contrast-enhanced CT with multiplanar reconstruction remains the diagnostic modality of choice, providing critical details for assessing tumour extent and vascular invasion, and guiding surgical planning.

Histopathological examination remains essential for the definitive diagnosis of pheochromocytoma, relying on cellu lar and architectural features such as capsular invasion, necrosis, and vascular thrombosis -hallmarks of malignancy that are critical for establishing prognosis and guiding further management.

En bloc adrenalectomy with nephrectomy and caval wall reconstruction was technically feasible but carried substantial perioperative risk due to the tumour's vascular involvement.

The patient's rapid postoperative deterioration emphasizes the importance of anticipating catecholamine-induced hemodynamic instability and ensuring intensive monitoring.

While adrenalectomy remains the only curative treatment, individualized perioperative strategies and proactive intraoperative management are critical in high-risk cases.

Each documented case of canine pheochromocytoma contributes to refining current diagnostic algorithms and surgical protocols, especially in the context of advanced imaging and perioperative care.

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PREVALENCE OF *LEPTOSPIRA* ANTIBODIES IN HORSES FROM ROMANIA

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Abstract

Leptospirosis, a zoonotic disease with a worldwide distribution, can affect horses causing reproductive disorders such as abortion, embryonic absorption and stillbirths. The aim of the current study was to determine the prevalence of Leptospira antibodies in mares and stallions belonging to a breeding farm in the southern region of Romania. Blood samples were collected from 91 horses and were analysed by the microscopic agglutination test (MAT) using 12 distinct live antigens. To determine if acute infection was present, the positive individuals were retested after 14 days. The results showed that Leptospira antibody prevalence was high, with 59.34% of samples testing positive. Of the 91 samples included in the study, 40 tested positive for one serotype, 11 samples for 2 serotypes, 2 samples for 3 serotypes and 1 sample reacted positive with 5 serotypes.

Key words*: equine leptospirosis, horses, MAT, reproduction, serological survey.*

INTRODUCTION

Leptospirosis is a zoonotic disease with a wide global distribution. The economic impact of leptospirosis on society stems from the expenses associated with farm animals and public health, including mortality, declining productivity, and abortions (Gurău & Drăgan, 2018). With over a million cases and almost 60,000 deaths annually from human infection, it is a major but underappreciated cause of morbidity and mortality (Costa et al., 2015); however, considering the nonspecific symptoms that many individuals present with, this number probably represents a severe underestimate of the disease burden (Sykes et al., 2022). The persistence of the aetiological agent in the environment is dependent on the interactions between humans, animals and ecosystems. The natural reservoir of *Leptospira* is wild animals, particularly rodents, which play an important role in the long-term maintenance and circulation of the causative agent in outbreaks (Burlacu et al., 2020). In many cases, wild, stray or domestic animals can carry the pathogen without exhibiting any noticeable symptoms. The stealthy carrier status facilitates dissemination of the bacteria in the environment, contaminating water sources and soil (Constantinescu et al., 2015).

Although horses have rarely been implicated in the spread of leptospirosis to humans or other animals (Hamond et al., 2013), seroreactivity is common in this species, and titres to multiple serovars have been reported, such as Copenhageni in Brazil (Hamond et al., 2013), Pomona in the USA (Timoney et al., 2011), and Icterohaemorrhagiae and Bratislava in North and South America, Europe and Asia (Turk et al., 2013; Verma et al., 2013).

While horses may present all the classical symptoms of leptospirosis (fever, anorexia, jaundice, anaemia, haemorrhages on the mucosa and depression) (Verma et al., 2013), a more common presentation is the subclinical chronic form, in which leptospiral colonization of the reproductive tract takes place. Also called "silent leptospirosis", it is characterized by low fertility, oestrus repetition and sometimes, late-term abortions. Often, this type of infection is caused by strains belonging to the Australis serogroup, particularly serovar Bratislava, which has been considered a non-pathogenic serovar, adapted to horses. (Di Azevedo & Lilenbaum, 2022).

The current study was performed to determine the prevalence of *Leptospira* antibodies in an equine breeding farm in order to gain insight into the possible reasons for reproductive underperformance exhibited by multiple mares.

MATERIALS AND METHODS

A total of 91 horses, 5 males and 86 females used for breeding, were included in the study. In the female group, both donors and recipients were tested. None of the horses had a history of vaccination against leptospirosis. During the clinical examination, no classic symptoms of leptospirosis were recorded in any of the horses; however, two of the recipient mares had suffered recent mid-term abortions. A large number of the mares included in the study presented with oestrus repetition and low fertility.

Blood samples were collected from each animal twice, 14 days apart. The samples were allowed to clot at room temperature for 4 hours, were centrifuged at 2000 rpm for 5 minutes, and the serum was collected in Eppendorf tubes and refrigerated until use. The serum samples were subjected to serological testing using the standard microscopic agglutination test (MAT). To detect the presence of *Leptospira* spp. antibodies, samples were tested against 12 reference serovars: Australis, Autumnalis, Ballum, Bataviae, Canicola, Grippothyphosa, Hardjo, Icterohaemorrhagiae, Pomona, Tarassovi, Sejroe and Wolffii. Prior to testing the equine serum samples, the identity of the antigens was confirmed against reference sera obtained from the Royal Tropical Institute in Amsterdam, The Netherlands. To reduce observer variability, the MAT was performed by one operator to limit inter-observer variations of the results.

Antigens were cultured in EMJH medium (Difco) for 7 days at 29°C, to a density of 2-3x10^8 CFU/ml. A screening test was performed using a 1/50 serum dilution, to which an equal volume of each antigen was added, making the final serum dilution 1/100. Testing was performed in microtitration plates, which were incubated for 2 hours at 29°C. The reactions were examined using a dark-field microscope, and samples showing ≥ 50% agglutination at the 1/100 dilution were considered positive. For each positive sample, serial dilutions were performed and tested against the respective antigen(s) to determine the final antibody titre. The endpoint was considered the final dilution showing 50% agglutination and 50% free *Leptospira* (WOAH, 2021).

RESULTS AND DISCUSSIONS

The first serological test revealed that 54 subjects (59.34%) were positive for *Leptospira* antibodies, including 20 recipient mares, 28 donor mares, 4 work horses and 2 stallions. Positive reactions were recorded for 10 of the 12 antigens included in the test. The majority of positive samples reacted to serovar Australis (83.33% of positive samples). The second most prevalent reaction was to serovar Icterohaemorrhagiae (22.22% of positive samples). The remaining serovars encountered lower prevalence: 7.40% for Grippothyphosa, 3.70% for Autumnalis, Bataviae, Canicola, Ballum and Sejroe, and 1.85% for Pomona and Tarassovi (Figure 1). No positive reactions were recorded for serovars Hardjo and Wolffii.

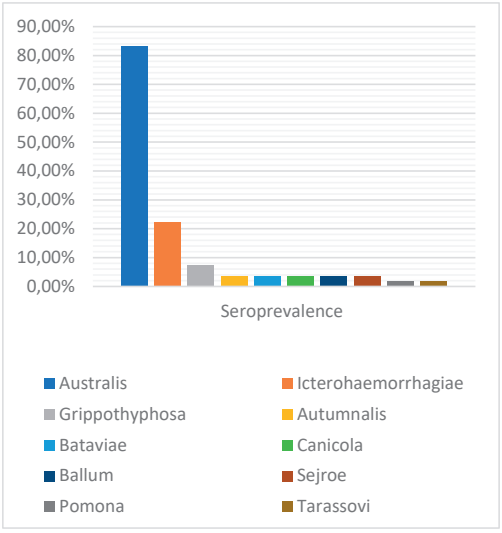

Figure 1. Prevalence of positive reactions to each *Leptospira* spp. serovar

The majority of the titres for most serovars were relatively low. Antibody titres of samples with positive reactions to Australis ranged from 1/100 to 1/1600. Samples positive for Icterohaemorrhagiae had titres of 1/100 to 1/200, as well as most of the samples with

positive reactions to the other serovars, only a few reaching 1/400. Table 1 presents the number of positive samples recorded for each serovar and their respective titres.

Table 1. Number of positive samples and antibody titres for each serovar

Number of samples/serovar										
Serovar/ Titre	Aus.	Ictero.	Gripp.	Aut.	Bat.	Can.	Ball.	Sej.	Pom.	Trs.
1/100	13	6	-	1	1	2	2	1	1	-
1/200	13	6	1	-	1	-	-	1	-	1
1/400	11	-	3	-	-	-	-	-	-	-
1/800	6	-	-	1	-	-	-	-	-	-
1/1600	2	-	-	-	-	-	-	-	-	-
Total	45	12	4	2	2	2	2	2	1	1

Legend: Aus. - Australis, Ictero - Icterohaemorrhagiae, Gripp. - Grippothyphosa, Aut. - Autumnalis, Bat. - Bataviae, Can. - Canicola, Ball. - Ballum, Pom. - Pomona, Trs. - Tarassovi.

The majority of samples were positive to 1 serovar (40 out of 54); however, we encountered several samples with positive reactions to 2 or more serovars (Table 2). While exposure to multiple serovars is possible, especially in a farm surrounded by wildlife, there is also the possibility of cross-reactivity occurring between different serovars (Barwick et al., 1998).

Table 2. Number of samples with positive reactions to one or more serovars

Number of serovars	Number of positive samples
1	40
2	11
3	2
4	0
5	1

The results of our study showed that a large number of horses had been exposed to *Leptospira* antigens. To determine if the horses were undergoing an active *Leptospira* infection, blood samples from the same animals were collected 14 days after the initial test samples and were subjected to the same protocol in order to determine any increase in the antibody titres. A fourfold increase in antibody titres in paired serum samples, collected in the acute and the convalescent phases of the disease, represents a definitive criterion for diagnosis (WOAH, 2021).

The second assessment confirmed most of the results obtained in the first test. The majority of the animals maintained positive reactions to the same serovars as detected during the first trial and with similar antibody titres. Out of the 91 horses included in the study, 6 recorded a fourfold rise in antibody titres during the trial. All 6 individuals had initially reacted positively to serovar Australis with titres from 1/100 to 1/400, and after 14 days, the titres were 4 times higher. None of the animals with increased antibody titres had any leptospirosis symptoms or any sign of disease. Lower increases in antibody titres were also recorded in 19 other individuals; however, only two- or three-fold, and not conclusive for acute disease diagnosis. The majority of the horses with negative samples in the first trial remained negative, with the exception of two mares, which were negative during the first trial and reacted positive to serovar Australis with a titre of 1/100 during the second trial (Table 3). The one mare which was positive to 5 serovars during the first MAT (Australis 1/100, Autumnalis 1/100, Ballum 1/100, Sejroe 1/100 and Tarassovi 1/200), during the second test reacted positive to only three serovars: Australis, with a titre of 1/400, Ballum 1/100 and Tarassovi 1/100. Leptospirosis in Romania has been intensely studied over the years, especially in humans and dogs (Romaniuc et al., 2001; Manciuc et al., 2018; Predescu et al., 2018; Sonea et al., 2024; Iorgoni et al., 2025), and in swine (Gurau and Dragan, 2018). However, in equids, there is very little to no information available on the prevalence of leptospirosis in our country. Equine leptospirosis is not only a potential cause of major economic losses but also an occupational hazard for the humans who come in contact with infected horses, as these animals

may be responsible for environmental contamination (Flores et al., 2017).

Numerous studies have been conducted worldwide to assess the prevalence of leptospirosis in equine populations. The reported prevalence rates vary considerably, reflecting differences in geographic location, environmental conditions, and study design.

Table 3. Number of positive samples and antibody titres for each serovar in the second test

Number of samples/serovar										
Serovar/ Titre	Aus.	Ictero.	Gripp.	Aut.	Bat.	Can.	Ball.	Sej.	Pom.	Trs.
1/100	10	6	-	-	-	-	1	1	1	1
1/200	8	5	3	-	-	1	-	-	-	-
1/400	16	1	1	-	-	1	-	-	-	-
1/800	6	-	-	1	-	-	-	-	-	-
1/1600	4	-	-	-	-	-	-	-	-	-
1/3200	1									
1/6400	1									
Total	46	12	4	1	0	2	1	1	1	1

Legend: Aus. - Australis, Ictero - Icterohaemorrhagiae, Gripp. - Grippothyphosa, Aut. - Autumnalis, Bat. - Bataviae, Can. - Canicola, Ball. - Ballum, Pom. - Pomona, Trs. - Tarassovi.

A study performed in Ukraine between 2007 and 2021 on 121,101 horses found that 10.8% tested positive for *Leptospira* antibodies. The dominant serovars were Copenhageni, with a prevalence of 32.8%; Bratislava - 16.1%; Grippothyphosa - 15.4%; and Canicola - 13.2%. Reactions to more than one serovar were found in 55.1% of positive cases (Ukhovskyi et al., 2023). In Switzerland, a study was performed on 615 horse sera against 15 *Leptospira* serovars. Results showed that 58.5% of samples were positive to one or more of the antigens. Pyrogenes had the highest prevalence, 22.6%, followed by Canicola (22.1%) and Australis (19.2%) (Blatti et al., 2011). Researchers from Croatia performed a 10-year-long study on apparently healthy horses and found that in the study period, seroprevalence of *Leptospira* antibodies varied between 5% and 15.94%. The dominant serovar was Pomona (41.98%), followed by Grippothyphosa (31.34%), Sejroe (8.03%), Icterohaemorrhagiae (7.05%), and Bratislava (6.47%) (Benvin et al., 2023). In Northern Italy, a study carried out on Bardigiano horses detected 67.2% seropositive animals, with the highest prevalence belonging to serovar Bratislava (41.8%), followed by Canicola, Tarassovi, Copenhageni, Pomona, and Hardjo (Vera et al., 2019).

In the present study, the most prevalent serovar was found to be Australis (83.33% of positive samples), followed by Icterohaemorrhagiae (22.22% of positive samples) and Grippothyphosa (7.40% of positive samples). Similar results were obtained by researchers in the USA, in the states of Missouri and Nebraska in 2016-2017, with a prevalence of 30.5% for serovar Australis, 25.4% for Grippothyphosa and 17.8% for Icterohaemorrhagiae (Trimble et al., 2018). In Colorado state, 82% of equine serum samples analysed for *Leptospira* antibodies were positive to at least one serovar. All samples were collected from apparently healthy horses, and serovar Bratislava had the highest prevalence (Fagre et al., 2020).

In New Zealand, 25% of equines subjected to MAT had *Leptospira* antibodies, with positive reactions to serovars Pomona (13%), Ballum (12%), Hardjo (9%), Copenhageni (22%), and Tarassovi (15%) (Bolwell et al., 2020). However, in Australia, serovar Arborea was serodominant in horses from Northern Queensland (Wangdi et al., 2013).

A high prevalence of *Leptospira* antibodies was found in numerous studies on horses from Brazil, with positive MAT results ranging from 16.2% in Brejo Paraibano (de Oliveira Filho et al., 2014), and 45.9% in the Southern States (Da Silva et al., 2020) to 61.6% in the State of Goiás (Romanowski et al., 2023).

The variability, prevalence and predominant serovars across the world highlight the complexity of understanding the global epidemiology of leptospirosis in horses and underscore the need for continued surveillance and standardized research approaches.

CONCLUSIONS

During the first serological test, 59.34% of samples were positive for antibodies, with a cut-off titre of 1/100. Of the positive samples, 20 were collected from recipient mares, 28 from donor mares, 4 from work horses and 2 from stallions. Serovar Australis had the highest prevalence (83.33% of positive samples), followed by Icterohaemorrhagiae (22.22% of positive samples), Grippothyphosa (7.40%), Autumnalis, Bataviae, Canicola, Ballum and Sejroe (3.70% each), and Pomona and Tarassovi (1.85% each). Results of the second microagglutination test were highly similar to the first, with the majority of the animals maintaining similar antibody titres. Out of the 91 horses included in the study, 6 recorded a fourfold rise in antibody titres during the trial.
The study highlights the presence of *Leptospira* antigen exposure in horses from our country. Given that infected horses often show no sign of leptospirosis, the findings of the current study underline the importance of routine testing and continued surveillance of equine populations, as these animals can serve as unnoticed carriers, posing a potential zoonotic risk.

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EFFECT OF CULTIVATION PARAMETERS ON BACTERIAL DENSITY AND TOXIN PRODUCTION OF A *CLOSTRIDIUM PERFRINGENS* TYPE C STRAIN

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Abstract

The purpose of the current study was to investigate the effect of certain cultivation parameters, such as pH adjustment, stirring and nutrient addition, on the bacterial density and toxin production of a Clostridium perfringens type C culture. Bacterial cultures were grown simultaneously on a 30-litre Bionet bioreactor and an industrial 500-litre bioreactor. Both experiments were performed using the same culture media, the same nutrients and the same inoculum and during the same timeframe. Differences in bioreactor equipment, such as homogenisation systems and pH measuring equipment, resulted in different final cultivation outcomes. The toxicity of the experimental Bionet culture was determined at 3200 LD_{50}, while the industrial bioreactor culture tested at only 800 LD_{50} toxicity.

Key words: *beta toxin, immunogenic, Clostridium perfringens, cultivation, vaccine.*

INTRODUCTION

Clostridium perfringens is an anaerobic, Gram-positive, spore-forming bacterium that, compared to other anaerobes, is relatively resistant in the presence of oxygen (McClane & Robertson, 2013). It is a major pathogen of both humans and animals, its virulence being largely due to secreted toxins but also to its very rapid doubling time, which allows it to quickly reach pathogenic burdens in the infected material (Gohari et al., 2021).

Clostridium perfringens type C (ClpC) is the etiological agent of necrotising enteritis in domestic animals, such as goats, pigs, cattle, horses, and humans. In small ruminants, the infection causes haemorrhagic enteritis in lambs and an acute enterotoxaemia, known as "struck", manifesting as sudden death in adult sheep (Diab et al., 2016). In piglets, calves, foals, and kids, type C strains produce haemorrhagic and necrotic enterotoxaemia. In poultry, ClpC strains can cause necrotic enteritis, resulting in increased mortality and the risk of contamination of products intended for human consumption (Caraman et al., 2024). Piglets are especially susceptible to the infection, which can result in mortality rates of 30 to 50% (Fisher et al., 2006).

C. perfringens is a rapidly multiplying bacterium and a prolific toxin producer (Anwar et al., 2019). Virulence factors of ClpC strains include beta toxin, alpha toxin and also other toxins such as perfringolysin O, beta2 toxin, and/or enterotoxin (Rood, 1998). The ability to sporulate grants them increased resistance in the environment, making them impossible to eradicate (Zaragoza et al., 2019). With the increasing concern regarding antibiotic resistance among bacterial strains in the intensive farming system (Șandru et al., 2023), active immunisation with toxoid vaccines remains the main protective method against infection (Zaragoza et al., 2019). Type C outbreaks determine massive economic losses and mortality rates that can reach 100% in unvaccinated herds (Nagahama et al., 2015). The efficacy of anaerobic vaccines depends largely on the toxin yield of the bacterial culture. Toxin production by ClpC depends on the presence of a peptide source, pH control, and the presence of nutrients in the form of fermentable

carbohydrates, such as glucose, fructose or dextrin, which have been shown to increase culture growth and toxin production (Ducan, 1979; Esmaeilnejad-Ahranjani, 2023).

The purpose of the current study was to investigate the efficacy of two different cultivation settings, a 30-litre Bionet bioreactor and a 500-litre industrial bioreactor, in regard to bacterial density and toxin production, for a vaccine strain of ClpC.

MATERIALS AND METHODS

The subject bacterial strain, *Clostridium perfringens* type C - Stamatin, belonged to the Seed Bank of Pasteur Institute, and the experiment was carried out within the Antibacterial Vaccines Department of the Pasteur Institute, in Bucharest. The culture media utilised in the experiment were prepared in-house. The inoculum was prepared using VF culture medium, containing *Clostridium perfringens* sporulation broth, liver hydrolysate and yeast extract, all three ingredients in atomised powdered form, to which L-cystine, glucose and liquid yeast extract (*Saccharomyces karlsbergensis*, neutralized to pH 7.2-7.4 and clarified by filtration) were added. The pH of the medium was adjusted to 7.6 using NaOH solution. The culture medium (*C. perfringens* medium) for the bioreactor cultivation was prepared after an original recipe, containing liver meat glucose cysteine broth, cooked meat medium broth, e.t. medium, *Clostridium perfringens* sporulation broth, casein yeast peptone, liver hydrolysate, yeast extract, liver extract, glucose, l-cysteine and purified water. The culture medium was sterilised by filtration with 0.2 µm pore size Millipore filters.

The lyophilised bacterial strain was reconstituted by adding 2 mL of sterile PBS solution and homogenising by repeated pipetting. The inoculum was obtained by seeding VF broth tubes with 0.5 mL reconstituted strain. The seeded tubes were incubated for 6 hours at 37°C under anaerobic conditions. The cultures obtained on VF medium were transferred into glass bottles containing 400 mL *C. perfringens* medium and incubated following the same protocol. In preparation of the experiment, the bioreactors were sterilised, filled with the filtered culture medium - 20 L of medium for the Bionet bioreactor and 450 L for the industrial bioreactor, and brought to 37°C. Four sterile glass bottles containing the inoculum, 50% glucose solution, 10N NaOH solution and liquid yeast extract were attached to each bioreactor.

Bionet cultivation. Anaerobic conditions during cultivation were ensured by introducing a gas mixture containing 85% nitrogen, 5% carbon dioxide and 10% hydrogen into the bioreactor at a pressure of 300 bar and stirring at 400 rpm for 30 minutes at a flow rate of 5 L per minute. Before inoculation, the temperature of the culture medium was brought to 36±2°C. When the set temperature was reached, yeast extract and glucose were added to the medium. Stirring was stopped and the medium was inoculated with the prepared inoculum. Throughout cultivation, the pH and temperature of the culture were monitored in real time. The Bionet cultivation parameters were as follows: temperature 37°C, homogenisation 200 rpm, pH 7.4. After inoculation, visible growth was detected at 2 hours p.i. At this time, the bioreactor began the automatic pH regulation. Nutrient addition was performed manually to support the growth of the culture, as required. Samples were collected at 1 h, 2 h, 3 h, 4 h, 5 h, and 6 h p.i.

Industrial bioreactor cultivation. The culture medium was brought to 37°C and the pH was adjusted to 7.4 using 10N NaOH solution. The same mixture of gases was used to create anaerobic conditions. Yeast extract and glucose solution were added and homogenised with the medium, after which the inoculum was added. Samples were collected every hour, and the pH was measured and adjusted manually. Homogenisation was performed using the bioreactor's vibration system, and nutrients were also added manually during cultivation. Samples for the comparison experiment were collected at the same times p.i. as the Bionet cultivation (1 h, 2 h, 3 h, 4 h, 5 h, and 6 h).

The collected samples from both cultivations were stored on ice until processing. Gram-stained slides were prepared from both cultures to microscopically assess the morphology of the bacilli. The cultures were seeded onto Columbia blood agar and Sabouraud agar and incubated at 37°C to ensure bacterial purity and fungal sterility.

To determine the differences between the experimental cultivations, the samples from each cultivation were tested for bacterial density and toxicity. Bacterial density was determined by inoculating 10-fold dilutions of the culture samples into plates with melted TSC agar. The plates were incubated for 24 h at 37°C in anaerobiosis, after which the resulting colonies were counted.

The ClpC toxin was prepared by culture centrifugation (4000 rpm, 30 minutes, 4°C) and supernatant filtration through 0.8, 0.45, 0.22 and 0.1 µm pore size Millipore filters. To determine the median lethal dose, dilutions in peptone water were made from whole toxins. The potency of bacterial toxins from each collected sample was tested on *BALB/c* mice of 21-24 grams. Each mouse received an i.v. injection of 0.5 ml of the supernatant dilution. The toxin dilutions used in the assay were 1/100, 1/200, 1/400, 1/800, and 1/1600. As negative controls, 3 mice received i.v. injections of sterile peptone water. The supernatant toxicity titre was calculated as twice the reciprocal of the highest dilution that induced mortality of at least 2 out of 3 mice within 72 hours of administration.

RESULTS AND DISCUSSIONS

The microscopic examination of the samples revealed Gram-positive rods, short, thick, with rounded ends, isolated or grouped in clusters (Figure 1). On blood agar, the colonies appeared smooth, greyish, with intense haemolysis.

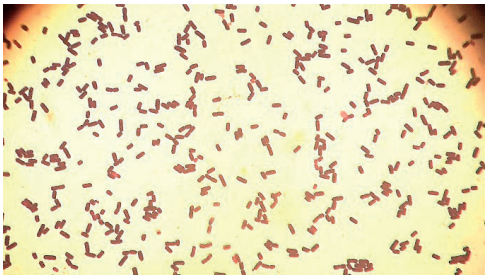

Figure 1. *Clostridium perfringens* type C, microscopic aspect

Culture parameters for the Bionet cultivation are presented in Table 1.

The density of the bacterial cultures obtained from the Bionet cultivation sampling was as follows: sample 1 collected 1-hour p.i. had a bacterial density of $4.5x10^2$, sample 2 collected at 2 hours p.i. - $5x10^3$, sample 3 (3 hours p.i.) - $7.8x10^4$, sample 4 (4 hours p.i.) - $9.2x10^5$, sample 5 (5 hours p.i.) - $3.4x10^7$, and sample 6 (6 hours p.i.) - $3.8x10^8$ (Table 2).

Table 1. Cultivation parameters for Bionet culture experiment

	pH	Stirring	NaOH	Nutrients
1 h	7.40	-	-	-
2 h	7.20	191 rpm	120 mL	-
3 h	7.35	198 rpm	150 mL	G - 40 mL Y- 40 mL
4 h	7.25	198 rpm	158 mL	-
5 h	7.28	199 rpm	170 mL	-
6 h	7.20	199 rpm	-	-

Legend:1 h, …, 6 h - hours post-inoculation, rpm - rotation per minute, G - Glucose 40%, Y - Yeast extract.

The industrial bioreactor cultivation was performed at the same time as the Bionet cultivation, using the same batches of culture media and nutrients. pH measurements and adjustments were performed manually on an hourly basis.

The industrial bioreactor samples had slightly lower bacterial density: sample 1 (1-hour p.i.) – $1.25x10^2$, sample 2 (2 hours p.i.) – $1.95x10^2$, sample 3 (3 hours p.i.) – $7x10^3$, sample 4 (4 hours p.i.) – $9.4x10^4$, sample 5 (5 hours p.i.) – $5.3\ x10^5$, and sample 6 (6 hours p.i.) – $3x10^7$ (Table 2).

Table 2. Bacterial density of the samples collected from the experimental cultivations

Sample	Bacterial density - Bionet cultivation	Bacterial density - Industrial cultivation
1	$4.5x10^2$ CFU/mL	$1.25x10^2$ CFU/mL
2	$5x10^3$ CFU/mL	$1.95x10^2$ CFU/mL
3	$7.8x10^4$ CFU/mL	$7x10^3$ CFU/mL
4	$9.2x10^5$ CFU/mL	$9.4x10^4$ CFU/mL
5	$3.4x10^7$ CFU/mL	$5.3x10^5$ CFU/mL
6	$3.8x10^8$ CFU/mL	$3x10^7$ CFU/mL

The mouse intravenous injection model was used to assess the lethality of the culture supernatants obtained from each collected sample. Sterile filtered culture supernatants were used to prepare dilutions in peptone water, which were injected into the tail vein of Balb/C mice.

The supernatant toxicity evaluation revealed superior results for the Bionet cultivation,

reaching a maximum toxin titre of 3200 LD_{50} within 5 hours of cultivation (Table 3). The industrial bioreactor cultivation only yielded a titre of 800 LD_{50} on samples collected 5-, and 6-hours p.i. (Table 4). The negative control group survived, without exhibiting any clinical signs of illness, after being administered peptone water.

Table 3. Toxicity test results for the Bionet culture (number of dead mice within 72 hours of administration)

Sample	Toxin dilutions				
	1/100	1/200	1/400	1/800	1/1600
1	-	-	-	-	-
2	-	-	-	-	-
3	3	3	2	1	-
4	3	3	3	1	-
5	3	3	3	3	2
6	3	3	3	2	2

The results of the Bionet experiment show that samples collected after 5 and 6 hours of cultivation had the highest toxicity levels. It also shows a direct correlation between the increasing bacterial density and the rise in toxicity levels, with the exception of the last sample, where LD_{50} remained the same as sample 5, despite having a higher CFU/ml than sample 5.

Table 4. Toxicity test results for the industrial culture (number of dead mice within 72 hours of administration)

Sample	Toxin dilutions				
	1/100	1/200	1/400	1/800	1/1600
1	2	1	-	-	-
2	3	3	2	-	-
3	3	3	1	-	-
4	3	3	2	-	-
5	3	3	2	-	-
6	3	3	3	1	-

Supernatants obtained from the industrial culture samples had a rather stable level of toxicity, showing little to no correlation with the increase of the bacterial density or the fluctuation of the pH levels.

For decades, researchers have investigated the optimum cultivation techniques for *C. perfringens* strains in order to increase bacterial density and toxin production. It has been reported in literature that toxin production of *C. perfringens* strains varies depending on a number of factors, such as the bacterial strain, the culture medium, pH, heat treatment, etc. (Saito, 1990). Studies have shown the importance of the nutrients contained by the culture medium regarding toxin production, proving that different media provide different toxicity outcomes when used to cultivate the same bacterial strain (Fernandez-Miyakawa et al., 2007). In regard to the optimum homogenisation technique during cultivation, Esmaeilnejad-Ahranjani et al. (2023) have found that increasing the stirring rate to 300 rpm decreased the lag time from 60 min to 30 min; however, enhancing it further had the opposite effect, increasing the lag time back to 60 min. In industrial conditions, due to the large volume of culture, stirring plays an important role, as it has been documented that an uneven distribution of a culture in a bioreactor may cause gradients in biomass, substrate and pH, which may lead to a decrease in productivity (Esmaeilnejad-Ahranjani et al., 2022; Madian et al., 2022). Maintaining a stable pH throughout cultivation is a crucial factor in determining the toxicity of the culture. Researchers have demonstrated that pH level is one of the factors which have a remarkable effect on *Clostridium* toxin activity (Ahmed et al., 2022; Khoshanabadi et al., 2022; Tariq et al., 2022). This may explain the results obtained in the current study, specifically the lower toxicity of the culture obtained in the industrial bioreactor which had a vibration homogenising system, compared with the Bionet culture, obtained with a constant stirring at 200 rpm.

CONCLUSIONS

Clostridium perfringens type C cultures were grown simultaneously in a 30-litre Bionet bench scale bioreactor and a 500-litre industrial bioreactor, using the same bacterial strain and culture medium, to assess the difference in bacterial density and toxin production. Samples were collected each hour post-inoculation and tested for bacterial density by incorporating 10-fold dilutions into melted TSC agar and for toxicity using the mouse intravenous injection model. Results showed that the Bionet culture had a final bacterial density of $3.8x10^8$ CFU/mL, while the industrial culture obtained only $3x10^7$ CFU/mL. Toxicity evaluation of the bionet

experiment showed a maximum toxin titer of 3200 LD_{50} within 5 hours of cultivation. The industrial cultivation had a toxin titre of 800 LD_{50} on samples collected 5-, and 6-hours p.i. Further research is necessary in order to optimise cultivation parameters for a more successful scaling-up process.

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CASE REPORT REGARDING THE OUTCOME OF SHOCKWAVE THERAPY IN THE TREATMENT OF TENDINOPATHIES WITH LOW HEALING RATES IN SPORT HORSES

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Abstract

Tendinopathies in sport horses, particularly those with low healing rates, represent a major challenge in veterinary medicine. Shockwave therapy has shown promising significant effects, stimulating tendon tissue healing and reducing pain compared to conventional treatments. Deep digital flexor tendon (DDFT) injuries are common in sport horses and are caused by repetitive biomechanical stress. This study examines the success of shockwave therapy in managing insertional injuries of the DDFT with low healing rates in a 14-year-old female Oldenburg show-jumping horse. Clinical and imaging assessments through MRI revealed significant improvements in approximately three months of therapy, including reduced pain, increased mobility, and improved healing of the affected tendon. This case report aims to demonstrate that Shockwave Therapy (ESWT) increases the healing rate, reducing recovery time in sport horses.

Key words: *Shockwave, tendinopathy, horse, DDFT.*

INTRODUCTION

Tendinopathies are among the most frequent musculoskeletal injuries in competitive sport horses, often causing significant performance deficits and prolonged downtime (Morris & Carter, 2015). The disorder is characterized by the disorganization of collagen fibers, increased interfibrillar spaces, and the formation of scar tissue, all of which contribute to reduced tensile strength and functional integrity of the tendon (Jones et al., 2017). In recent years, attention has turned toward innovative treatment approaches such as shockwave therapy (Gonzalez & Reed, 2018). This treatment option has gained popularity in human and equine medicine due to its potential to stimulate neovascularization, promote collagen synthesis, and induce anti-inflammatory effects (Sherry et al., 2022; Smith et al., 2016).

Traditional management of equine tendinopathies has relied on rest, controlled exercise, and various medical treatments including nonsteroidal anti-inflammatory drugs and regenerative therapies (Morris & Carter, 2015). Despite these interventions, the healing process is often protracted, with a high incidence of re-injury reported in sport horses (Thompson et al., 2019). In contrast, shockwave therapy uses sound waves to mechanically stimulate cellular responses that facilitate tissue repair (Jones et al., 2017). Recent experimental studies have shown promising results, highlighting rapid improvements in tendon architecture and function when combined with standard rehabilitation protocols (Gonzalez & Reed, 2018; Sara Toner and Candice Crosby , 2023). However, clinical documentation of shockwave therapy in naturally occurring cases remains relatively scarce, particularly with low healing rates (Thompson et al., 2019).

MATERIALS AND METHODS

To characterize the diagnosis and evolution of tendinopathy treatment, the following method were used: orthopaedic examination, radiographic examination, Magnetic Resonance Imaging (MRI), Pro Stride therapy, shockwave therapy, and orthopaedic bracing.

The period analysed in this study was August-October 2024.The results obtained from the

methods applied in the clinic were processed by analysing the data, identifying trends, and evaluating the outcomes of each treatment. The data were statistically analysed to build a comprehensive overview of the treatment evolution for tendinopathies (Gagnier et al., 2013).

Patient History and Signalment

The subject, a 14-year-old female Oldenburg horse actively competing in show-jumping events, presented with left forelimb lameness that had been persistent since the previous year. Initially, the condition responded positively to coffin joint injections with PRP; however, by spring, the efficacy of the treatment diminished, with symptom relief lasting less than one month. Following a competition, the lameness reappeared. Diagnostic imaging, including MRI and ultrasonography, confirmed the involvement of the deep digital flexor tendon (DDFT) and the navicular bursa. The primary issue was ongoing lameness, which significantly impaired both the horse's athletic performance and training capabilities.

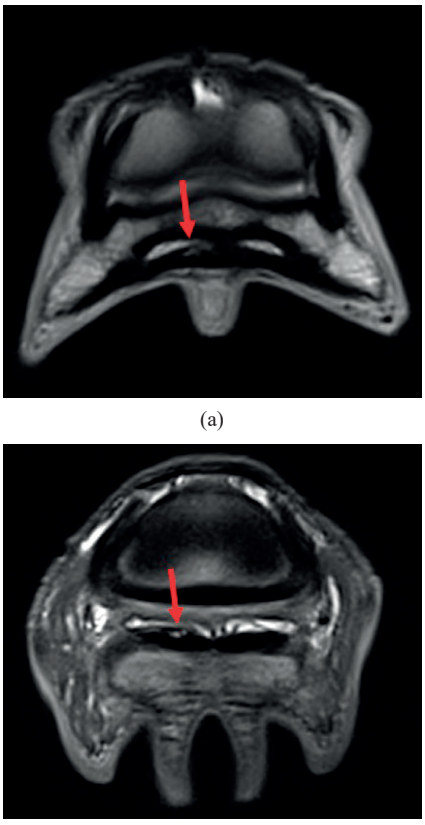
RESULTS AND DISCUSSIONS

Clinical examination and diagnostic work-up

A comprehensive clinical examination revealed moderate to severe lameness in the affected limb. The pre-treatment lameness score for the left forelimb (LF) was assessed as 1/5 on a straight line.

On a hard circle, the lameness score was recorded as 2/5 on both sides, the horse showing increased lameness on a hard surface. In addition to the physical examination, a diagnostic MRI revealed moderate active tendinopathy of the DDFT with two core lesions at the supra- and infra-navicular levels (Figures 1, 2). There was also moderate proliferative navicular bursitis with possible adherence formation, mild navicular bone changes suggestive of navicular disease, and mild bone oedema of the distal phalanx indicating aseptic pedal osteitis (Figure 3). Additionally, mild osteoarthritis of the distal interphalangeal joint was observed.

At the orthopedic consultation conducted in early August, including the SLEIP application for (objective gait analysis) evaluation, an increase in lameness severity was observed (Figures 4, 5). A treatment protocol was implemented, consisting of intrabursal regenerative therapy with Pro Stride, orthopedic shoeing and Shockwave therapy



(a)

(b)

Figure 1. MRI depicting core lesions in the deep digital flexor tendon (arrow). The lesions are at the infra-navicular (a) and supra-navicular level (b)

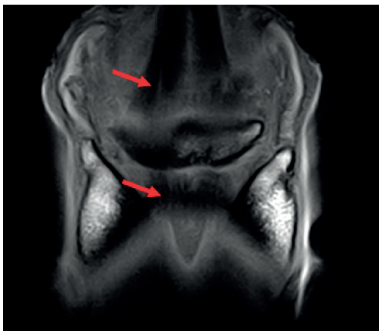


Figure 2. Two core lesions in the medial lobe of the DDFT at different levels (arrows)

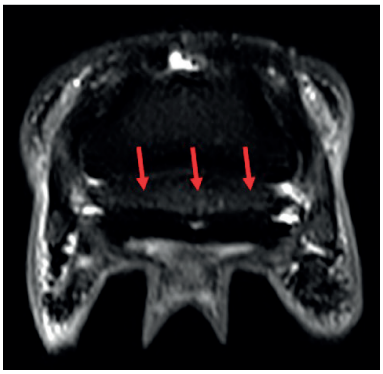


Figure 3. Navicular bone edema (arrows)

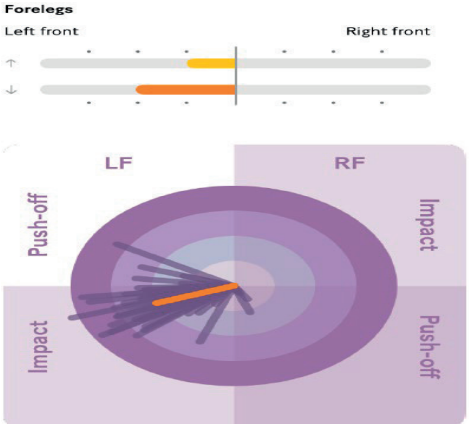


Figure 4. The image shows a SLEIP gait analysis, objectively confirming lameness progression in the left forelimb

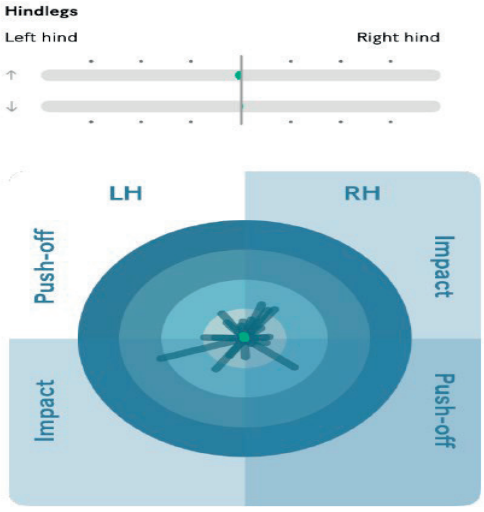


Figure 5. SLEIP gait analysis, confirms that the hind limbs show no signs of lameness

Treatment procedure

Considering the chronic nature of the condition and poor healing history, with the previously mentioned treatments, shockwave therapy was recommended as an adjunct treatment. The therapy was administered using a focused shockwave generator, calibrated to deliver an energy flux density between 0.12 and 0.20 mJ/mm². The treatment was administered every 7 days throughout 5 sessions, with each session consisting of 1000 pulses at a rate of 180 pulses per minute.

Selected treatment parameters were those recommended in recent veterinary studies referring to shockwave therapy (Sherry et al., 2022).

Pro Stride procedures were performed under aseptic conditions, using radiographic guidance to precisely target the podotrochlear bursa (Figure 6).

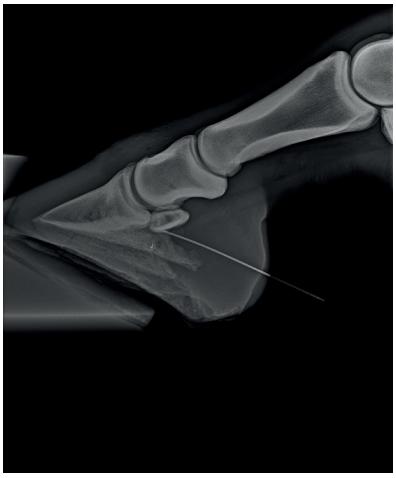


Figure 6: The X-ray image shows radiographic guidance for precise Pro-Stride injection into the podotrochlear bursa, ensuring accurate placement and effective treatment

The horse was sedated, and blood collection was performed only after asepsis of the site by well-trained personnel. Blood collection is performed by drawing 60 ml from the jugular vein into a syringe containing ACD-A anticoagulant. The first centrifugation step is conducted at 3200 rpm for 15 minutes, facilitating the separation of cellular components. Following this, platelet-poor plasma is removed, while 2 ml of platelet-rich plasma (PRP) is extracted from the side port. This PRP is subsequently transferred into

the APS Concentrator, where it is mixed with specialized beads designed to optimize protein enrichment. A second centrifugation step at 2000 rpm for 2 minutes further refines the solution. The final product, consisting of 2-3 ml of Autologous Protein Solution, is extracted via syringe and prepared for therapeutic application.

Rehabilitation Protocol

The shockwave sessions were integrated into a rehabilitation program. Following the first treatment session, the horse was placed on a controlled exercise regimen using hand-walking and gradually increasing on straight line. Pain management was strictly regulated with the transient administration of nonsteroidal anti-inflammatory drugs – Phenylbutazone 10 ml (2.2 mg/kg) for 7 days – during the initial post-treatment period. Consistent monitoring was implemented through serial lameness scoring SLEIP, as well as ultrasonographic, MRI, and radiographic evaluations.

After three months of treatment and a structured rehabilitation plan, significant improvements were observed. SLEIP gait analysis confirmed the effectiveness of the treatment protocol and the added benefit of Shockwave therapy, showing a substantial reduction in lameness in the left forelimb (Figure 7), MRI comparisons demonstrated notable healing, with improvements in the deep digital flexor tendon tear and a reduction in static navicular bursitis (Figures 8, 9).

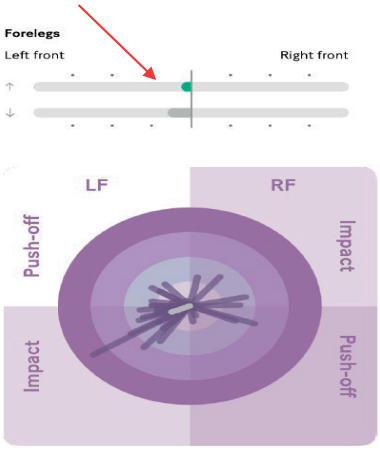


Figure 7. SLEIP gait analysis, highlighting the effectiveness of the treatment protocol and the added benefit of Shockwave therapy, confirming a significant reduction in lameness in the left front limb (arrow)

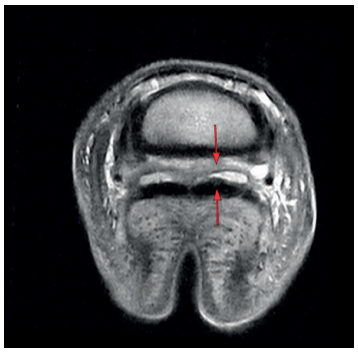


Figure 8. Improved deep digital flexor tendon tear, static navicular bursitis

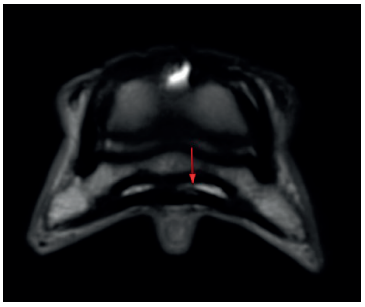


Figure 9. Prior MRI comparisons, based on previous imaging that initially revealed lesions at the infra-navicular and supra-navicular levels, two core lesions in the medial lobe of the DDFT at different levels, and navicular bone oedema, showed a significant improvement in the deep digital flexor tendon tear

This case report demonstrates the potential effectiveness of shockwave therapy in managing chronic tendinopathies in sport horses, particularly those with inherently low healing rates. Improvements were observed after three months of treatment due to a structured rehabilitation plan. Thus, significant SLEIP gait analysis confirmed the effectiveness of the treatment protocol and the added benefit of Shockwave therapy, showing a substantial reduction in lameness in the left forelimb. MRI imaging has demonstrated by comparison a notable healing process, with improvements in the deep digital flexor tendon tear and a reduction in static navicular bursitis. The subject's improvement, demonstrated by reduced lameness scores and improvements on MRI and ultrasonography, supports preliminary studies suggesting that mechanical stimulation induced by shockwaves can promote a regenerative response. The clinical findings reported herein are consistent with the

hypothesis that shockwave therapy facilitates the reorganization of collagen fibres and promotes biomechanical stability in injured tendon tissues (Jones et al., 2017). The application of shockwave therapy in this case not only expedited the reduction of pain and lameness but also provided objective improvements in tendon structure as visualized on ultrasonography.

In a comparative context, conventional management strategies including rest, anti-inflammatory medications, Pro-stride, and controlled exercise have been shown to yield variable healing times and a substantial risk of re-injury.

The mechanism of shockwave therapy is believed to be multifactorial. Biologically, the induced mechanical forces are theorized to stimulate growth factors such as vascular endothelial growth factor (VEGF) and promote the proliferation of fibroblasts essential for collagen matrix formation. Furthermore, by triggering a localized inflammatory response, shockwave therapy may catalyse the natural healing process, overcoming the stagnation observed in chronic injuries. In this context, the case findings suggest that targeted shockwave therapy could shift the tendon microenvironment towards one that is conducive to repair.

CONCLUSIONS

In this detailed case report, shockwave therapy demonstrated significant potential in the treatment of chronic tendinopathy in a sports horse with a history of low healing rates. Over three months of treatment, the combined objective improvements in lameness scoring - from a pre-treatment score of 1/5 and 2/5 to lame-free - and the significant reduction in lesion size observed on MRI highlight the effectiveness of this therapeutic protocol. In conclusion, the integration of shockwave therapy into a comprehensive rehabilitation program may accelerate functional recovery and improve structural tendon repair in sports horses suffering from chronic tendinopathies. This case provides a valuable reference point for future studies and contributes to the evolving understanding of advanced therapeutic strategies in veterinary sports medicine.

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A RETROSPECTIVE STUDY ON THE INCIDENCE OF CRYSTAL TYPES IN CATS

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Abstract

Cats exhibit a high susceptibility to the formation of urinary calculi, which can lead to severe urinary complications. This study was conducted on a batch of 46 cats from a private veterinary clinic in Bucharest, consisting of 15 females and 31 males. Among the females, 14 were spayed, while 27 of the males were neutered.
The most frequently identified type of urolith was magnesium ammonium phosphate (struvite), observed in 25 cases, followed by amorphous crystals in 13 cases, calcium oxalate in 3 cases, and mixed urolithiasis (magnesium ammonium phosphate and calcium oxalate) in 5 cases. Shorthairs showed the highest prevalence of struvite crystals. Middle-aged cats were the most affected, with a higher incidence in neutered males. These findings underscore the importance of early urinalysis and breed-specific preventive strategies to reduce the risk of surgical intervention.

Key words*: uroliths, cats, calcium oxalates, magnesium ammonium phosphates, urinalysis.*

INTRODUCTION

Urolithiasis is the second most common cause of clinical signs associated with feline urinary tract disease. The term "urolithiasis" refers to the presence of uroliths in any part of the urinary tract, although it is most frequently observed in the bladder and urethra. Uroliths are classified according to the mineral composition of their structure. As such, both quantitative and qualitative analyses of the uroliths are essential for determining an appropriate therapeutic approach (Gomes et al., 2018).
The formation of urine supersaturation with crystalloids is influenced by several factors, including the volume of crystals in the urine, renal crystal excretion, urine concentration, pH, and the presence or absence of urolith-promoting substances or inhibitors (Defarges et al., 2020; Kovaříková et al., 2021).
Urolithiasis is a prevalent condition in both dogs and cats, with struvite and calcium oxalate being the most commonly identified mineral types. However, other mineral compositions may also contribute to the formation of uroliths. Certain types of uroliths, such as struvite, purines, and cysteine, are amenable to medical dissolution, whereas others, including calcium oxalate and compound uroliths, are resistant to this form of treatment (Bartges, 2018; Jepson, 2023; Canello et al., 2017)

MATERIALS AND METHODS

To evaluate the incidence and distribution of various types of uroliths in felines, we conducted a retrospective study between 2022 and 2024 at a private veterinary clinic in Bucharest. The research encompassed a cohort of 47 domestic cats, comprising both males and females, presenting with varying clinical manifestations. While some exhibited overt lower urinary tract signs such as hematuria, dysuria, pollakiuria, or urinary obstruction, others were asymptomatic and diagnosed incidentally.
Comprehensive diagnostic assessments were performed, including abdominal ultrasonography and urinalysis, which facilitated the identification of urolithiasis localized to the urinary bladder. Urine samples were aseptically collected via ultrasound-guided cystocentesis to ensure sample integrity and minimize contamination risk. These specimens underwent thorough analysis in the clinic's in-house laboratory.

Following sample collection, the urine was analyzed immediately. The sample was centrifuged for 5 minutes at 2000 rpm. After centrifugation, the supernatant was discarded, leaving approximately 0.5 mL of fluid to retain the sediment. The sediment was then gently resuspended using a pipette. A single drop of the resuspended sediment was placed on a microscope slide, covered with a cover slip, and examined microscopically. Microscopic evaluation was performed using a 10× objective for initial scanning and a 40× objective for the identification of uroliths

RESULTS AND DISCUSSIONS

Of the total number of felines included in the study, 15 were females and 31 were males. Among the females, 14 were spayed, which represents 93% of the total number of females, while only one remained intact, 7% of the total number of females. Regarding the males, 27 were neutered, which represents 87%
of the total number of males and only four were unneutered, which accounts for 13%.

A key aspect of this study was the systematic classification of feline subjects based on breed in order to evaluate potential breed-specific predispositions to urolithiasis.

Of the total number of cats included in the study, 32 were European Shorthair, representing 70% of the total population, followed by 8 British Shorthair cats, accounting for 18%. Additionally, there were 2 individuals from each of the Persian, Birman, and Russian Blue breeds, each constituting 4% of the total (Table 1). Identifying breed-related susceptibility is essential for understanding genetic or physiological factors that may contribute to the pathogenesis of urinary stone formation. By categorizing felines according to their breed, this study aimed to assess whether particular breeds exhibit a higher incidence of urolithiasis, which could have implications for targeted preventative measures, breed-specific dietary recommendations, and early diagnostic strategies.

To compare with our study, a study conducted on a larger sample of animals has been cited in the specialized literature, highlighting similar trends or notable differences regarding the incidence of urolithiasis in different cat breeds, this study was conducted over eight years, analyzing a cohort of 143 feline cases, identified a predominant incidence of urolithiasis among specific feline breeds. The vast majority of affected individuals were domestic shorthair cats, accounting for 99.69% of cases, followed by domestic longhair cats at 21.15% and domestic medium hair cats at 6%. These findings suggest a potential breed predisposition, with domestic shorthair cats being disproportionately affected. Furthermore, the mean age of cats diagnosed with urolithiasis was determined to be approximately seven years, indicating that middle-aged felines may be at an increased risk for urinary stone formation (Dear et al., 2011).

Table 1. The classification of cats based on their breed

Breed	Total number
Persian Cat	2
European shorthair	32
British Shorthair	8
Birman	2
Russian Blue	2

Age was a fundamental demographic variable evaluated in this study to investigate its potential contribution as a predisposing factor in the pathogenesis of urolithiasis in felines. The correlation between age and the incidence of urolithiasis is of important clinical significance, as physiological, metabolic, and dietary changes associated with aging may influence urinary tract health and the propensity for urolith formation.

Among the 46 feline subjects included in the study, 20 individuals (44%) were classified within the 0-5-year age group, indicating that a significant proportion of cases occurred in younger cats. The second-largest group comprised felines aged 6-10 years, accounting for 18 cases (39%), suggesting that middle-aged cats may also be at considerable risk for urolithiasis. Furthermore, 8 cats (17%) fell within the 11-16-year age category, representing the geriatric population in this study (Figure 1). These findings provide valuable insights into the age-related distribution of urolithiasis and may support the need for age-specific management strategies, including dietary modifications and routine urinalysis screening in at-risk populations.

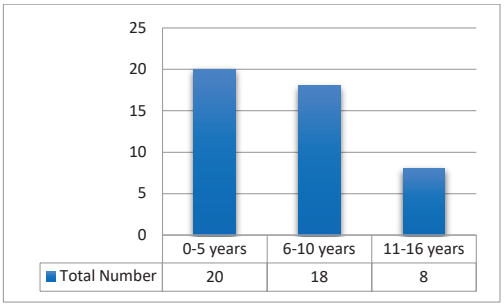

Figure 1. The classification of felines based on age

A total of 46 urine samples were analysed to determine the prevalence and distribution of different types of urinary crystals associated with urolithiasis in felines. The most frequently identified crystalline composition was ammonium-magnesium phosphate, commonly known as struvite, which was present in 25 samples, accounting for 54% of the total cases.

In 13 samples (28%), amorphous crystals were detected. These crystals, which may consist of a mixture of various mineral components, are often considered a nonspecific finding. However, their presence can indicate urinary supersaturation and a predisposition to urolith formation, particularly in felines with underlying metabolic imbalances or dietary influences that affect urine composition.

A mixed composition of both ammonium-magnesium phosphate and calcium oxalate crystals was identified in 5 samples, representing 11% of the total cases. The coexistence of these two types of urolithogenic minerals suggests fluctuating urinary pH conditions, potentially influenced by dietary intake, hydration status, or concurrent metabolic alterations. Such cases may be of particular clinical concern, as they indicate a complex pathophysiological process requiring individualized therapeutic and dietary management strategies.

Calcium oxalate crystals were identified as the sole mineral component in only 3 urine samples, comprising 7% of the total cases.

These findings provide valuable insight into the distribution of urinary crystals in felines diagnosed with urolithiasis, highlighting the predominance of struvite crystalluria while also acknowledging the occurrence of mixed and calcium oxalate compositions. The data emphasize the importance of urine analysis in the early detection of crystal formation, allowing for timely intervention through dietary modifications, increased water intake, and urinary pH regulation to mitigate the progression to clinical urolithiasis (Figure 2).

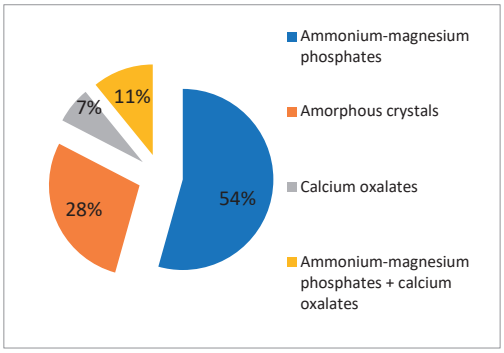

Figure 2. Classification based on the percentage of uroliths identified in the analysed samples

A comprehensive study conducted at the Minnesota Urolith Centre provided valuable epidemiological data regarding the incidence and demographic distribution of urolithiasis in felines. The findings indicated that struvite urolithiasis was most frequently diagnosed in cats between the ages of 2 and 7 years, suggesting that younger to middle-aged felines may be at an increased risk for the development of struvite-based urinary calculi. This pattern may be associated with dietary factors, urine pH, and differences in metabolic activity that influence urinary supersaturation with magnesium, ammonium, and phosphate ions, the primary constituents of struvite crystals.

Furthermore, the study highlighted a notable sex predisposition, with a higher prevalence of struvite urolithiasis observed in female cats compared to their male counterparts.

Breed predisposition was also examined, revealing that Siamese cats exhibited an increased susceptibility to struvite urolith formation compared to other feline breeds. This suggests a possible genetic or breed-related metabolic factor influencing urinary mineral composition, warranting further investigation into breed-specific risk factors and preventative measures.

In contrast, the incidence of calcium oxalate urolithiasis followed a distinct pattern. The mean age of affected felines ranged between 8 and 12 years, indicating that this type of urinary

stone is more commonly diagnosed in older cats. This trend may be linked to age-related physiological changes, including alterations in calcium homeostasis, renal function, and urine acidification, all of which are known to contribute to calcium oxalate crystallization.

Moreover, calcium oxalate urolithiasis demonstrated a pronounced male predisposition, with a significantly higher incidence reported in male cats compared to females. This finding aligns with existing research that suggests male cats may have an increased risk due to factors such as lower urinary tract anatomy, hormonal influences on calcium metabolism, and potential differences in dietary intake or water consumption patterns.

These results underscore the importance of demographic factors, including age, sex, and breed, in the epidemiology of feline urolithiasis. Understanding these predispositions is crucial for developing targeted prevention strategies, including age-specific dietary recommendations, sex-based risk assessments, and breed-focused urinary health monitoring. Further research is warranted to elucidate the underlying pathophysiological mechanisms driving these observed trends and to refine management approaches for reducing the incidence of urolithiasis in high-risk feline populations (Grauer, 2015).

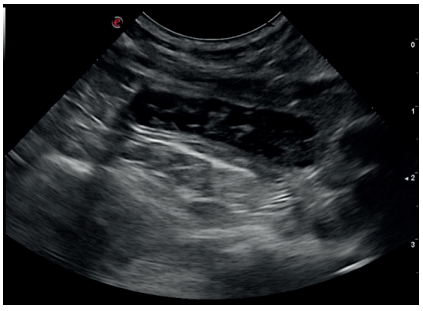

Figure 3. Ultrasound image of bladder urolithiasis in a cat involved in the study

Abdominal ultrasonography assisted in the diagnosis of urinary stone disease in the feline subjects included in this study. This imaging technique is widely recognized for its high sensitivity in detecting urinary tract abnormalities, including the presence of uroliths, bladder sand (crystalline sediment), and associated structural changes within the urinary bladder. Ultrasonography offers several advantages, such as being non-invasive, readily accessible in clinical practice, and capable of visualizing radiolucent uroliths that may not be detectable on conventional radiographs (Figures 3).

Despite these benefits, ultrasonography alone has limitations in determining the precise composition of uroliths. While certain sonographic characteristics, such as echogenicity and acoustic shadowing, may provide indirect clues regarding the mineral composition of urinary stones, definitive identification cannot be established through imaging alone. The accurate classification of urolith type is essential for guiding appropriate therapeutic interventions, including dietary modifications, pharmacologic management, or surgical removal when indicated.

To overcome these diagnostic limitations, urine samples were obtained via ultrasound-guided cystocentesis for further laboratory analysis. This technique, which involves the percutaneous aspiration of urine directly from the urinary bladder under ultrasonographic guidance, ensures a sterile sample collection method, minimizing the risk of contamination from the lower urinary tract. The collected urine was subsequently subjected to detailed laboratory evaluations, including urinalysis, urine sediment examination, and crystallographic analysis, to determine the specific mineral composition of the uroliths present (Figures 4, 5).

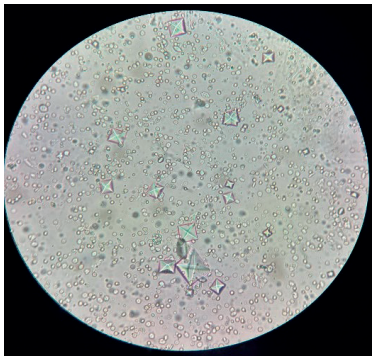

Figure 4. Microscopic image of calcium oxalates

By integrating ultrasonographic assessment with laboratory-based urine analysis, this study aimed to achieve a comprehensive diagnostic approach for feline urolithiasis. This multimodal strategy not only facilitated the detection of

urinary calculi but also allowed for the precise characterization of their composition, which is critical for selecting the most effective management and prevention strategies tailored to each individual case.

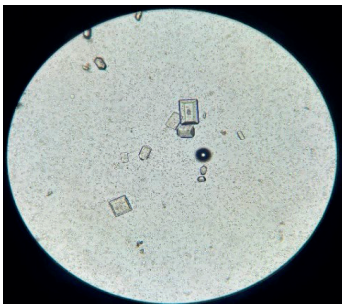

Figure 5. Microscopic image of magnesium ammonium phosphate

We also focused on assessing potential breed-related predispositions to urolithiasis. The most frequently detected crystalline composition was ammonium-magnesium phosphate, commonly referred to as struvite. Struvite crystals were identified in 18 European Shorthair cats, 6 British Shorthair cats, and 1 Russian Blue cat. These findings suggest a higher prevalence of struvite crystalluria in European Shorthairs, potentially indicating an underlying breed-specific susceptibility. This predisposition may be influenced by genetic factors, metabolic differences, or dietary habits that contribute to urinary supersaturation with struvite-forming minerals. The relatively high incidence of struvite crystalluria in British Shorthair and Russian Blue cats, though lower than in European Shorthairs, further underscores the need for targeted urinary health management in these breeds.

In addition to struvite, amorphous crystals were identified in multiple feline subjects, with the highest occurrence observed in European Shorthairs (9 cases). Other breeds in which amorphous crystals were detected included the Persian (1 case), British Shorthair (1 case), Birman (1 case), and Russian Blue (1 case).

Furthermore, a mixed composition of ammonium-magnesium phosphate (struvite) and calcium oxalate crystals was detected in 5 European Shorthair cats. This coexistence of differing crystalline types within the same urine sample suggests a dynamic urinary environment in which pH levels and mineral concentrations fluctuate over time, leading to the formation of mixed uroliths. Such cases may present a unique clinical challenge, as the management strategies for struvite and calcium oxalate stones differ significantly.

About calcium oxalate crystalluria, this type of urinary crystal was identified in the urine samples of 1 Persian cat, 1 British Shorthair cat, and 1 Birman cat. Compared to struvite, the overall prevalence of calcium oxalate crystals was lower in this study population. This discrepancy may reflect variations in urinary pH, as calcium oxalate tends to form in more acidic urine, whereas struvite formation is favoured in alkaline conditions (Ericksen, 2021).

These findings provide crucial insights into the breed-specific distribution of urinary crystals in felines diagnosed with urolithiasis. The predominance of struvite crystalluria, particularly in European Shorthair cats, highlights the necessity for breed-oriented preventative strategies, including regular urinalysis screening, appropriate dietary formulations, and hydration optimization. Furthermore, the detection of mixed and calcium oxalate crystals underscores the complexity of feline urolithiasis and the importance of individualized treatment approaches. Future research should focus on elucidating the genetic and environmental factors influencing urolith formation in different feline breeds to enhance preventive and therapeutic measures for this common urinary disorder (Table 2).

A large-scale study conducted over a six-year period, encompassing a total of 7,866 feline and canine subjects, identified 36 distinct cat breeds affected by urolithiasis. Among these, European Shorthair cats exhibited the highest prevalence of urinary stone formation, indicating a potential breed-specific predisposition. The most frequently identified urolith composition in this breed was ammonium-magnesium phosphate, commonly known as struvite. This finding aligns with previous research suggesting that European Shorthairs may have a metabolic or dietary susceptibility to struvite crystallization, necessitating targeted preventive measures such as urinary pH modulation and dietary adjustments.

In contrast, calcium oxalate uroliths were more frequently diagnosed in British Shorthair,

British Longhair, Birman, and Persian cats. These breeds exhibited a markedly higher incidence of calcium oxalate stones compared to struvite, suggesting differing pathophysiological mechanisms contributing to urolith formation.

These findings reinforce the importance of breed-specific risk assessments in the management of feline urolithiasis. Understanding the prevalence of different urolith types across various breeds can inform tailored preventive and therapeutic approaches, ultimately reducing the recurrence and clinical complications associated with urinary stone disease in felines (Burggraaf et al., 2020).

Table 2. Prevalence of urolith type within breed

Urolith type	Breed				
	Persian Cat	European shorthair	British Shorthair	Birman	Russian Blue
Ammonium-magnesium phosphates	0	18	6	0	1
Amorphous crystals	1	9	1	1	1
Ammonium-magnesium phosphates + Calcium oxalates	0	5	0	0	0
Calcium oxalates	1	0	1	1	0

CONCLUSIONS

This retrospective study highlights the predominance of struvite urolithiasis in cats, particularly among European Shorthair breeds, suggesting a potential breed-specific predisposition influenced by metabolic or dietary factors. In contrast, calcium oxalate crystals were more frequent in British Shorthair, Birman, and Persian cats, indicating distinct etiopathogenic mechanisms.

Age and sex were also relevant factors, with middle-aged cats being more commonly affected and a notable sex distribution observed across different crystal types. The identification of mixed urolith compositions further emphasizes the multifactorial nature of feline urolithiasis and the need for individualized diagnostic and therapeutic strategies.

These findings support the clinical utility of routine urinalysis and ultrasonography in early detection, particularly in predisposed breeds. Preventive approaches - focused on hydration, dietary management, and regular screening - are essential to minimize recurrence and reduce the need for surgical intervention.

Future research should explore genetic and environmental contributors to breed-associated risks and refine targeted, evidence-based management protocols to improve outcomes in feline urinary tract health.

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EMERGENCY MANAGEMENT IN A DOG WITH ETHYLENE GLYCOL INTOXICATION: CASE REPORT

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Abstract

A 1-year-old, 20 kg, intact female mixed-breed dog was admitted to the clinic for a nephrology consultation following ethylene glycol ingestion. Upon examination, the patient displayed severe hypertension (202/154 to 205/156 mmHg, measured by oscillometric method), abdominal pain, conjunctivitis, advanced dehydration, a rectal body temperature of 38.9°C, and dry mucous membranes. Laboratory tests revealed elevated BUN 151 mg/dL, CRE 10.2 mg/dL, PHOS 9.9 mg/dL, and GLU 123 mg/dL. Urinalysis showed a borderline proteinuria (UPC 0.5-2.0), pH of 5.0, and microalbumin ≥25 mg/L. To preserve renal function, haemodialysis was indicated as extracorporeal renal replacement therapy. Over eight days, four haemodialysis sessions were performed following the placement of a central venous catheter under light sedation with oxygen therapy supplementation. These therapeutic interventions, including intensive fluid management, were crucial in improving renal function. Significant biochemical improvements were observed: BUN decreased to 66 mg/dL, CRE to 3.7 mg/dL and PHOS to 8.0 mg/dL. Additionally, Ca levels rose from 11.3 mg/dL to 13.5 mg/dL. This case emphasizes the importance of timely intervention in acute kidney injury following ethylene glycol toxicity. Continued monitoring is essential for long-term renal recovery.

Key words*: BUN, CREA, haemodialysis, canine, ethylene glycol.*

INTRODUCTION

Ethylene glycol (EG) is a dihydric alcohol (HO–CH_2–CH_2–OH) with a sweet-bitter taste. It is colourless, odourless, water-soluble, and possesses antifreeze properties. Ethylene glycol is commonly found in various products, including antifreeze, brake fluids, and industrial solvents. (Leth & Gregersen, 2005). Ethylene glycol poisoning is a medical emergency characterized by rapid progression to acute kidney injury, metabolic acidosis, and multi-organ dysfunction. This condition is well-documented in both veterinary and human toxicology literature due to its severe outcomes and the need for rapid intervention (Grauer et al., 2008; Connally & Thrall, 1999). Its sweet, palatable taste makes ethylene glycol intoxication frequent in dogs (Tarr, Hayton et al., 1985) If treatment is not initiated within a few hours (within 6-12 hours), the prognosis becomes severe, with progression from acute kidney injury to acute renal failure (Schweighauser & Francey, 2015). Following ingestion, it is rapidly absorbed from the gastrointestinal tract, with peak plasma concentrations in dogs occurring 2-3 hours post-ingestion. The compound is then primarily metabolized by the liver. The toxicity is not attributed to the primary compound itself, but rather to the formation of toxic metabolites via the enzyme alcohol dehydrogenase (ADH). Ultimately, oxalic acid binds to calcium, resulting in the formation of insoluble calcium oxalate complexes. These complexes are freely filtered by the kidneys and deposited in the renal tubules, causing acute kidney injury. To a lesser extent, deposition also occurs in the vasculature of the brain, heart, and other organs (Schweighauser & Francey, 2015). Clinical signs progress through neurologic, cardiopulmonary, and renal stages, often culminating in acute renal failure if untreated (Segev & Baneth, 2013). Ethylene glycol toxicosis manifests in three stages. The first stage (30 minutes to 12 hours post-ingestion) is characterized by central nervous system signs, including neurologic depression, ataxia,

seizures, coma, or death, likely due to aldehyde metabolites, hyperosmolarity, and metabolic acidosis. The second stage (12 to 24 hours post-ingestion) involves resolving neurologic symptoms and the onset of cardiopulmonary signs such as tachypnoea and tachycardia, with common necropsy findings including pulmonary oedema and congestion. The final stage (24 to 72 hours post-ingestion) is marked by oliguric renal failure and signs of uraemia, including anorexia and vomiting, which may develop within 12 hours of ingestion (Scherk, Koenig et al., 2013).

MATERIALS AND METHODS

A 1-year-old intact female mixed-breed dog was referred for a specialized nephrological consultation on the 22nd of June, 2024. The dog presented with a history of suspected ethylene glycol intoxication. The owner reported that the dog had access to an unknown quantity of antifreeze, approximately 6 hours before presentation. Before the incident, the dog was healthy, with no known medical issues or history of renal disease. Upon initial examination, the dog showed signs of neurologic depression, including ataxia and mild disorientation. The owner noted that the dog had become lethargic and somewhat unresponsive in the hours following ingestion. The dog was observed to have a decreased level of activity and appeared weak. A complete blood count (CBC) was performed using the Vetscan HM5 Haematology system, which evaluated white blood cells (WBC), lymphocytes, monocytes, neutrophils, eosinophils, basophils, red blood cells (RBC), haemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), red blood cell distribution width (RDW), platelets (PLT), and platelet indices, including mean platelet volume (MPV) and platelet distribution width (PDW). Serum biochemistry was performed using the Vetscan VS2 Chemistry Analyzer, measuring albumin (ALB), alkaline phosphatase (ALP), alanine aminotransferase (ALT), amylase (AMY), total bilirubin (TBIL), blood urea nitrogen (BUN), calcium (CA), phosphorus (PHOS), creatinine (CRE), glucose (GLU), sodium (NA^+), potassium (K^+), total proteins (TP), and globulin (GLOB). Abdominal ultrasonography was performed using the Esaote MyLabX7 ultrasound system. Blood pressure was determined by the oscillometric method (High-Definition Oscillometry). Urinalysis was conducted using the Vetscan UA Urine Analyzer to assess leukocytes, ketones, nitrites, urobilinogen, bilirubin, glucose, protein, specific gravity, pH, blood, ascorbic acid, microalbumin, calcium, creatinine, and the protein-to-creatinine ratio. In this study, the B. Braun Dialog+ device was used to perform haemodialysis. The therapeutic approach focused primarily on haemodialysis, hydro-electrolytic rebalancing, and partial parenteral nutrition. To support rehydration and metabolic stabilization, a continuous rate infusion (CRI) of Ringer's solution was administered. Partial parenteral micronutrition was ensured through the administration of levo-microamino acids. Additionally, to aid renal function, the treatment protocol included enteric dialysis supplements, calcium-based phosphorus binders, a specialized renal diet, and nutritional support as adjuvant therapies.

RESULTS AND DISCUSSIONS

On June 22nd, 2024, the patient presented with severe metabolic acidosis, elevated serum creatinine, blood urea nitrogen, and phosphorus levels, consistent with advanced stage 5 acute kidney injury. Similar findings have been reported in acute ethylene glycol toxicity in both canine and feline patients (Bartges et al., 1998). The biochemical parameters revealed a significant elevation in creatinine (CREA 10.2 mg/dL-RR: 0.4-1.2 mg/dl), blood urea nitrogen (BUN 151 mg/dL-RR: 7-25 mg/dl), and phosphorus (PHOS 9.9 mg/dl- RR: 2.9-6.6 mg/dl), while glucose (GLU 123 mg/dl- RR: 60-110 mg/dl) was mildly elevated. The complete blood count (CBC) showed a decreased mean corpuscular haemoglobin concentration (MCHC 29.3 g/dl- RR: 31-39 g/dl) and mean corpuscular haemoglobin (MCH) within the normal range (MCH 19.1 pg - RR: 14-20 pg). Urinalysis conducted through ultrasound-guided cystocentesis, demonstrated a urine protein-to-creatinine ratio (UPC) of $\geq$0.5 to <2.0 (proteinuric), a pH of 5.0, microalbumin $\geq$25 mg/L, and creatinine at 8.8 mmol/L. At the ultrasound evaluation, bilateral renal alterations were noted, specifically characterized by the

presence of a hyperechoic cortical rim in both kidneys. Arterial blood pressure was elevated, with values ranging between 202/154 (174) mmHg and 205/156 (176) mmHg, using the oscillometric method (High Definition Oscillometry). The decision to initiate haemodialysis was based on critical biochemical abnormalities and the progression of clinical signs. Haemodialysis is considered the most effective treatment for severe ethylene glycol intoxication, as it directly removes both the parent compound and its nephrotoxic metabolites. Numerous clinical case studies and reviews have demonstrated the effectiveness of haemodialysis in treating ethylene glycol toxicosis in small animals (Hall & Grubb, 2001; Grauer et al., 2008). The procedure was performed after placing a central venous catheter under light sedation and supplemental oxygen. The procedure was performed using strict aseptic techniques, including the application of a surgical scrub, sterile gloves, and a sterile environment. Special care was taken in handling the catheter line throughout the process to ensure the highest standards of hygiene and safety. Each lumen of the catheter was scrubbed with 0.5% chlorhexidine for 2 minutes to maintain aseptic conditions.

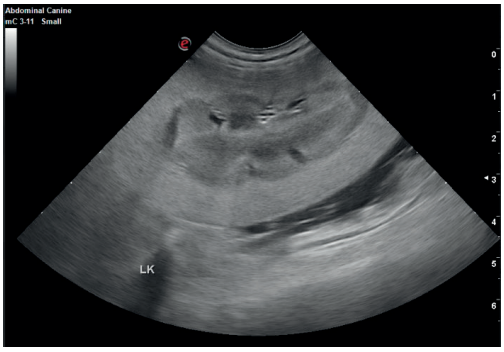

Figure 1. The presence of a cortical rim in the left kidney

Twenty-four hours after the first session of haemodialysis, the blood biochemistry results were as follows: creatinine (CREA) level was 7.4 mg/dl (reference range: 0.4-1.2 mg/dl), blood urea nitrogen (BUN) decreased to 73 mg/dl (reference range: 7-25 mg/dl), and phosphate (PHOS) showed a slight increase to 10.0 mg/dl (reference range: 2.9-6.6 mg/dl). During the haemodialysis session, the patient remained stable without any significant clinical events. After the second session of haemodialysis on the 24th of June, bloodwork results were: CREA 5.1 (RR: 0.4-1.2 mg/dl), BUN 41 (RR: 7-25 mg/dl), PHOS 8.4 (RR: 2.9-6.6 mg/dl) and GLU 118 (RR: 60-110 mg/dL). The blood biochemistry results after the 24-hour pause and after the third session of haemodialysis, were as follows: CREA 4.3 (RR: 0.4-1.2 mg/dl), BUN 43 (RR: 7-25 mg/dl), PHOS 7.6 (RR: 2.9-6.6 mg/dl), GLU 119 (RR: 60-110 mg/dL) and CA 12.6 (RR: 8.6-11.8 mg/dL). On the 28th of June, after another 24-hour pause and following the fourth session of haemodialysis, the blood biochemistry values were as follows: CREA 4.1 (RR: 0.4-1.2 mg/dl), BUN 40 (RR: 7-25 mg/dl), PHOS 9.1 (RR: 2.9-6.6 mg/dl), GLU 127 (RR: 60-110 mg/dL) and CA 13.1 (RR: 8.6-11.8 mg/dL). The latest set of tests from June 29th showed the following values: CREA 2.2 (RR: 0.4-1.2 mg/dl), BUN 32 (RR: 7-25 mg/dl), PHOS 6.8 (RR: 2.9-6.6 mg/dl), GLU 114 (RR: 60-110 mg/dL) and CA 12.5 (RR: 8.6-11.8 mg/dL). After two days of hospitalisation without any haemodialysis sessions but with fluid therapy, oral and intravenous drug therapy, the blood biochemistry values were as follows: CREA 2.0 (RR: 0.4-1.2 mg/dl), BUN 30 (RR: 7-25 mg/dl), PHOS 6.1 (RR: 2.9-6.6 mg/dl) and CA 11.5 (RR: 8.6-11.8 mg/dL).

After discontinuing haemodialysis, the patient-maintained stability with fluid therapy and medication, suggesting partial renal recovery and a favourable prognosis with continued supportive care.

Between the haemodialysis sessions, supportive therapy was implemented to optimize renal function, and systemic stabilization such as fluid therapy with Ringer solution was administered via continuous rate infusion (CRI) at a dosage of 7 mL/kg/h.

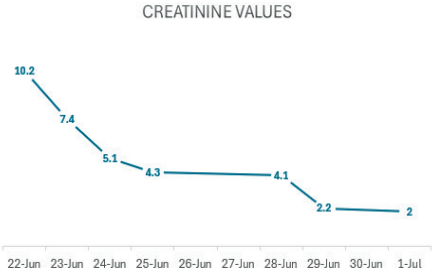

Figure 2. Creatinine values during hospitalization

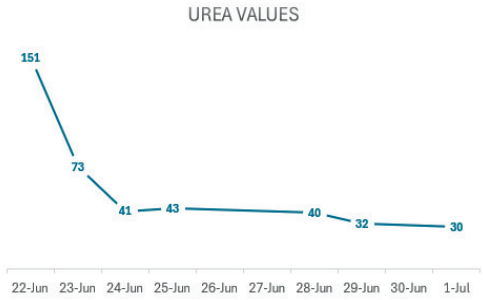

Figure 3. Urea values during hospitalization

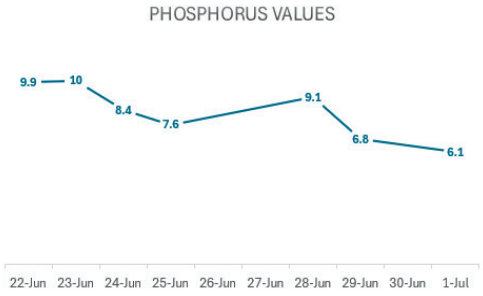

Figure 4. Phosphorus values during hospitalization

The fluid requirements were meticulously calculated based on the patient's individual needs, taking into account the degree of hydration, ongoing losses, and overall fluid balance. This approach aimed to ensure optimal rehydration, maintain electrolyte homeostasis, and support hemodynamic stability throughout the treatment. Partial parenteral micronutrition was provided using levo-microamino acids at a rate of 6 mL/kg/24 h to support metabolic demands. Additionally, enteric dialysis supplements and calcium-based phosphorus binders were incorporated to aid in toxin elimination and phosphate regulation. A specialized renal diet, and targeted nutritional supplements, were introduced as adjunctive therapy to further support kidney function and promote overall recovery. Such multimodal therapeutic strategies are consistent with current recommendations in veterinary emergency protocols (Davis et al., 2007). After eight days of hospitalization and four haemodialysis sessions, the patient was discharged with a prescribed treatment plan.

Ethylene glycol intoxication is a life-threatening condition in dogs, commonly caused by the ingestion of antifreeze. Once absorbed, ethylene glycol is rapidly metabolized into toxic compounds that induce severe metabolic acidosis and acute kidney injury. Kidney damage occurs due to calcium oxalate crystal deposition, leading to renal failure if left untreated. The sooner the patient undergoes haemodialysis, the higher the chances of recovery. Haemodialysis efficiently removes ethylene glycol and its toxic metabolites while correcting metabolic imbalances and supporting kidney function during recovery.

The clinical progression observed in this case underscores the critical importance of early intervention in ethylene glycol intoxication. According to Schweighauser and Francey (2016), initiating treatment within the first 8 hours post-ingestion significantly improves prognosis and reduces the risk of irreversible acute kidney injury (AKI). In the case presented, prompt initiation of haemodialysis contributed to the gradual decrease in creatinine and blood urea nitrogen (BUN) levels, helping stabilize renal function.

This outcome is consistent with findings reported in similar case studies, where extracorporeal therapy proved effective in removing ethylene glycol and its toxic metabolites, while correcting metabolic derangements and supporting renal recovery (Schweighauser & Francey, 2016).

In addition, the observed elevations in phosphorus and calcium may reflect renal deposition of calcium oxalate crystals - a well-documented complication of ethylene glycol toxicity. Careful monitoring and supportive interventions, including phosphate binders and fluid therapy, were essential to mitigating these secondary effects.

Although haemodialysis was highly beneficial, it is important to note that full normalization of renal parameters was not achieved within the hospitalization period. Previous reports suggest that in severe cases, persistent renal damage may occur even with timely intervention (DiBartola et al., 1985). Nonetheless, the progressive improvement in laboratory values and the patient's stable condition at discharge are promising indicators of partial renal recovery.

Overall, this case reinforces the value of haemodialysis as a life-saving therapeutic option in veterinary nephrology and highlights the necessity of early diagnosis and intervention

to prevent long-term renal dysfunction. Expanding access to extracorporeal therapies such as haemodialysis in critical care veterinary settings could significantly improve survival rates in cases of acute toxicosis (Langston, 2002).

CONCLUSIONS

This case report underscores the critical importance of early diagnosis and timely initiation of hemodialysis in ethylene glycol intoxication, even in patients presenting with advanced stage 5 acute kidney injury. The extracorporeal intervention enabled the progressive stabilization of renal function, as evidenced by the consistent decline in serum creatinine, blood urea nitrogen, and phosphorus levels over the course of therapy.

Hemodialysis proved effective not only in eliminating ethylene glycol and its nephrotoxic metabolites but also in correcting severe metabolic acidosis and preventing irreversible nephron damage associated with calcium oxalate deposition. Adjunctive therapies - including fluid resuscitation, phosphate binders, partial parenteral nutrition, and a renal-specific diet - played an essential role in restoring metabolic balance and supporting renal recovery.

Although full normalization of biochemical parameters was not achieved during hospitalization, the observed clinical improvement and sustained reduction in azotemia at discharge indicate a favorable prognosis and partial preservation of renal function. The outcome validates hemodialysis as the most effective therapeutic approach for severe ethylene glycol poisoning when initiated promptly.

Beyond its individual clinical relevance, this case emphasizes the broader implications for emergency veterinary care. Improved access to extracorporeal renal replacement therapies and heightened awareness of their efficacy could significantly enhance survival and long-term outcomes in patients with acute toxicologic nephropathies. These findings support the integration of early hemodialysis into standardized treatment algorithms for companion animals exposed to nephrotoxic agents.

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THE CLINICAL AND LESIONAL PRESENTATION OF *ERYSIPELOTHRIX RHUSIOPATHIAE* INFECTION IN SHEEP

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Abstract

Erysipelothrix rhusiopathiae is a bacterium that poses a significant health threat in sheep, manifesting through a range of clinical signs and lesions. Clinical presentations of infection include fever, joint swelling characterized by arthritis, and systemic signs such as erythema and necrosis of the skin. Affected sheep may exhibit difficulty in movement or lameness, indicating discomfort and severe illness. Lesion formation often involves pronounced inflammation of the skin, and in advanced cases, there may be indications of septicaemia. Furthermore, complications such as fibrinous pericarditis and endocarditis can arise in severe instances of the infection. Understanding the clinical and lesional manifestation of Erysipelothrix rhusiopathiae is crucial for effective diagnosis and management in sheep populations.

Key words*: Erysipelothrix rhusiopathiae, sheep, clinical signs, lesions, septicaemia.*

INTRODUCTION

A wide variety of vertebrates, including pigs, sheep, and humans, are commensal hosts for the Gram-positive rod-shaped bacteria *Erysipelothrix rhusiopathiae* (Shimoji, 2000; Wang et al., 2010). There are many sources of infection of this bacterium in nature, as it may survive for weeks/months, even in extreme environments. In healthy pigs, the bacteria may be found in lymphoid tissues, especially in the tonsils, in 30-50% of cases, making them the most significant reservoir of infection (Opriessnig, 2012). Bacteria may be passed from infected to uninfected animals by faeces, urine, saliva, and nasal secretions (Mavrot et al., 2020; Opriessnig et al., 2012). Carriers, such as wild birds or other animals, may spread illness and infection via the soil, water, food, and bedding that animals eat or make direct contact with.

The pathogenicity of *E. rhusiopathiae* is associated with cellular attachment, intracellular invasion, and survival; the bacteria are capable of reproducing in phagocytic cells (Shimoji, 2000; Wang et al., 2010). Polyarthritis, which usually manifests in lambs between two and six months of age, is the most prevalent clinical presentation in sheep. The onset of chronic alterations in the joints is a key point in the clinical symptomatology of this illness, which is marked by significant morbidity and low mortality (Mavrot et al., 2020). Infections of the skin, heart, lungs, and blood (septicaemia) are among the other symptoms (Griffiths et al., 1991; Mavrot et al., 2020; Rad et al., 1998). As a result, the condition may have a major effect on animal welfare and the economy because of its associations with wastefulness, stunted development, and slaughterhouse condemnation.

There are several potential entry points for infection in lambs, including the skin (e.g., after molesting, shearing, or tail docking), the mouth, or the umbilical cord (Mavrot et al., 2020). Joints involving subchondral bone are the only sites of infection in chronic ovine erysipelas, according to the available information (Thompson et al., 2007; Wang et al., 2010). Joint pathology is the primary emphasis of the few histologic descriptions available for sheep (Ersdal et al., 2015).

The purpose of this study is to comprehensively investigate the clinical presentation, gross and microscopic lesions, and therapeutic response associated with *Erysipelothrix rhusiopathiae* infection in sheep. By documenting the progression of clinical signs, characterizing the pathological changes in affected tissues, and evaluating the efficacy of treatment strategies.

MATERIALS AND METHODS

The studies were conducted from 2021 to 2023 at a non-professional sheep breeding farm located in Domnesti, Teghes village, Ilfov County.

Animals

The farm on which the studies were carried out is a mixed-breed sheep farm. The flock totals 164 head, consisting of 6 breeding rams, about 80 breeding ewes, the remainder up to 164 being lambs, and cull ewes. The animals are farmed in a semi-intensive system, mainly for cheese production and secondarily for meat production, and are kept on pasture. The pastures used for sheep farming are natural pastures.

In the cold season, when calving takes place, the animals are kept in stalls in order to protect them from adverse climatic conditions (cold wind, snow, frost). The bedding in the stalls is made of straw and plant waste from fodder, which together with the animal's excrements form a straw bedding that provides a soft and warm bedding for the animals' rest and well-being. Pigs for family consumption are also kept on the same holdings, but in different sheds.

Sampling

Samples for bacteriologic and PCR examination from living animals were blood and joint fluid obtained by puncture, and from slaughtered animals, from joints, lymphoid structures, and the spleen.

Bacteriology

Samples were inoculated onto blood agar (BA). All samples were incubated in oxygen at 37°C for approximately 24 hours prior to initial examination. Isolates assessed as being *E. rhusiopathiae* after the first isolation were replated on Columbia Agar and reevaluated after 24 hours of incubation at 37°C. Isolates from all cases were confirmed by Api Coryne. Antibiograms were also performed for the isolates obtained.

PCR

DNA was isolated from approximately 200 µL of EDTA-blood or tissue samples by using the Qiagen QIAamp Cador (IndiSpin) Pathogen Mini Kit.

The detection kit used was EryRhu dtec-qPCR, with the amplification regime: Activation - 2 min at 95°C, Denaturation - 40 cycles of 5 sec at 95°C, Hybridisation/Extension - 20 sec at 60°C.

RESULTS AND DISCUSSIONS

Clinical Features

The farmer indicated that all the animals suffering from lameness were lambs of primiparous ewes that had difficulties in bonding and feeding them.

Four lambs had previously been slaughtered at this farm because of recumbency, swollen joints, and poor condition; 14 other lambs from those farms exhibited symptoms of lameness.

Although adult sheep did not exhibit any symptoms of pneumonia or lameness, the afflicted lambs did walk stiffly and had swollen joints.

Lameness was observed in lambs and ewes before this outbreak of polyarthritis about 3-4 years ago, the time corresponding with the first year of pig rearing on the farm.

In the clinical examination, the following parameters were assessed:

Body Condition Score (BCS):

A 5-point scale was used to evaluate the nutritional and physical condition of the animal:

1 - Emaciated: The animal is extremely thin with visible bones, no palpable fat, and signs of muscle wasting; typically, indicative of severe malnutrition or chronic illness, and may be near death.

2 - Thin: Bones are still prominent, but some fat may be palpable. Muscle mass is reduced. The animal appears underweight and may be clinically compromised.

3 - Moderate: Ideal body condition. The animal has a good balance of fat and muscle; bones are not visible but can be felt with slight pressure.

4 - Overweight: Noticeable fat deposits over ribs and tail head; the waist is less defined. There may be a risk of developing health complications.

5 - Obese: Excessive fat coverage with no palpable ribs or bone landmarks; movement may be impaired and there is an increased risk of metabolic disorders.

Affected Joints: All joints showing signs of inflammation, pain, or structural change were

identified and recorded, noting whether involvement was unilateral or bilateral and whether multiple joints were affected.
Pain on Palpation of Joints: A subjective 5-point pain scale was employed to quantify the animal's response during physical manipulation of the joint:
1 - Minimal pain: Mild discomfort with palpation, no overt behavioural signs of distress.
2 - Mild pain: Slight withdrawal or flinch response; discomfort noted but not severe.
3 - Moderate pain: Definite reaction to palpation such as pulling away, vocalization, or visible discomfort.
4 - Severe pain: Strong withdrawal, guarding behavior, or significant distress on palpation.
5 - Extreme pain: Intense reaction with inability to palpate thoroughly due to aggression, vocalization, or complete avoidance; indicates very high sensitivity and joint pathology.
Macroscopic Characteristics of Synovial Fluid: Joint fluid obtained via arthrocentesis was examined visually to assess indicators of inflammation or infection, including:
Color: Clear, yellow, or turbid.
Viscosity: Normal (stringy, high viscosity) vs. reduced (watery or thin).
Volume: Increased volume may indicate effusion.
Clarity: Transparent, mildly cloudy, or opaque, suggesting varying degrees of cellular infiltration.
Level of Joint Swelling: The degree of swelling observed or palpated was graded on a 3-point scale:
Slight: Minimal enlargement of the joint capsule; may only be noticeable on close inspection or palpation.
Moderate: Clearly visible swelling with some restriction of joint movement or function.
Severe: Pronounced swelling with significant joint distortion and likely loss of normal range of motion.
The age of the affected lambs was between 1 week and 4 months (Table 1), the most severe clinical manifestations being present in the youngest lambs, these also having the highest pain score (PS: 3-4), the most frequently affected joint was the carpal joint, found in 50% of the lambs. Only one lamb showed swelling of the area associated with the atlanto-occipital joint (*Articulatio atlantooccipitalis*) and inability to turn/roll the head.
It is known that the joint fluid from *Erysipelothrix rhusiopathiae* infection may have different appearances depending on the duration of the investigation. In this batch of lambs, the most prevalent appearance was serofibrinous, present in 61% of the animals.

Table 1. Clinical findings

ID	Sex	Age	Body Score	Affected joints	Pain Score	Swelling Level	Appearance of joint fluid
1	♀	2-month-old	3	Left + right carpal	2	Slight	Serofibrinous
2	♀	2-month-old	2	Left + right stifle	2	Moderate	Serofibrinous
3	♂	3-week-old	1	Left + right carpal	4	Moderate	Cloudy, fibrinous
4	♂	3-month-old	2	Left + right stifle	2	Moderate	Serofibrinous
5	♂	4-month-old	4	Left + right carpal	1	Slight	Cloudy, fibrinous
6	♀	3-month-old	2	Left + right carpal	2	Moderate	Serofibrinous
7	♀	2-week-old	1	Left + right tarsocrural	3	Severe	Cloudy, fibrinous
8	♀	3-month-old	2	Left + right carpal	2	Moderate	Serofibrinous
9	♀	4-week-old	2	Left + right stifle	3	Moderate	Serofibrinous
10	♀	2-week-old	1	Left + right tarsocrural	3	Severe	Cloudy, fibrinous
11	♀	4-week-old	2	Left + right stifle	3	Severe	Cloudy, fibrinous
12	♂	2-month-old	2	Left + right carpal	2	Moderate	Serofibrinous
13	♀	3-month-old	4	Left + right carpal	1	Slight	Serofibrinous
14	♀	1-week-old	1	Atlanto-occipital joint	3	Severe	-

♂ – male; ♀ – female

In general, polyarthritis in lambs is caused by microorganisms with the ability to cause bacteraemia and septicaemia. *Erysipelothrix rhusiopathiae* has this capacity, and its evolution in this flock could be related to a colostrum deficiency, the farmer mentioning in the case history that the lambs came from mothers who had difficulties in accepting and feeding the newborns.

Pathology

There was a large volume of fibrinous exudate present at joint level and numerous necrotic lesions of the synovial membranes in the joints of the lambs that were slaughtered. Generally, there was an increased amount of cloudy synovial fluid, the joint capsule wall was thickened, and the synovial membrane was marked by hyperaemic lesions with a villous proliferation. In the most severely affected joints, there were erosions/ulcerations in the synovial cartilage. All lambs displayed abnormalities in carpal and tarsal joints; the majority were afflicted bilaterally. Just like the other lambs, the youngest one exhibited the same type of alterations in the atlanto-occipital joint and an accumulation of fibrinous liquid in the pericardium.

Therapy

After performing an antibiogram of the collected materials, sensitivity to the following antibiotics was established: penicillin, ampicillin, amoxicillin-clavulanic acid, ceftiofur, cefovecin, cefquinome, enrofloxacin, marbofloxacin, florfenicol and resistance to: doxycycline, tetracycline, erythromycin, clindamycin, sulfamethoxazole - trimethoprim, and vancomicyn.

Because of the relatively small and varied weight between 10 and 30 kg, it was opted to administer ampicillin at a dose of 8 mg/kg for 5 days and, as an anti-inflammatory and analgesic, meloxicam at a dose of 1 mg/kg, for 3 days.

Although some of the lambs were left with a slight difficulty in walking following the treatment, it did not significantly influence their weight gain.

It was recommended either to remove the pigs from the farm, to vaccinate the flock against *Erysipelothrix rhusiopathiae* infection, or to implement working measures to prevent the transmission of the bacterium from pigs to sheep.

CONCLUSIONS

Erysipelothrix rhusiopathiae is a Gram-positive bacterium that affects a variety of vertebrates, including pigs, sheep, and humans. It is particularly found in the tonsils of pigs and is the main reservoir for the bacteria.

Sheep, especially lambs, are highly susceptible to infections caused by this bacterium, leading to conditions such as polyarthritis, septicaemia, and joint infections. These infections often manifest in young lambs and lead to symptoms like swollen joints, lameness, and severe pain.

The infected lambs showed clinical signs such as stiffness, swollen joints (especially the carpal joint), and pain. The joint fluid was mostly serofibrinous in appearance, indicating inflammation, and many lambs had cloudy, fibrinous fluid, indicative of bacterial infection and immune response.

Lesion analysis revealed fibrinous exudate, necrotic lesions in the synovial membranes, thickened joint capsules and, in severe cases, erosions in the synovial cartilage. These findings indicate significant joint damage due to the bacterial infection.

The condition, marked by significant morbidity, can affect animal welfare by causing pain and lameness, leading to difficulty in feeding. It can also have an economic impact due to weight loss, reduced growth, and condemnation at slaughter due to the infection. This highlights the importance of addressing both the clinical and environmental factors that contribute to the spread of infections like *E. rhusiopathiae*.

In conclusion, the outbreak of *Erysipelothrix rhusiopathiae* in lambs appears to be linked to the introduction of pigs to the farm, emphasising the importance of controlling the interaction between different animal species and implementing preventive measures such as vaccination and biosecurity protocols.

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BONE MARROW SAMPLING IN VETERINARY MEDICINE. LITERATURE REVIEW

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Abstract

Bone marrow (BM) evaluation is critical in diagnosing haematological disorders and staging different types of cancer. However, recent studies suggest its role in detecting disseminated tumour cells. This literature review aims to highlight the importance of BM evaluation and aid practitioners in collecting high-quality samples, focusing on domestic carnivores, horses, and laboratory animals, emphasising indications, types of specimens, and sampling techniques. Various indications for BM evaluation include peripheral blood abnormalities, lymphoma staging, myeloproliferative disorders and toxicological analysis. The main types of specimens are BM aspirate and BM core, each requiring a different type of needle. For domestic carnivores, the most accessible sites for collection are the proximal humerus and the iliac crest, while for horses, collection is performed from the sternum. In laboratory animals, BM aspiration is typically performed using the femur, tibia or the iliac crest as the collection site. BM sampling differs between species and can be challenging to obtain in some cases. Choosing the proper sampling technique and corroborating results with clinical and haematology data is important to maximise BM evaluation.

Key words: *bone marrow, collection techniques, domestic carnivores, horse, laboratory animals.*

INTRODUCTION

Bone marrow (BM) evaluation is a routine diagnostic tool in human medicine, but less used in veterinary practice. The difficulty of obtaining bone marrow samples can impact the quality of the specimens. BM samples include aspiration and core biopsy: each method offers its own advantages, and using both techniques for a comprehensive diagnosis is recommended (Das et al., 2023). It is also important to note that the instruments used for collecting samples from animals are often adapted from those used in human medicine. This fact can make it challenging to apply these techniques effectively in very small animals.

Bone marrow examination in animals is crucial for diagnosing and managing various conditions, especially haematological malignancies and disorders. These examinations provide important insights that guide therapeutic approach and, in laboratory settings, enhance our understanding of drug toxicity, highlighting their significance in both clinical and research fields.

It is important to emphasize that studies indicate that this procedure, although seemingly invasive and labour-intensive, can be performed under suitable conditions with minimal trauma to the animal. Recovery is typically swift, and complications are rare (Woods et al., 2021).

This literature review highlights the significance of BM evaluation and assists practitioners in collecting high-quality samples, particularly focusing on domestic carnivores, horses, and laboratory animals. It emphasizes the indications, types of specimens, and sampling techniques.

MATERIALS AND METHODS

In order to review the literature on bone marrow sampling, relevant materials were gathered from the current database using the following keywords: bone marrow, collection techniques, domestic carnivores, horses, laboratory animals, literature review, and indications for BM examination. Selected articles were chosen to address the most pertinent aspects of bone marrow collection. The information obtained was compared with data available in reference books on veterinary pathology, clinical pathology, haematology, and oncology. The

following sections outline key aspects of bone marrow collection in veterinary practice.

Indications for BM sampling

Bone marrow evaluation is primarily indicated when changes in peripheral blood are noted. Several such abnormalities include: multilineage changes observed in peripheral blood combined with morphological atypia, most cases of pancytopenia (Mylonakis & Hatzis, 2017), persistent neutropenia, unexplained thrombocytopenia, and non-regenerative or poorly regenerative anaemia (Harvey, 2012). Another important role of bone marrow examination is lymphoma staging (Harvey, 2001). Pinto et al., in 2024, found that some dogs classified as stage V revealed peripheral blood abnormalities in the absence of bone marrow infiltration. Other authors report similar findings: lymphoma-related blood abnormalities suggest bone marrow infiltrates, but this is not certain (Graff et al., 2014), highlighting the importance of bone marrow evaluation as a tool for staging this neoplasm. Bone marrow aspirates are also valuable for identifying infectious or parasitic agents like *Leishmania infantum*, *Cytauxzoon felis*, and *Histoplasma capsulatum* (Wellman & Radin, 2004). Paparcone et al. (2013) concluded that bone marrow aspirate from the sternum is a practical method for the diagnosis of leishmaniasis in dogs. Also, BM evaluation can aid in identifying the cause of hyperproteinaemia associated with multiple myeloma, lymphoma, leishmaniasis, or systemic fungal infections (Harvey, 2001; Harvey, 2012). Similarly, hypercalcemia can be explained in the context of multiple myeloma or lymphoma (Roodman, 1997; Kohart et al., 2017). In dogs, iron stores can also be evaluated by examining the bone marrow (Phiri et al., 2009; Pawsat et al, 2021; Pawsat et al, 2023). In horses, bone marrow is a common source for harvesting mesenchymal stromal cells (MSC), which are used to treat various musculoskeletal diseases (Taylor & Cleg, 2011). Other studies show the utility of bone marrow in detecting disseminated tumour cells. Research in human medicine highlights the significance of bone marrow examination for detecting disseminated tumour cells (DTC) in mammary cancer, as it serves as an independent prognostic factor (Braun et al., 2000; Siddappa et al., 2013). Bone marrow is considered the most accessible tissue for DTC analysis, as it can be collected during primary surgery (Vincent-Salomon et al., 2008). Marconato et al. (2019) suggest that disseminated tumour cells from the blood and bone marrow could serve as a potential biomarker for canine mammary cancer. The study found that dogs with detectable tumour cells in their bone marrow had a shorter survival time compared to those without detectable tumour cells. Additionally, bone marrow can be useful in identifying the cause of fever of unknown origin (Das et al., 2023). According to a case study, the authors concluded that bone marrow can be used to detect toxic chemicals in forensic veterinary medicine, particularly for investigating exposure to organophosphates and carbamates (Marcelino et al., 2020). Furthermore, bone marrow fat analysis can provide a post-mortem diagnostic tool that can give insights regarding antemortem starvation in emaciated animals (Raglus, 2019).

Types of bone marrow specimens

Bone marrow samples can be represented by either an aspirate or a core biopsy. For aspirates, the Ilinois needle is usually used, while for core biopsy, the Jamshidi type needle is needed (Figure 10). Different types of needles are summarised in Table 2. In the field of veterinary medicine, bone marrow aspiration is preferred over bone marrow biopsy, primarily due to its cost-effectiveness and faster processing time (Harvey, 2012). The choice between aspiration and biopsy depends on the clinician's differential diagnoses and the specific information sought from the examination of the bone marrow. In many cases, aspiration and biopsy complement each other, and both may be necessary (Reeder et al., 2013). Cytology yields detailed information about the morphology of bone marrow cells that can be difficult to observe in histological preparations (Wellman & Radin, 2004). Aspirate samples are primarily used for cytology evaluation, but they can also be applied for flow cytometry or the preparation of paraffin section of bone-marrow aspirate, referred to as a clot section (Ong et al., 2015). Core samples, on the other hand, are utilized for histopathology (Figure 1) and immunohistochemistry.

Additionally, a rolling imprint can be performed from a core biopsy, which can then be used for cytology evaluation (Harvey, 2012). Imprints can also be obtained from post-mortem samples (King et al., 2014).

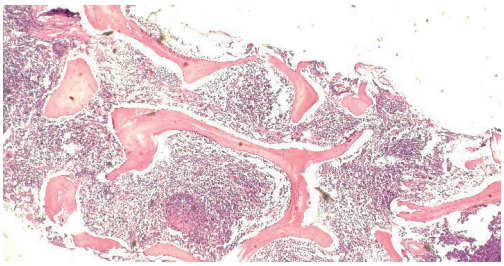

Figure 1. Microscopic aspects of a histological section of a core biopsy, haematoxylin and eosin stain, 50x magnification (Original)

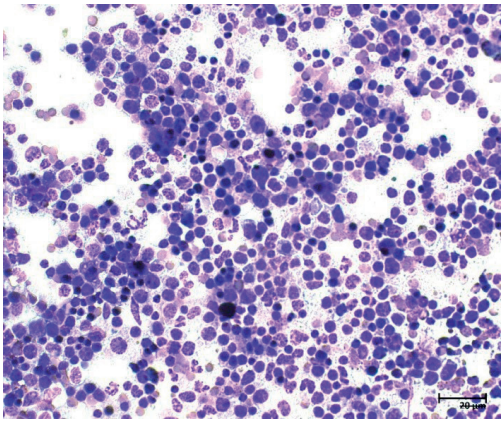

Figure 2. Microscopic aspects of a smear from an aspirate, May-Grünwald stain, 400x magnification (Original)

Cytology (Figure 2) effectively assesses the M: E ratio (myeloid to erythroid ratio) and cell morphology (Reeder et al., 2013), including dysplastic changes (Mori et al., 2020).

Examples of pathologies that cytology aids in diagnosis include myelodysplastic syndrome, different types of leukaemia, lymphoma staging, multiple myeloma, precursor-targeted immune-mediated anaemia and other immune-mediated cytopenia.

Cytology can also be helpful for detecting microorganisms (Paparcone et al., 2013; Harvey, 2012). Multiple smears should be done from the aspirate, and they must be done rapidly after sampling. Otherwise, the aspirate should be placed in an EDTA tube (Ayala-Trejo et al., 2015).

Histopathology is very useful for assessing bone marrow cellularity, localized changes such as inflammation, focal neoplasia or necrosis (Grindem et al., 2002), vascular lesions or examining bone tissue (Harvey, 2012). Such lessons include osteomyelitis, vascular amyloidosis, myelofibrosis or metastatic neoplasia (Grindem et al., 2002).

Another strong indicator for core biopsy is when an unsuccessful aspirate is encountered, a situation referred to as “dry taps” (Harvey, 2012). A core specimen should be at least 0.5 cm long (Weiss & Smith, 2002) and have enough active marrow. Grossly, cortical bone is white, while active marrow is red (Byers, 2017).

After performing a rolling imprint, samples submitted for histopathology or immunohistochemistry should be submitted in 10% buffered formalin.

Further sample processing implies decalcification, paraffin embedding and sectioning, which results in a longer turnaround time for these specimens.

Molecular tests are helpful for the determination of clonality in lymphoma or leukaemia (Avery, 2009). Immunohistochemistry is typically done in combination with histological assessment and is helpful in differentiating reactive from neoplastic lesions, characterisation of metastatic neoplasm and characterisation and classification of lymphoid neoplasms (Kremer et al., 2005).

Flow cytometry can be done in combination with cytology, or it can replace the cytological examination (Reagan et al., 2011). It is performed mainly in cases of myeloproliferative or lymphoproliferative disorders. In human medicine, it has been reported that flow cytometry can detect early myeloma or atypical cases and plays an important role in the differential diagnosis of different types of lymphomas (Yuan & Stetler-Stevenson, 2011). Samples submitted for flow cytometry should be placed in a sodium heparin tube (Lee et al., 2008).

Different types of bone marrow samples are summarised in Table 1 and different types of slides are shown in Figure 3.

Table 1. Sample types, advantages, disadvantages and uses

Sample Type	Advantages	Disadvantages	Used for
Core samples	Overall architecture Sections can be used for immunohistochemistry Accurate cellularity	More expensive Longer sampling time Longer processing time (decalcification) More invasive	Histopathology Immunohistochemistry Cytology (Rolling Imprints)
Aspirate	Cost effective Faster sampling time Faster processing time Easier to asses cell morphology and detect dysplastic changes Easier to asses myeloid to erythroid ratio and cell count	Difficult to asses overall cellularity Focal lesions may be missed	Cytology (Smear) Cloth section Flow cytometry

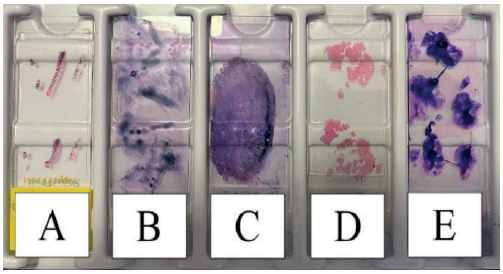

Figure 3. Different slides for bone marrow evaluation: A. Histological section from a core biopsy; B. Roll Imprint from a core sample; C. Smear from a BM aspirate; D. Clot section; E. Imprint from BM harvested from the femur, post-mortem (Original)

Election sites

The main sites for bone marrow collection in animals include the iliac crest, sternum, and proximal humerus, each presenting specific advantages and considerations for veterinary practitioners.

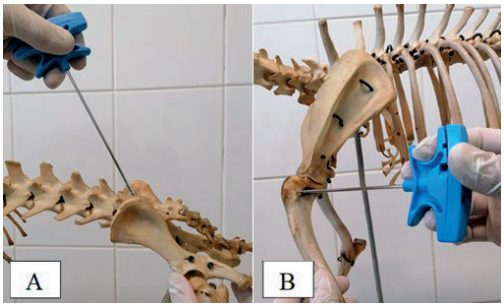

Figure 4. Election sites for BM samples in dogs - A. Iliac crest; B. Proximal humerus

The iliac crest (Figure 4A) is a common site in normal-weight dogs because it is easily accessible and the lateral approach is preferred in small dogs and cats with a thin iliac crest (Raskin and Messik, 2012). The proximal humerus (Figure 4B) is frequent used in domestic carnivores, especially in obese dogs (Grindem, 1989). In small dogs and cats, the femur can also be used for sampling, including both aspirate and core biopsy; however, core samples are more challenging to obtain (Raskin & Messick, 2012). The sternum can be used in large breed dogs and horses (Harvey, 2012). One study suggests that, in dogs, the sternum is easier to aspirate than the proximal humerus or the iliac crest, with no notable differences in specimen quality among the three (Defarges et al., 2013). Additionally, it has been indicated that ilium samples tend to provide higher quality core specimens (Nicolae et al., 2023). Furthermore, in small dogs, using a 15 g needle for sampling the humerus is more practical than using a 13 g needle (Abrams Ogg et al., 2012). Studies have shown that appropriately sized needles enhance the quality of specimens obtained from small dog breeds (Abrams-Ogg et al., 2012; Raskin & Messick, 2012). Additionally, the use of rotary-powered devices can increase the efficiency and quality of aspiration, resulting in better cellular integrity and reduced pain (Tappin et al., 2014; Swords et al., 2010).

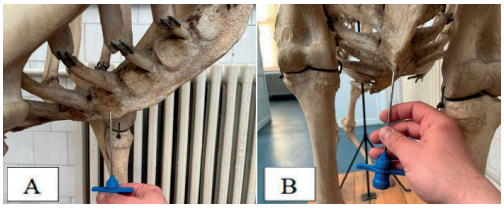

Figure 5. Sternum of a horse, showing the fourth vertebra: A. Lateral view; B. Frontal view

The coxal tuberosity can also be used as a sampling site in young horses and foals (Harvey, 2012). It has been concluded that aspirate samples from the sternum are of higher quality than those sampled from the coxal tuberosity in horses (Delling et al., 2012).

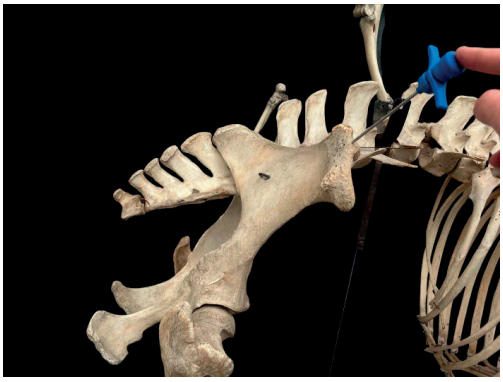

Figure 6. Coxal tuberosity from a horse (Original)

Sampling bone marrow from horses, whether from the sternum (Figure 5) or the coxal tuberosity (Figure 6), implies some risk for practitioners, mainly because of the position of the person collecting the sample (Ribitsch et al., 2010; Delling et al., 2012). In rats, samples usually are harvested from the iliac crest, trochanteric fossa or tibial crest (Brooks et al., 2022).

Sampling technique

Bone marrow sampling is typically performed under sedation. Local anaesthesia through infiltration of the subcutis and periosteum is also required, and may suffice for the aspiration of bone marrow if the animal is cooperative (Mylonakis & Hatzis, 2017).

The procedure must be performed in a sterile manner, which involves clipping hair and surgically scrubbing the selected site (Raskin & Messick, 2012).

When selecting the iliac crest as a collection site, the animal is positioned in sternal recumbency with the pelvic limbs fully flexed and the pelvic acropodium moved cranially under the axilla. The optimal puncture site is located just caudal to the most dorsal point of the dorso-cranial iliac crest (Townsend, 2008). Ensure that the needle is aligned parallel to the long axis of the iliac crest (Harvey, 2012). For the proximal humerus approach, the animals are placed in lateral decubitus, on the side of the animal that matches the predominant hand of the practitioner, then, the shoulder joint is flexed and rotated to expose the site of collection (Villiers et al., 2016). After palpating the greater tubercle of the humerus, the needle is placed into the craniolateral aspect of the proximal humerus (Harvey. 2001) (Figures 7 and 8).

For the femur procedures, the animal is positioned in lateral decubitus, and the needle is aligned parallel to the axis of the bone (Raskin & Messick, 2012). For aspirating the sternum in dogs, Paparcone et al. (2013) placed the animals in right lateral right lateral decubitus with the right thoracic limb pulled cranially and the left thoracic limb pulled caudally, both being parallel to the animal's body.

When collecting samples from the sternum of a horse, the animal should be sedated and restrained appropriately. A stab incision is made in the skin over the sternum, and a bone marrow aspirate needle is introduced through this incision until it contacts the sternebrae, slightly lateral to midline (Sellon, 2006).

Ultrasonography can aid in identifying the right sternebrae (Eydt et al., 2016). An 11-gauge, 10 cm Jamshidi needle can be advanced through the stab incision and advanced to touch the ventral surface of the 5th sternebrae in the midline (Kasashima et al., 2010). Rowland et al. (2018) found that during sternal bone marrow aspiration in horses, there was no significant difference in pain response or reaction compared to a SHAM procedure, when assessed using salivary cortisol, heart rate variability, and sedation parameters. This was true both during the procedure and for two hours afterward.

In rats, a 26-gauge needle was initially used in a study by Vlahovic (1964) to access the femur's marrow cavity through the knee joint, with its position confirmed by extending a wire stylet.

A more recent technique from Ordodi et al. (2006) improves upon this method. In this technique, the rat is placed in dorsal recumbency, and the investigator stabilizes the leg. The needle is inserted perpendicularly through the skin and muscle above the knee joint until it contacts the femur. A rotating motion creates a hole at the junction of the epiphysis and diaphysis, then the needle is repositioned to enter the diaphysis channel.

In mice, Chung (2014) describes the technique for bone marrow aspirate from the femur. The authors describe the method as follows: first, the tibia is bent; the syringe is held with the thumb and index finger for stability; the needle is inserted through the patellar tendon, positioning it between the femoral condyles and into the femur's shaft, then it is rotated to be aligned with the shaft; the needle is slowly advanced and rotated while checking its placement by gently moving the syringe laterally.

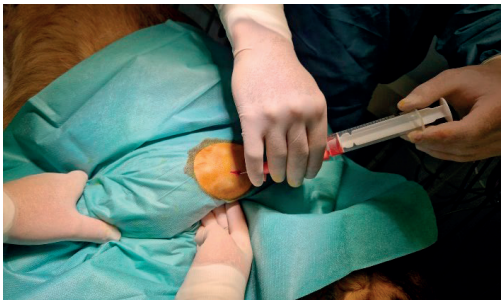

Figure 7. BM aspiration from the proximal humerus of a dog (Original)

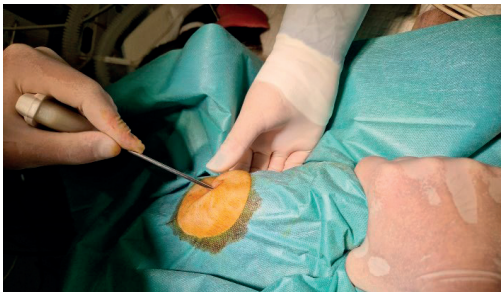

Figure 8. BM core sampling from the proximal humerus of a dog (Original)

Both the Illinois and Jamshidi needles are suitable for aspiration procedures. According to Villiers et al. (2016), in dogs, the procedure is described as follows: the needle is inserted into the sampling site, using alternating rotational movements and firm pressure to penetrate the bone cortex; once in the medullary cavity, the needle should remain stable; after removing the stylet, a 20 ml syringe is attached, and the syringe plunger is pulled sharply 2-3 times to collect the bone marrow; the syringe is removed, and the fluid is quickly transferred to microscope slides before it coagulates.

To collect a core sample, the Jamshidi needle is advanced 2-3 cm into the medullary cavity without a stylet and performed rapid rotational movements to section the specimen. The core sample is extracted by introducing the blunt probe of the needle pack in the tip of the needle (Harvey, 2012) (Figure 9).

For post-mortem collection of BM samples, according to King et al. (2014), any large bone can be broken obliquely or sawed to extract the bone marrow; touch imprint is made first; the remaining tissue can be submitted for histopathology.

Reeder et al. (2013) compared bone marrow samples obtained using two different techniques: the combined technique, which involves collecting an aspirate and a biopsy from the same site consecutively, and the direct technique, which involves obtaining a biopsy only, without prior aspirate. The study concluded that the histological specimens obtained from the combined technique were shorter in length and more frequently diluted with peripheral blood compared to those obtained from the direct technique. The appropriate size of the needle according to the animal weight is shown in Table 3.

Table 2. Different types of needles used for BM sampling (data from Veterinary Instrumentation UK Catalogue 2020)

Needle type	Specifications
Jamshidi	Aspirate/Core Biopsy Disposable
Klima	Aspirate Autoclavable
Rosenthal	Aspirate Autoclavable Similar to Klima
Ilinois	Aspirate Disposable

Table 3. Choosing the size of the needle according to the weight of the animal (data from Veterinary Instrumentation UK Catalogue 2020)

Animal weight	Needle size
<5 kg	18G
5-15 kg	15-16G
15-30 kg	13G
30-50 kg	11G
>50 kg	8G

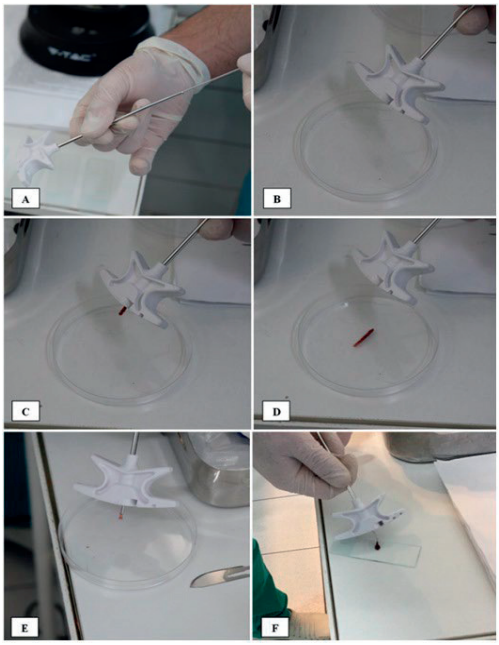

Figure 9. Bone marrow core samples: A-D. Evacuation of the BM core from the needle; E-F. Inappropriate samples: specimen too small (E), specimen missing, only BM liquid is present (F) (Original)

Figure 10. Bone marrow samples and needles. From top to bottom: Jamshidi type needle, Ilinois type needle, Syringe with BM aspirate, rolling imprint smears and core sample (on the second smear) (Original)

CONCLUSIONS

The evaluation of bone marrow in veterinary practice is a critical diagnostic tool with significant implications for both clinical and research fields. Although less commonly utilized compared to human medicine, its importance cannot be overstated. Choosing the right specimen is crucial for effective BM evaluation. To achieve an accurate diagnosis, it is essential to correlate BM results with clinical and haematological data.

Through this literature review, it is evident that indications for bone marrow sampling extend beyond identifying blood dyscrasias, as it plays a pivotal role in lymphoma staging, detection of infectious agents, and understanding conditions such as hyperproteinaemia and hypercalcemia. The examination of bone marrow also opens avenues for advanced research, particularly in understanding the behaviour of disseminated tumour cells, which may serve as significant prognostic markers in companion animals. For practitioners working with domestic carnivores, horses, and laboratory animals, prioritizing high-quality sample collection methods and adhering to established protocols can lead to improved diagnostic outcomes. Finally, bone marrow evaluation in veterinary medicine can enhance our capacity to manage complex health conditions effectively and contribute to advancements in veterinary pathology and oncology.

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INCIDENCE OF RESPIRATORY DISEASES IN POST-WEANED HEIFERS ON A DAIRY FARM

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Abstract

Bovine respiratory disease (BRD) is a leading cause of morbidity and mortality in cattle worldwide, significantly affecting both beef and dairy production systems. This article explores the epidemiology, risk factors, and management strategies linked to BRD. It emphasises the multifactorial nature of the disease, with key contributions from infectious agents like Mannheimia haemolytica, Pasteurella multocida, and viral pathogens, including infectious bovine rhinotracheitis (IBR). Environmental and management factors such as poor ventilation, high animal density, and stress during transport or weaning further increase the disease's incidence and complicate prevention efforts. The study shows that calves aged 2 to 4 months are most vulnerable, particularly during the post-weaning phase when immune defence is weakened, making them more susceptible to infection. Early detection through clinical observation and advanced diagnostic tools is essential, alongside preventive measures like vaccination, proper colostrum management, and stress reduction. The article highlights the significant economic impact of BRD, including slower growth, reduced productivity and higher veterinary costs, and advocates for integrated approaches that combine management, diagnostics, and vaccination to control the disease effectively and reduce long-term losses.

Key words*: Bovine Respiratory Disease, morbidity, mortality, economic impact.*

INTRODUCTION

Bovine respiratory disease (BRD) is a leading cause of illness and death in cattle worldwide, posing significant challenges to both beef and dairy production systems. Characterised by its multifactorial nature, BRD is influenced by an intricate interplay of infectious agents, environmental conditions, and management practices. Pathogens such as *Mannheimia haemolytica*, *Pasteurella multocida*, and the infectious bovine rhinotracheitis (IBR) virus are commonly implicated, often acting synergistically to exacerbate disease severity (Griffin et al., 2010; Fulton, 2009).

Environmental and management factors - such as overcrowding, inadequate ventilation, and stress from transport or weaning - further heighten susceptibility, particularly in calves aged 2 to 4 months during their vulnerable post-weaning phase (Taylor et al., 2010).

Early diagnosis, coupled with preventive strategies like vaccination, colostrum management, and stress reduction, has been emphasised as key to mitigating the disease's impact (Cusack et al., 2003; Chirase & Greene, 2001).

The economic repercussions of BRD are substantial, including reduced growth rates, diminished productivity, and increased veterinary expenditures, making it a priority for producers and veterinarians alike (Duff & Galyean, 2007).

The prevalence of BRD varies by farm; results from Bach (2011) also showed that 4 or more clinical BRD events before weaning doubled the odds that a calf would leave the herd before completing her first lactation. Lung consolidation (because of an inflammatory process) may also increase the risk of preweaning mortality and decrease the likelihood of pregnancy and first-lactation milk production (Buczinski et al., 2014; Buczinski et al., 2018; Dunn et al., 2018). Moreover, the upper respiratory resident bacterial population is known to play a crucial role in respiratory health (Caswell, 2014); however, the characterisation of its role in the pathogenesis of BRD, due to perturbations of the microbiota, is not well understood (McMullen et al., 2019). Early

diagnosis is essential for control of BRD, as late identification leads to a greater risk of treatment failure, chronic disease, and a higher number of calves weaning with "dirty lungs" (i.e., lung consolidation; Ollivett, 2016). Clinical scoring systems facilitate recognition of calves with clinical BRD; however, these systems are insensitive to detecting calves with subclinical disease and do not reliably correlate with lung lesions identified at necropsy (Love et al., 2014; Maier et al., 2019; Berman et al., 2021).

Ultrasonography (US) has become an essential diagnostic tool for evaluating pulmonary abnormalities in calves with respiratory disease, providing a non-invasive, real-time assessment of lung pathology. Unlike traditional auscultation, which may be subjective, lung ultrasonography (LUS) offers a more objective and sensitive method to detect early and subclinical pulmonary lesions. Lung ultrasonography (US) is a non-invasive diagnostic tool that is highly accurate in identifying lung consolidation (Rabeling et al., 1998; Maier et al., 2019). Integrating clinical respiratory scoring (CRS) and US can improve detection of BRD by capturing subclinical cases that may be missed by physical examination alone (Maier et al., 2019; Cramer and Ollivett, 2019).

The purpose of this study is to assess the impact of bovine respiratory disease (BRD) on the health of dairy cows in a large-scale farm by using clinical examination, ultrasound, and necropsy. Microbiological evaluation was done for subjects with suspicion of bacterial infections at necropsy. It was retrospectively analysed data from 2023 and 2024 from a Romanian dairy farm with approximately 4,500 cattle.

MATERIALS AND METHODS

Farm and herd health management

The farm operates an intensive rearing system, with detailed records maintained for each animal, including primary data such as identification, health status, production parameters, and reproduction records. This dairy milks 1,100 Holsteins and has an average of 13,000 kg of milk/305 milking days. Environmental indicators, such as temperature, humidity, ventilation efficiency, and air quality, are also monitored regularly, focusing more on adult cows.

This study was conducted on 2710 calves. In this farm, sexed semen is predominantly used, leading to a greater number of female calves; this study is made on females. Case selection was based on everyone who has a respiratory clinical sign. All the calves included in the evaluation are kept outdoors in individual pens (Figure 1).

In 2023, out of a total of 1335 births, 1195 were female calves, accounting for 89.5% of the total. In 2024, from 1375 births, 1250 were females, 90.9% of the total.

The dataset was compiled from monthly farm records, encompassing critical metrics related to births, deaths, and respiratory health outcomes. The primary data points included:

- reproductive metrics - total births, categorised into male and female calves;
- mortality data - total deaths, disaggregated by gender (e.g., female and male deaths) and age groups (0-20 days, 20-60 days, and > 60 days).

All data were recorded as part of routine farm management practices, with records maintained by trained personnel.

During gestation, all dams received vaccination against *E. coli*, rotavirus, and coronavirus to safeguard against neonatal diarrhoea. Newborn calves were fed about 4 litres of colostrum within their first 6 hours of life and were then placed in individual stalls lined with straw bedding.

At birth, calves are fed exclusively colostrum, verified by a refractometer to have a Brix value of at least 22. In our study, no female calf exhibited a serum protein level below 5. On the second day of life, along with blood sample collection to assess colostrum effectiveness, the calves were administered a dose of iron as a preventive measure.

Between January 2023 and January 2024, the vaccination protocol for calves was carefully designed to ensure comprehensive protection against respiratory and clostridial diseases:

- Intranasal vaccine (7-10 days of age): calves were administered an intranasal vaccine targeting Bovine Respiratory Syncytial Virus (BRSV) and Parainfluenza-3 Virus (PI3);
- Subcutaneous respiratory vaccine (14-21 days of age): a subcutaneous vaccine was given to target BRSV, PI3, and *Mannheimia haemolytica*

serotypes A1 and A6. A booster dose was administered approximately 28 days later to strengthen and prolong immunity;
- Clostridial vaccine (14-21 days of age): during the same period (ideally between 14-21 days), calves received a vaccine against *Clostridium perfringens*. A booster dose was provided about 4 weeks after the initial vaccination to ensure robust and lasting protection.
Since February 2024, the vaccination protocol has been updated, discontinuing the use of intranasal vaccination in favour of a subcutaneous vaccine. This new vaccine is administered starting on the 10th day of life and targets the same respiratory pathogens - Bovine Respiratory Syncytial Virus (BRSV) and Parainfluenza-3 Virus (PI3). The subcutaneous administration provides systemic protection, and a booster dose is given at 21 days of age to ensure robust and long-lasting immunity.
This revised protocol is implemented alongside the existing clostridial vaccination schedule. The clostridial vaccine, targeting *Clostridium perfringens* and related pathogens, is also administered during the 14-21-day window, with a booster approximately 4 weeks later.

Weaning and post-weaning management
Calves underwent a structured weaning and post-weaning protocol designed to minimise stress, ensure a smooth dietary transition, and optimise immunity development.
The management practices were as follows:
1. Weaning phase (60-65 days of age): the process included gradually reducing milk feedings over several days to minimise digestive and behavioural stress. During this phase, calves were introduced to dry feed, allowing them to adjust to a solid diet before complete weaning.
2. Post-weaning transition/grouping (66-80 days of age): after weaning, calves were moved into groups of three animals. Dry feed was the sole dietary component during this period, with rations formulated to meet the nutritional needs of growing calves. Feed intake was monitored daily to ensure proper adaptation. Calves were closely observed for signs of stress, such as reduced feed intake, weight loss, or respiratory symptoms, which could indicate disease or difficulty adapting to the new diet.
3. Integration into larger groups (81-100 days of age): calves were transitioned into larger groups of 30 animals to promote socialisation and prepare them for group housing in later production phases. Environmental conditions, such as ventilation, bedding quality, and stocking density, were optimised to reduce stress and the risk of respiratory infections during this period. Calves were regularly checked for signs of illness, with particular attention to respiratory symptoms, given the increased risk associated with larger group housing.
4. Vaccination Protocol: initial Herpesvirus Vaccination (100-120 days of age): All calves received their first dose of the vaccine for bovine herpesvirus. Booster Dose (130-150 days of age): A booster dose was administered one month after the initial vaccination to ensure robust and long-lasting immunity. Vaccination records were meticulously maintained to ensure compliance with the schedule.
5. Supportive care: additional measures, such as providing adequate bedding, access to clean water, and consistent handling practices, were implemented to reduce stress during weaning and group transitions. During the transition periods, calves received nutritional supplements, including vitamins and trace minerals, to support their immune system and overall health. This systematic approach aimed to reduce the risk of disease outbreaks, including respiratory infections, during critical growth periods. The combination of gradual dietary changes, group housing strategies, and timely vaccination ensured that calves developed strong immunity and adapted well to their production environment.

Clinical and paraclinical evaluations
Animals were sourced from a dairy farm and case selection was based on everyone who has a respiratory subclinical or clinical sign.
The subjects (2445 female calves) were monitored daily, and veterinary consultation was conducted whenever clinical signs of illness were observed. The clinical manifestations of respiratory disease in calves include coughing, nasal discharge, pyrexia, tachypnoea, dyspnoea, lethargy, and reduced feed intake. The calf's body temperature was measured using a digital rectal thermometer, followed by pulmonary auscultation to assess respiratory sounds. Subsequently, thoracic ultrasonography was

performed to evaluate lung health and detect potential lesions associated with respiratory disease. According to Feitoza et al. (2025), "TUS provides superior accuracy in detecting both subclinical and advanced respiratory conditions, particularly in high-risk populations".

Pulmonary ultrasonography was performed on 308 live animals using a specific ultrasound machine and probe, a 7.5 MHz linear transducer. Thoracic ultrasound examinations were conducted throughout the weaning period. Examinations were conducted bilaterally across defined thoracic zones following established protocols. Findings such as comet-tail artefacts, pleural irregularities, consolidations, and pleural effusions were recorded and classified according to severity. In ultrasonography, bovine respiratory disease (BRD) severity is classified based on lung lesions. In the mild stage, stage 1, minimal changes are seen, with slight consolidation or fluid that does not significantly impact lung function. In the moderate stage, stage 2, more consolidation and pleural effusion are present, with visible tissue changes. The severe stage, stage 3, shows significant lung damage with extensive consolidation and pleural effusion. In the advanced stage, stage 4, fibrosis or scarring may occur, along with abscesses or cavities. These findings were later correlated with post-mortem lesions to assess diagnostic accuracy.

Pathological evaluation

Necropsies were performed on 22 animals following standard procedures, with special attention to the respiratory system. Lungs, trachea, bronchi, and associated lymph nodes were collected and examined for pathological changes. Macroscopic examination of the lungs was conducted to assess the presence of lesions such as consolidation, pleuritis, abscess formation, and fibrin deposition. Lesions were scored based on distribution, severity, and type using a standardised grading system.

Microbiological evaluation

Samples were collected from 19 animals. Lung tissue and tracheal swabs were cultured on selective and non-selective media (e.g., blood agar, MacConkey agar) and incubated under aerobic and anaerobic conditions. Colony morphology, Gram staining, and biochemical tests were used for bacterial identification. These analyses were performed in an authorised laboratory.

RESULTS AND DISCUSSIONS

Clinical exam findings

In 2023, out of a total of 1195 females, 274 (22.9%) exhibited respiratory signs. When categorised by age, 28 (2.3%) affected females were between 0 and 20 days old, 30 (2.5%) were between 20 and 60 days old, and the majority, 216 (18.1%), were older than 60 days.

In 2024, in contrast, out of 1250 females, only 99 (7.9%) showed respiratory signs. The age distribution of affected females was as follows: 1 (0.08%) in the 0-20-day group, 6 (0.48%) in the 20-60-day group, and 92 (7.4%) in the over 60-day group.

Over the course of 2023 and 2024, a total of 308 cases of calves presenting with pulmonary pathology were documented beyond 60 days of age. The affected calves exhibited a range of clinical signs characteristic of respiratory disease, including nasal discharge in 54 cases (17.53%), coughing in 44 (14.29%), laboured breathing in 16 cases (5.19%), fever in 72 cases (23.38%), lethargy in 6 cases (1.95%), poor appetite in 18 cases (5.84%), ocular discharge in 45 cases (14.61%), abnormal lung sounds in 36 cases (11.69%), and weight loss in 86 cases (27.92%). These symptoms varied in severity, with some calves displaying mild nasal discharge and intermittent coughing, while others exhibited more pronounced respiratory distress and systemic illness. Fever was present in 72 cases (above 39.5°C), indicating an active inflammatory response. Lethargy and reduced feed intake were particularly notable in severely affected calves, contributing to weight loss and overall poor body condition. Calves that showed lethargy were most often hypothermic, even in the summer months, and when the temperature dropped below 38°C, all bodily functions progressively deteriorated, ultimately leading to death.

In auscultation, abnormal lung sounds, such as wheezing and crackles, were detected, suggesting lower respiratory tract involvement, possibly due to bronchopneumonia or interstitial pneumonia.

Laboured breathing and abnormal lung sounds suggest lower airway involvement, potentially due to pulmonary oedema, bronchoconstriction, or mucus accumulation. The presence of nasal and ocular discharge indicates excessive mucus production, which is a typical response to viral.
The progression to weight loss and poor body condition (Figure 6) highlights the metabolic toll of chronic inflammation and reduced nutrient absorption. In this study, we observed in 42.6% of cases a weight loss, comparing the body size of calves of the same age and from the same group.

Ultrasonographic findings

In this study, ultrasonographic examination of the thorax revealed several characteristic pulmonary abnormalities, including:
B-lines (Lung Rockets): These hyperechoic, vertical artefacts extending from the pleural line indicate interstitial syndrome, commonly associated with pulmonary oedema, inflammation, or early pneumonia (Figures 2 and 4).
These B-lines appeared also in several scanned calves, which over time did not materialise into clinical signs of respiratory pathology. Also appeared in all pulmonary cases (100%).
Pleural Irregularities and Thickening: Disruptions in the normally smooth pleural interface suggest pleuritis or consolidation, in agreement with findings from previous studies on bovine respiratory disease (Timsit et al., 2020). In our research, it appeared in 10.07% (31 cases) of all 308 cases.
Lung Consolidation (Figure 3): Hypoechoic, non-aerated lung regions were detected in calves with severe respiratory symptoms, indicating bronchopneumonia. We found lung consolidation in 6.82% (21 cases).
Pleural effusion: some cases exhibited anechoic fluid accumulation within the pleural space, consistent with secondary pleuritis or fibrinous pneumonia. We diagnosed 22 (7.15%) cases with pleural effusion from all calves older than 60 days with respiratory disease.
The severity of lung ultrasonographic findings correlated positively with clinical symptoms such as laboured breathing, coughing, fever, and abnormal lung sounds on auscultation. Calves exhibiting severe dyspnoea and lethargy were more likely to have extensive lung consolidation and pleural thickening, suggesting a more advanced disease state (Figures 3 and 5).

Pathological findings

Post-mortem examination identified lung consolidation in 21 (95.45%) of cases, with pleuritis and abscess formation observed in 20 cases (90.9%).
Histopathology revealed neutrophilic infiltration, alveolar damage, and fibrin deposition, consistent with severe respiratory disease. Lesion severity was significantly associated with clinical signs, emphasising the importance of early detection.
In post-mortem findings we found:
Lungs: lobar pneumonia in 14 cases (63.64%) with firm, dark red areas with fibrinous exudates in cranio-ventral lobes, suggesting acute bacterial infection; bronchopneumonia we get it in 6 cases (27.28%); purulent exudate and inflammation around bronchi, likely caused by *Mannheimia haemolytica* or *Pasteurella multocida*; interstitial pneumonia 1 case (4.55%): diffuse congestion, oedema, and thickened septa;
Trachea and bronchi: tracheitis with mucosal congestion with mild catarrhal exudate; bronchitis with mucus accumulation and haemorrhagic exudates, indicative of secondary bacterial infection, presented in 6 cases (27.28%);
Pleura: pleuritis consisting of fibrinous exudate and mild adhesions, commonly secondary to bacterial pneumonia, presented in 7 cases (31.82%).

Comparative analysis of pulmonary ultrasonography with necropsy findings

Pulmonary ultrasonography revealed abnormalities in all of the animals with clinical signs of respiratory diseases, including lung rockets`, B lines (Figure 4), pleural effusions, and pulmonary consolidations (Figure 5).
These findings showed a high correlation with post-mortem lesions, with a sensitivity of (100%), and to calculate specificity, a control group of healthy calves confirmed lesion-free on post-mortem would be required.

Microbiological findings

Bacterial cultures identified *Mannheimia haemolytica* in 2 cases, *Pasteurella multocida* in

4 cases, and *Histophilus somni* in 4 cases. Not for all the cases could we identify the bacteria. The pathogenicity could not be determined for all specimens.

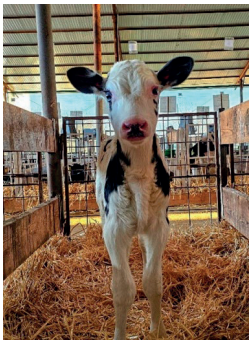

Figure 1. A young, healthy calf with his own pen

Figure 2. Post-mortem appearance of the lungs of a calf with ante-mortem lung ultrasound multiple B-lines (lung rockets), indicating alveolar-interstitial syndrome

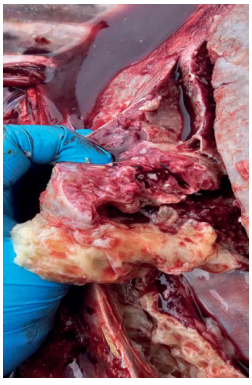

Figure 3. Following the post-mortem examination, this finding is consistent with lung consolidation

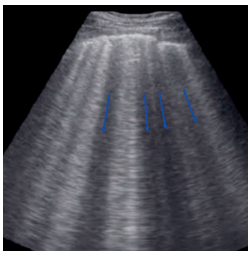

Figure 4. Ultrasonographic exam with B-lines (lung rockets) found in the early stages of pneumonia

Figure 5. Following the necropsy examination, fluid was identified in the thoracic cavity, along with fibrin and pleuritis in the calf

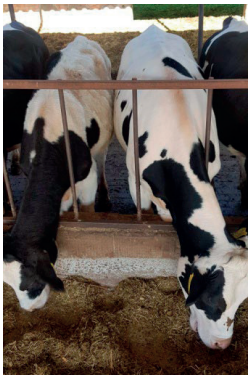

Figure 6. Two calves of the same age group exhibited notable differences in growth. The left calf has a documented history of respiratory diseases, while the right calf has no such medical records

Our findings align closely with prior research on BRD and other respiratory infections in calves. Nasal discharge and coughing are among the earliest clinical indicators of respiratory disease, often progressing to abnormal lung sounds and weight loss if left untreated. Similarly, Lisuzzo et al. (2024) emphasised that fever and respiratory distress correlate strongly with bacterial pneumonia, particularly in cases involving *Mannheimia haemolytica* or *Pasteurella multocida*.

However, some discrepancies exist between our findings and previous reports. While Wilson et al. (2017) found that severe weight loss was uncommon in BRD cases managed with early antibiotic intervention, our study observed significant weight loss in some calves, likely due to delayed treatment initiation and reduced feed intake. This suggests that early detection and prompt therapeutic intervention play a crucial role in mitigating disease severity and economic impact.

According to Bowen et al. (2021), calves with a history of respiratory disease before weaning exhibit significantly lower weaning weights and reduced lifetime productivity, emphasising the need for early intervention and preventive measures.

Environmental monitoring helps correlate conditions that increase the risk of respiratory diseases in calves. Studies have shown that housing calves outdoors, particularly in individual hutches with proper shelter, is associated with a decreased risk of respiratory diseases compared to indoor group housing.

Outdoor housing provides better ventilation and reduces exposure to airborne pathogens, which are common risk factors for respiratory issues in calves. Like on this farm, all the calves are housed outdoors. This study highlights the significant role of pulmonary ultrasonography as a valuable diagnostic tool in detecting and monitoring respiratory disease in cattle. The correlation between ultrasonographic findings and post-mortem examinations confirms its reliability in assessing lung pathology. The identification of bacterial and possible viral pathogens reinforces the necessity of targeted antimicrobial and preventive strategies. Implementing ultrasonography in routine veterinary practice can enhance disease management, reduce treatment failures, and improve animal welfare.

CONCLUSIONS

The clinical signs observed, including nasal discharge, coughing, fever, and weight loss, align with previous studies on BRD, underlining the importance of early detection, proper vaccination, and improved management strategies to mitigate disease impact.

Ultrasonographic examination of the lungs proved to be a valuable, non-invasive diagnostic tool for detecting pulmonary pathology in calves with respiratory disease. Findings such as B-lines, pleural irregularities, lung consolidation, and pleural effusion provided objective evidence of disease severity, complementing clinical assessment.

Following an integrated analysis of the results from clinical exam, ultrasound, necropsy, and microbiology, measures were implemented to adjust the vaccination protocol, leading to a reduction in the incidence of bovine respiratory disease (BRD). A notable decrease in respiratory disease prevalence from 2023 to 2024, dropping from 22.9% to 7.9%. The reduction was observed across all age groups, with the most significant decline occurring in females older than 60 days (from 18.1% in 2023 to 7.4% in 2024). This downward trend suggests a potential improvement in health management or environmental conditions influencing respiratory disease incidence due to changes in the vaccination protocol.

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ATLANTOAXIAL INSTABILITY IN A YORKSHIRE DOG - CASE REPORT

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Abstract

Atlanto-axial instability is a condition that primarily affects small breed dogs, especially toy breeds, with a higher prevalence in young animals. These abnormalities can secondarily cause neurological disorders such as cervical pain and tetraplegia. This study aims to highlight the challenges in diagnosing atlantoaxial instability due to multiple causes, using advanced imaging techniques. The study was conducted on a 1.3-year-old female Yorkshire Terrier presenting neurological signs suggestive of atlantoaxial instability. Radiographic examination, CT scans, and MRI were performed, confirming the diagnosis based on developmental/congenital abnormalities at the occipito-atlanto-axial level.

Key words*: Imaging Diagnosis; atlantoaxial instability; MRI; toy breed; CT.*

INTRODUCTION

Atlantoaxial instability represents a common cause of cervical pain and neurological disorders characterised by tetraplegia or paraplegia (Slanina, 2016). This condition was first reported in 1967, primarily affecting small breeds, especially toy breeds such as Yorkshire Terrier, Pomeranian, Miniature or Toy Poodle, and Chihuahua, with the highest prevalence in young individuals - over 50% of cases occurring in dogs under six months of age (Slanina, 2016; Cummings et al., 2018).

This condition may have congenital or acquired causes, often resulting from cervical trauma (Slanina, 2016). Congenital causes include aplasia or hypoplasia of the odontoid process, dorsal angulation or degeneration of the odontoid process, and the absence of ligamentous support. Additionally, incomplete ossification of the atlas is considered a predisposing factor in large breeds (Wheeler, 1992; Slanina, 2016). In congenital cases, symptoms appear before the age of two years and may progress to cervical hyperesthesia, ataxia, and severe tetraplegia.

Atlantoaxial joint instability frequently results in excessive flexion, leading to the dorsal displacement of the axis relative to the atlas and subsequent spinal cord injury. Clinical signs range in severity from neck pain to paralysis, depending on the degree of spinal cord compression and contusion caused by the displacement. In the most severe cases, this subluxation can cause respiratory failure and death. Neurological symptoms may develop either suddenly or gradually in individuals with atlantoaxial instability (Slanina, 2016; Aikawa et al., 2013).

Atlantoaxial instability is typically diagnosed using standard radiographs; however, advanced imaging methods such as computed tomography (CT) and magnetic resonance imaging (MRI) provide valuable supplementary details and assist in planning for surgical treatment (Slanina, 2016; Aikawa et al., 2013).

This case study highlights the challenges of diagnosing atlantoaxial instability using advanced imaging techniques.

MATERIALS AND METHODS

The study involved a 1.3-year-old female Yorkshire Terrier puppy displaying cervical pain and paraplegia. A suspected diagnosis of atlantoaxial luxation was established following a neurological examination, and a specialized imaging examination was recommended.

An EXAMION radiology device was used for the radiological examination. The animal was

positioned in right lateral recumbency, ensuring a mild flexion of the cervical region.

The computed tomography (CT) examination was performed using a General Electric (GE) Revolution Evo 64-slice system, with a transverse helical CT series at the level of the skull and cervical region, reformatted with the following algorithms: native-bone/soft tissue; post-contrast-bone/soft tissue.

For the magnetic resonance imaging (MRI) examination, a Siemens Magnetom system with a power of 1.5 Tesla was used. The imaging protocol consisted of T1-weighted Spin Echo (SE) and T2-weighted Fast Spin Echo (FSE) sequences, performed with the animal placed in sternal recumbency under general anaesthesia. (Neagu et al., 2018).

Premedication was administered intravenously with Butorphanol at a dose of 0.2 mg/kg, followed by induction using Propofol at 3-5 mg/kg IV. After intubation, anaesthesia was sustained with Isoflurane and 100% oxygen. A volume-cycled ventilator provided spontaneous breathing or intermittent positive-pressure ventilation (IPPV), delivering 12-15 breaths per minute to maintain an end-tidal CO_2 level between 35-45 mmHg.

Following induction, oxygen was initially administered at 2 L/min, with the anaesthetic vaporizer set to achieve a target end-tidal isoflurane level of 2.0% within 10 minutes. Once this concentration was stabilized, the oxygen flow was decreased to (500 + 10/kg) mL/min, and the isoflurane concentration was maintained at 1.5% for the remainder of the procedure to ensure adequate anaesthesia. (Tudor et al., 2019; Pavel et al., 2021).

RESULTS AND DISCUSSIONS

The study's patient is a 1.5-year-old female Yorkshire Terrier. The clinical examination revealed cervical hyperesthesia when moving the head and neck. The neurological examination identified paraplegia and delayed proprioceptive sensitivity in the hind limbs, but with normal reflexes and a preserved normal mental status.

As a result, the neuroanatomical localization was identified at the cervical spinal level. Based on the VITAMIND acronym, the potential aetiologies considered included inflammatory, traumatic, and degenerative causes. Following the neurological assessment, radiographic imaging and computed tomography of the cranio-cervical region were recommended to evaluate the osseous structures, along with magnetic resonance imaging to assess the degree of spinal cord involvement. Radiographic evaluation of the C1-C2 cervical region demonstrated an increased distance between the ventral arch of the atlas and the odontoid process, as well as a widened space between the dorsal arch of C1 and the spinous process of C2. It was not possible to determine whether spinal cord compression was present.

Figure 1. Right lateral radiograph of the cranio-cervical junction showing an increased distance between the ventral arch of the atlas (C1) and the odontoid process, along with a widened space between the dorsal arch of C1 and the spinous process of C2

Computed tomography identified abnormalities at the atlantooccipital joint, including hypoplastic occipital condyles, mild dorsoventral elongation of the foramen magnum, and a slight ventral displacement of the atlas. At the atlantoaxial joint, findings included misalignment of the vertebral lamina characterized by significant widening of the space between the dorsal tubercle of C1 and the spinous process of C2 - partially corrected by positioning during imaging - as well as mild hypoplasia of the odontoid process

During the MRI examination at the C1-C2 level on the sagittal images of the occipito-atlanto-axial segment, an increase in the distance between the base of the C1 vertebra and the odontoid process can be observed. Additionally, an increase in the distance from the C1 dorsal arch to the C2 spinous apophyse is noted. There is also the disappearance of the T2 hyperintense

signal from the dorsal and ventral subarachnoid space, as well as spinal cord modifications such as dorsal deviation, along with compression. This is due to hypoplasia/aplasia of the dens or changes in its position.

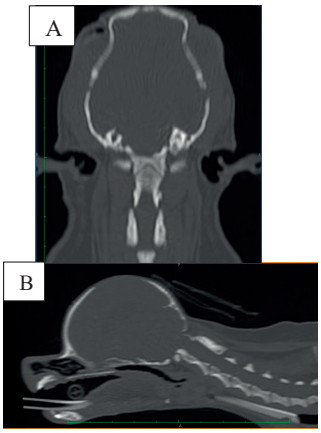

Figure 2. Dorsal (A) and sagittal (B) reconstructions of the cranio-cervical junction reveal hypoplastic occipital condyles, a mild dorso-ventral elongation of the foramen magnum, and a subtle misalignment of the atlas with ventral displacement. At the atlantoaxial joint, there is malposition of the vertebral lamina, characterized by significant widening of the space between the dorsal tubercle of C1 and the spinous process of C2, which spontaneously corrected with CT positioning. Additionally, a slight hypoplasia of the dens is noted

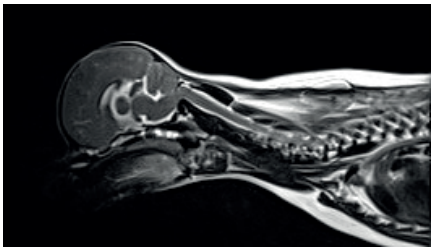

Figure 3. Sagittal image - C1-C2 segment - shows a noticeable widening of the space between the base of the C1 vertebra and the odontoid process. Additionally, an increased gap is evident between the posterior arch of C1 and the spinous process of C2. The T2 hyperintense signal is absent at both the dorsal and ventral subarachnoid levels. Furthermore, the spinal cord shows a dorsal displacement accompanied by compression

Atlantoaxial instability is a condition that tends to occur most commonly in small breeds, particularly Yorkshire, Pomeranian, Chihuahua, and Pekinese breeds (Cerda-Gonzales et al., 2010). A study published by Tudury et al. in 2018 highlighted that among toy breeds, the prevalence is highest in Yorkshire Terriers (30.30%), Poodles (21.21%), mixed-breed dogs (12.12%), and Miniature Pinschers (9.09%). However, it can also be found in large breeds such as Rottweilers (Wheeler et al., 2008). The age of affected animals ranges from 3 to 108 months, but 72.72% of affected animals were 12 months old or younger, indicating that this condition is more common in young animals, especially during the first year of life, as observed in the current study (Beckmann et al., 2010; Tudury et al., 2018). The primary causes are usually linked to congenital abnormalities of the C1 and C2 vertebrae, such as malposition of the vertebral lamina leading to an enlarged gap between the dorsal tubercle of C1 and the spinous process of C2, along with underdevelopment or absence of the odontoid process. Additionally, minor cervical traumas can trigger or worsen the symptoms of instability, leading to neurological changes (Seim, 2008; Stalin et al., 2014).

The diagnosis of atlantoaxial instability relies on the patient's medical history and neurological assessment. It is confirmed with the help of advanced imaging methods, which are particularly valuable as they allow evaluation of the angulation between the C1 and C2 vertebrae, identification of potential anomalies in this region, and, through MRI, assessment of the extent of spinal cord compression (Waschk et al., 2019; Stalin et al., 2014).

An essential indicator in diagnosing atlantoaxial instability is the widened space between the posterior arch of the atlas and the spinous process of the axis (Kealy & McAllister, 2005; Fernandez et al., 2010). Seim describes in 2010 that a space of over 4 to 5 mm between the C1 lamina and the dorsal spinous tubercle of C2 generally allows for the diagnosis to be established in small breed dogs. Despite the numerical values described by Seim (2008), these are imprecise due to the significant variability in the size of dogs, thus maintaining subjectivity.

The diagnosis through radiographic examination is recommended to be performed without general anaesthesia if the patients are cooperative, to avoid causing further spinal cord trauma through the head flexion manoeuvre

(Lorenz & Kornegay, 2006; Fernandez et al., 2010).

Radiographic and CT examinations are valuable techniques for diagnosing conditions localised to the bony structures, whether traumatic or congenital in nature. However, to confirm the diagnosis of spinal cord involvement, MRI remains the most valuable method (Stalin et al., 2014; Cerda-Gonzales et al., 2010).

The clinical signs presented in this study, the age of the animal, and the breed are consistent with previous studies on this topic.

CONCLUSIONS

Atlantoaxial instability predominantly affects dogs, particularly those of small and toy breeds, with Yorkshire Terriers showing the highest incidence. Clinical data are correlated with the neurological examination and supplemented with specialised imaging to confirm the diagnosis.

Radiology and CT examination are the most useful techniques for visualising the bony changes underlying atlantoaxial instability, while MRI helps determine the degree of spinal cord involvement.

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COMPARATIVE STUDY OF THE USE OF AMNIOTIC MEMBRANE SUSPENSION (EYEQ AMNIOTIC EYE DROPS®) IN NON-VASCULARIZED AND VASCULARIZED INDOLENT CORNEAL ULCERS IN DOGS

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Abstract

Indolent corneal ulcers in dogs, also known as canine spontaneous chronic corneal epithelial defects (SCCEDs), represent a significant challenge for veterinarians due to their frequency and tendency to recur, particularly in brachycephalic breeds. Debridement plays a crucial role in the healing process of indolent corneal ulcers in dogs. Over time, combinations of antibiotic eye drops and corneal healing agents have been employed.
This study compared the effectiveness of an amniotic membrane suspension (EyeQ Amniotic Eye Drops®, Vetrix, Cumming, GA, USA) in 22 dogs diagnosed with non-vascularized and vascularized indolent corneal ulcers following multiple debridement procedures. The results of the study demonstrated that the presence of corneal vascularization reduces the healing period compared to non-vascularized indolent corneal ulcers.

Key words*: indolent corneal ulcer, amniotic membrane suspension eye drops, debridement, dog.*

INTRODUCTION

Indolent corneal ulcers in dogs that fail to heal and are refractory to conventional therapy are common in veterinary practice. Known as canine spontaneous chronic corneal epithelial defects (SCCEDs) can be superficial characterized by loss of corneal epithelium and exposure of corneal stroma without stromal loss (Gelatt et al., 2013; Eaton et al., 2017) or can be deep corneal ulcers and involve stromal defect (Bentley, 2005; Gellat et al., 2021).
Clinically, patients present ocular pain, blepharospasm, epiphora, corneal oedema peripheral to the lesion and the fluorescein test is positive (Maggs et al., 2013). Examination with a magnifying glass reveals a non-adherent anterior corneal epithelium under which fluorescein stain test is positive (Figure 1). Debridement of the non-adherent epithelium is a mandatory procedure before initiating local therapy. Cotton-tip debridement, scalpel blade debridement and superficial grid keratotomy (Boutin et al., 2020; Ionașcu, 2023) are inexpensive and efficient methods for the treatment of SCCEDs, performed under local anaesthesia.
In many cases (Figure 2), the anterior corneal epithelium is removed over a significant surface area.
Landrevie et al. (2023) utilized combinations of cotton-tip epithelial debridement and corneal thermal cautery, with or without diamond burr debridement, in the treatment of this condition.

Figure 1. OS Four-years-old French Bulldog with central indolent corneal ulcer, bordered by a lip of non-adherent epithelium

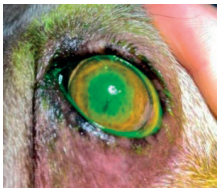

Figure 2. OS Previous case after cotton-tip debridement

After debridement, local treatment is carried out using antibiotic eye drops and corneal healing agents, administered 3-4 times per day (Maggs et al., 2017).

In indolent corneal ulcers, following debridement, daily disposable human contact lenses (Acuvue Oasys®) or bandage lenses specifically designed for dogs (An-Vision, GmbH®) can be used. These lenses protect the cornea, reduce pain, and consequently shorten the healing time (Ionașcu, 2021; Ionașcu, 2023). Another study demonstrates that a cross-linked, modified HA hydrogel provides further benefit by accelerating time to corneal wound closure compared to a non-cross-linked HA solution (Williams et al., 2017).

Over the past few years, a new type of matrix therapy agent named ReGeneraTing Agent (RGTA®) has provided encouraging results, accelerating the healing of chronic skin ulcers of diabetic or vascular origin (Martinez et al., 2019). RGTA® is a set of molecules, chemically engineered polymers, that are specifically designed to replace degraded heparan sulphate molecules in the injured matrix compartment. Therefore, they are considered as heparan sulphate mimetics based on their chemical structures and functions. RGTA® protects naturally existing structural and signalling proteins, and in doing so, creates a cellular microenvironment favourable to healing, thereby enhancing the speed and quality of tissue repair (Barritault et al., 2017; Hayek et al., 2016*)*.

In the domain of ophthalmology, an RGTA® family compound named OTR4120, a heparan sulphate mimetic, has been reported to show encouraging results for the treatment of corneal ulcers and dystrophies of various aetiologies (Chebbi et al., 2008).

The amniotic membrane (AM) has a long history of use in the treatment of various diseases of the ocular surface in human. It contains pluripotent cells, highly organized collagen, anti-fibrotic and anti-inflammatory cytokines, immune-modulators, growth factors, and matrix proteins (Murri et al., 2018). Recently, AM extract and AM extract eye drops have been successfully used in clinical applications, including dry eye and chemical burns in human (Murri et al., 2018). This is due to its ability to preserve the biochemical properties of the corneal material while delivering essential extracellular matrix components to the damaged cornea (Guo et al., 2011; Ledbetter et al., 2006).

Using the results obtained in the treatment of corneal conditions in humans with eye drops containing amniotic membrane suspension as a reference, a new product (EyeQ Amniotic Eye Drops®, Vetrix, Cumming, GA, USA) has been developed for use in veterinary ophthalmology in recent years.

In the specialized literature, experimental studies have been conducted using amniotic membrane eye drops (EyeQ Amniotic Eye Drops®, Vetrix, Cumming, GA, USA) in cases of superficial corneal ulcers in horses (Lyons et al., 2020) and stromal ulcers in rats (Lee et al., 2024).

The studies conducted by Lyons, 2020 aimed to evaluate the effect of amniotic membrane extract on re-epithelialisation of equine corneal ulcers compared with ulcers treated with antibiotic, antifungal and mydriatic medical therapy alone, and to evaluate equine corneal healing after experimentally induced superficial ulceration.

Lee et al., 2024 conducted a comparative study on the effect of topical amniotic membrane suspension (AMS) and ReGeneraTing Agent (RGTA) on surgically induced deep stromal ulcers in rats.

To the best of our knowledge, there are no studies investigating the efficacy of amniotic membrane suspension in non-vascularized and vascularized indolent corneal ulcers in dogs. The aim of this study is to present the clinical efficacy of amniotic membrane suspension (EyeQ Amniotic Eye Drops®, Vetrix, Cumming, GA, USA) in superficial and deep indolent corneal ulcer in dogs.

MATERIALS AND METHODS

A total of 22 dogs were presented at the Veterinary Teaching Hospital (Faculty of Veterinary Medicine Bucharest) for nonhealing corneal ulcers after failure of primary treatment by the referring veterinarian, usually by epithelial debridement, without evidence of defect resolution for several weeks to months. A total of 22 dogs (22 eyes) were evaluated in these case reports. All dogs were privately owned pets. Owners reviewed and signed an informed consent form before samples were

collected, as well as providing consent for the use of (EyeQ Amniotic Eye Drops®, Vetrix, Cumming, GA, USA) after debridement of indolent corneal ulcer.

To assess the efficacy of EyeQ Amniotic Eye drops®, Vetrix, Cumming, GA, a total of 22 dogs referred for non-healing ulcers were enrolled in this study.

All dogs underwent complete clinical evaluation and 10 dogs were diagnosed with superficial non-vascularized indolent corneal ulcer (Figures 3 and 4), 5 dogs were diagnosed with deep non-vascularized indolent corneal ulcers (Figure 6), and 7 dogs were diagnosed with deep vascularized indolent corneal ulcers (Figure 8).

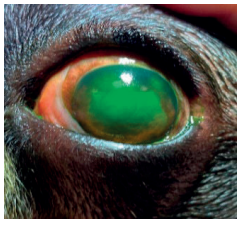

Figure 3. OS Seven-years-old French Bulldog with superficial non vascularized indolent corneal ulcer

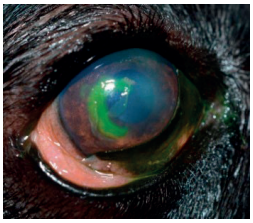

Figure 4. OD Eleven-years-old Terra Nova with superficial non vascularized indolent corneal ulcer

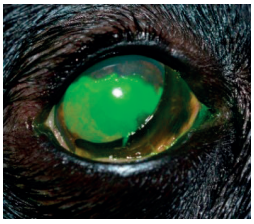

Figure 5. Case from figure 4, eleven-years-old Terra Nova with superficial non vascularized indolent corneal ulcer, clinical aspect after cotton-tip debridement

All dogs underwent sterile cotton-tip debridement under local anaesthesia with oxybuprocaine hydrochloride 0.4% (Benoxi®, Unimed Pharma).

The cotton-tipped swab was passed over the ulcer in multiple circular motions, removing the non-adherent epithelium (Figure 5, Figure 7, and Figure 9).

Figure 6. OD Nine-years-old English Bulldog with deep non vascularized indolent corneal ulcer

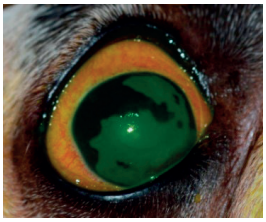

Figure 7. Case from figure 6, nine-years-old English Bulldog with deep non vascularized indolent corneal ulcer, clinical aspect after cotton-tip debridement

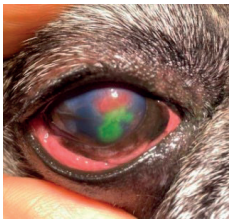

Figure 8. OD Ten-years-old English Bulldog with deep vascularized indolent corneal ulcer

Figure 9. Case from figure 6, ten-years-old English Bulldog with deep vascularized indolent corneal ulcer clinical aspect after cotton-tip debridement

After debridement, the dogs were locally treated with Tobramycin eye drops (Tobrom®, 3 mg/ml, Rompharm Company SRL) și EyeQ

Amniotic Eye drops®, Vetrix, Cumming, GA, three times a day. The patients were examined every 7 days for a period of 2 months, and debridement of the ulcers was performed when the fluorescein test revealed the denuded anterior epithelium.

The corneal ulcers were considered healed when the cornea showed a negative fluorescein test and there was no evidence of blepharospasm or ocular discharge.

The data were analyzed for breed, age, duration of clinical signs before referral, number of cotton-tip debridements, number of weeks before corneal vascularization appeared, number of weeks before healing was achieved, and any and complications.

RESULTS AND DISCUSSIONS

The corneal ulcers had been treated by referring veterinarians for a median of 13 weeks (range 2 weeks - 24 weeks) prior to enrollment in this study. Medical treatment consisted of topical and systemic antibiotics, as well as topical artificial tear solutions containing hyaluronic acid.

Dog breeds included French Bulldog (n=5), Shih-Tzu (n=3), English Bulldog (n=2), Bichon (n=2), Pug (n=2), Pekingese (n=2), crossbreds (n=2), Terra Nova (n=1), Samoyed (n=1), Jack Russel Terrier (n=1), Amstaff (n=1). The median age was 7 years, with a range between 1 year and 13 years. Eight patients were female (36.36%) and 14 were male (63.64%). Ten patients were treated in the right eye (45.45%) and 12 in the left eye (54.55%).

From the total number of dog patients (n=22), 5 dogs (22.72%) were diagnosed with non-vascularized deep indolent corneal ulcers (DN), 10 dogs (45.45%) with non-vascularized superficial indolent corneal ulcers (SN) and 7 dogs (31.82%) with vascularized deep indolent corneal ulcer (DV). The number of debridements varied: 3 dogs underwent 1 debridement, 5 dogs had 2 debridements, 10 dogs had 3 debridements, 2 dogs had 4 debridements, 1 dog had 5 debridements, and 1 dog had 6 debridements (Table 1).

Table 1. Patients' data included in the study

Case number	Breed	Age (years) Sex	Affected eye	Type of ulcer	Number of debridements	Time until vascularization	Healing time (weeks)	Complication
1.	Samoyed	11/M	OD	DN	3	3	8	pigmentation
2.	Bichon	12/F	OD	DN	2	3	5	None
3.	Jack Russel Terrier	13/M	OS	DN	2	3	5	pigmentation
4.	English Bulldog	7/M	OS	DN	4	1	5	pigmentation
5.	Shih Tzu	6/M	OD	DN	3	1	4	None
6.	French Bulldog	7/M	OD	SN	6	4	8	None
7.	English Bulldog	6/M	OS	SN	3	4	6	None
8.	French Bulldog	3/M	OD	SN	3	2	5	None
9.	Terra Nova	11/F	OD	SN	5	5	12	pigmentation
10.	Pug	6/M	OS	SN	3	1	5	None
11.	French Bulldog	7/F	OD	SN	2	1	3	pigmentation
12.	French Bulldog	10/F	OS	SN	3	3	12	pigmentation
13.	French Bulldog	1/F	OS	SN	1	without	2	None
14.	Amstaff	10/M	OS	SN	4	4	6	None
15.	Pug	2/M	OD	SN	1	without	3	None
16.	Bichon	3/F	OS	DV	1	present	3	None
17.	Shih Tzu	12/M	OS	DV	3	present	4	None
18.	Shih Tzu	12/F	OS	DV	3	present	4	None
19.	Pekingese	8/M	OD	DV	2	present	3	None
20.	Crossbreds	9/M	OS	DV	3	present	5	None
21.	Crossbreds	12/M	OD	DV	2	present	4	pigmentation
22.	Pekingese	13/F	OS	DV	3	present	5	None

*SN - superficial non vascularized indolent corneal ulcer; DN - deep non-vascularized indolent corneal ulcer; DV - deep vascularized indolent corneal ulcer.

After 2 weeks of treatment, 4.54% (1/22) of cases with superficial non-vascularized indolent corneal ulcers (SN) healed (Figure 10).

Figure 10. OS One-year-old French Bulldog with superficial non vascularized indolent corneal ulcer (SN) healed after 2 weeks and one debridement

After 3 weeks of treatment 18.18% (4/22) of cases healed, including two with superficial non-vascularized indolent corneal ulcers (SN) and two with deep vascularized indolent corneal ulcers (DV).
After 4 weeks of treatment 18.18% (4/22) of cases healed, including one with deep non-vascularized indolent corneal ulcer (DN) and three with deep vascularized indolent corneal ulcers (DV).
After 5 weeks of treatment 31.81% (7/22) of cases healed, including two with superficial non-vascularized indolent corneal ulcers (SN), three with deep non-vascularized indolent corneal ulcers (DN), and two with deep vascularized indolent corneal ulcers (DV).
After 6 weeks of treatment 9.09% (2/22) of cases, both with superficial non-vascularized indolent corneal ulcers (SN), healed.
After 8 weeks of treatment 9.09% (2/22) of cases healed, including one with superficial non-vascularized indolent corneal ulcer (SN) and one with deep non-vascularized indolent corneal ulcer (DN).
After 12 weeks of treatment 9.09% (2/22) of cases with superficial non-vascularized indolent corneal ulcers (SN) healed.
In patients with superficial non-vascularized indolent corneal ulcer (SN) și deep non-vascularized indolent corneal ulcer (DN) corneal vascularization appeared within a time frame ranging from 1 to 4 weeks. The vascularization was localized at the ulcer's margin (Figure 11) or, in some cases, very abundant, covering the entire surface of the ulcer (Figure 12).

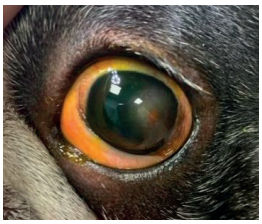

Figure 11. Clinical appearance of the vascularization in superficial non vascularized indolent corneal ulcer (SN) after 5 weeks of treatment

Figure 12. OS Ten-years-old French Bulldog, clinical appearance of the vascularization in superficial non vascularized indolent corneal ulcer (SN) after 6 weeks of treatment

The patients with deep vascularized indolent corneal ulcer (DV) healed at 3 weeks (2/7), 4 weeks (3/7) and 5 weeks (2/7). The cases (Table 2) with superficial non vascularized indolent corneal ulcer (SN) developed vascularization within a time frame ranging from 1 to 5 weeks, and healing occurred at 6 weeks (2/10), at 8 weeks (1/10) and at 12 weeks (2/10).
Two out of ten (2/10) cases with superficial non vascularized indolent corneal ulcer (SN) healed without the appearance of vascularization.

Table 2. Patients' data included in the study

Healing period in weeks	Number of cases with SN	Number of cases with DN	Number of cases with DV
2	1	0	0
3	2	0	2
4	0	1	3
5	2	3	2
6	2	0	0
8	1	1	0
12	2	0	0
TOTAL	10 cases	5 cases	7 cases

Fifteen out of twenty-two (68.2%) had healed without complications (Figure 13). The complications encountered in this case series were corneal pigmentation (Figure 14) in 7 cases (31.81%).

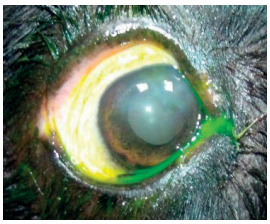

Figure 13. Case from figure 4, OD Eleven-years-old Terra Nova clinical appearance after 12 weeks of treatment for superficial non vascularized indolent corneal ulcer

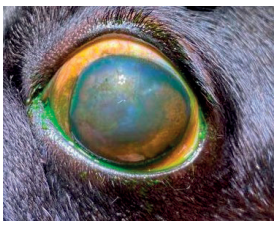

Figure 14. Case from figure 12 OS Ten-years-old French Bulldog, 12 weeks after the treatment. Corneal opacity and mild pigmentation

The local treatment with antibiotics (Tobrom®, 3mg/ml, Rompharm Company SRL) și EyeQ Amniotic Eye drops®, Vetrix, Cumming, GA administered three times a day, along with the debridements, led to the appearance of vascularization in superficial non-vascularized indolent corneal ulcers (SN) and deep non-vascularized indolent corneal ulcers (DN). Corneal vascularization accelerated the healing process.

Amniotic membrane extract eye drops (AMEED) showed a potential benefit in acute corneal injuries when human corneal epithelial cells and human limbal cells treated with AMEED healed faster after mechanical insult. In vitro and in vivo studies reveal that AMEED supports proliferation and differentiation of corneal epithelial cells, enhances epithelial wound healing, and inhibits corneal neovascularization and was as effective as transplanted AM in healing corneal damage in a rabbit model (Murri et al., 2018; Choi et al., 2011).

In addition, amniotic membrane extract (AME) can be combined with umbilical cord (UC) blood as another type of AM-derived solution (Tighe et al., 2017). This combination may be advantageous because the active-matrix component responsible for exerting AM's anti-inflammatory effects (HC-HA/PTX3 complex).

The AM has a long history of use in the treatment of various diseases of the ocular surface. It contains pluripotent cells, highly organized collagen, anti-fibrotic and anti-inflammatory cytokines, immune-modulators, growth factors, and matrix proteins. It is used to promote corneal healing in severely damaged eyes (Murri et al., 2018).

The amniotic membrane has long played a pivotal role in human corneal surgery (Meller et al., 2011), contributing to both the structural fortification of the cornea and the acceleration of the healing process. This is due to its ability to preserve the biochemical properties of the corneal material while delivering essential extracellular matrix components to the damaged cornea (Guo et al., 2011; Shimmura et al., 2002). Additionally, amniotic membrane exhibits antifibrotic and antiangiogenic effects, mitigating corneal scarring and maintaining transparency (Shimmura et al., 2002).

Most of the beneficial effects of AM are attributed to biomolecules, such as fibronectin, hepatocyte growth factor (HGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), transforming growth factor (TGF), and collagen types I, III, IV, and V, which are potent sources for corneal regeneration (Mamede et al., 2012). They combine to facilitate migration, adhesion, and differentiation of the model specifically designed to evaluate deep stromal ulcers (Lee et al., 2024).

We must emphasize here the need for careful selection of the patients.

In our study the patients did not show any local or systemic side effects. Owner compliance was very good, except for the fact that the product needs to be stored in the refrigerator between administrations. Some owners reported that the patient keeps its eye closed and exhibits minimal blepharospasm after administration.

This case series is limited by the heterogeneity of the cases and the lack of a comparative control group. Further research is needed using OCT for accurate diagnosis of the depth and extent of the lesions, as well as for assessing the healing process. Further studies are necessary to compare (EyeQ Amniotic Eye Drops®, Vetrix,

Cumming, GA, USA) with other therapies in corneal pathologies in dogs.

CONCLUSIONS

The results of this study suggest that the (EyeQ Amniotic Eye Drops®, Vetrix, Cumming, GA, USA) may be a safe and effective treatment for indolent corneal ulcers in dogs.

The (EyeQ Amniotic Eye Drops®, Vetrix, Cumming, GA, USA) is well tolerated, and the corneal healing is achieved with only 3 administrations per day.

The appearance of corneal vascularization reduces the healing time. In non-vascularized indolent corneal ulcers, the healing period is longer.

Further studies are necessary to determine the clinical efficacy in larger trials using OCT for classifying indolent ulcers based on their depth and extent.

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CLEFT PALATES IN BRACHYCEPHALIC DOGS

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Abstract

This study aimed to assess the quality of life and survival rate of brachycephalic puppies with congenital orofacial clefts (cleft palate and lip) during their first month of life. Conducted at the Faculty of Veterinary Medicine in Bucharest, the research included 12 puppies from brachycephalic breeds, with a majority of French Bulldogs. The study found that 4 puppies died immediately after birth, 2 within the first 48 hours, and the remaining 6 were monitored for 30 days. The puppies faced significant health challenges, including feeding difficulties, aspiration pneumonia, and malnutrition, leading to a high mortality rate. The results emphasize the need for better breeding controls, genetic screening, and early intervention to reduce congenital defects and improve outcomes for these dogs.

Key words*: cleft palates, brachycephalic, dog.*

INTRODUCTION

Brachycephalic breeds such as the English Bulldog, French Bulldog, Shih Tzu, and Pug have become extremely popular both in Romania and globally. These breeds are highly appreciated due to their docile temperament and affection towards their owners. However, this popularity is accompanied by a series of health problems induced by their lack of genetic diversity. As demand for such dogs has increased, so have health issues, often due to the disorganized breeding of these dogs, with inadequate control (Estevam, Marina et al., 2022).

Brachycephalic breeds are characterized by a short skull shape and flat nose, and are prone to a variety of congenital malformations, most frequently in the cranial area. Orofacial clefts may occur during embryonic or fetal development as a result of incomplete fusion of the anatomical structures in this area (Van den Berghe et al., 2010; Bar-Am, 2013). The upper lip and the incisive bone form the primary palate, while the secondary palate consists of the palatal fold and hard palate which is represented by palatal process of the maxilla and the palatine bone (Warzee et al., 2001; Nelson, 2003; Dyce et al., 2009; Van den Berghe et al., 2010). As a result, a cleft palate is defined as a congenital oro-nasal fistula caused by incomplete fusion of the structures that separate the oral cavity from the nasal cavities (Krzyżewska & Max, 2008; Fossum 2009).

Cleft disorders include cleft lip (cheiloschisis), characterized by the lack of union between the maxillary prominence and the medial nasal process, and cleft palate (palatoschisis), which can be primary or secondary. Primary palatoschisis is caused by incomplete fusion of the medial nasal processes and affects the incisive bone, almost always accompanied by cheiloschisis. Secondary palatoschisis affects the palatal process of the maxilla and the palatine bone, leading to incomplete fusion of these structures. Both defects can occur simultaneously, with varying severity, from incomplete unilateral to complete bilateral (Bar-Am, 2013).

Cleft palate can occur separately or alongside developmental abnormalities of other organs, leading to comorbidities that threaten the survival of the puppies (Nelson, 2003; Ingwersen, 2012).

This study aimed to investigate the quality of life and survival rate of brachycephalic canines with palatoschisis during their first month of life.

MATERIALS AND METHODS

The study was conducted at the University Emergency Hospital of the Faculty of Veterinary Medicine in Bucharest and included 12 female brachycephalic dogs that required

assistance during delivery or needed an emergency cesarean section. These 12 females gave birth to a total of 53 offspring, of which 16 presented primary and secondary cleft palates of varying degrees (Figures 1 and 2).

Figure 1. Primary cleft palate

The offspring included in the study were subjected to the APGAR test immediately after resuscitation manoeuvres. The test included 5 parameters, and based on the obtained score, the offspring were categorized into 3 risk groups: 0-3 points, puppies with low vitality; 4-6 points, puppies with moderate vitality; and 7-10 points, puppies with normal vitality.

Figure 2. Incomplete primary cleft palate

A total of 6 offspring, from the moment of extraction, were classified in Group I (with low vitality) and died within the following 6-10 hours due to respiratory failure and severe bradycardia (63-82 rpm and heart rate under 70 bpm). All remaining offspring in the study were weighed and hospitalized for a 48-hour period for care and monitoring. Feeding was provided using milk replacers (Royal Canin 1st Age Milk), ensuring a comfortable position for the puppies to prevent the occurrence of complications related to aspiration.

After 48 hours, the offspring were discharged and handed over to the owners, who were given instructions on feeding methods, proper feeding position, the need for daily weighing, and maintaining a body weight chart. Communication with the owners occurred daily or at least once every 3 days to monitor the offspring's progress and identify any potential medical issues requiring immediate attention.

Out of the 10 offspring discharged after 48 hours, the sudden death of 4 was reported, seemingly without clinical symptoms during the first 6 days after discharge. Another 3 offspring presented to the hospital as emergencies 10 days after discharge, with acute respiratory failure that developed suddenly, hypothermia, increased heart rate, weak pulse, and apathy. The daily body weight chart for these 3 offspring showed a sawtooth pattern, with 2 of them remaining stationary, and the third offspring's weight dropped below the discharge limit. After clinical and radiological evaluation, it was determined that these 3 offspring had developed aspiration pneumonia.

Only 3 offspring survived without major medical issues until the age of 30 days. The body weight chart assessment revealed a very slow but favourable development, with the animals remaining below the average weight for their age (Table 1, Figure 3).

Table 1. The body weight chart over the course of the 30 days of monitoring.

Day	Puppy 1 (Weight in g)	Puppy 2 (Weight in g)	Puppy 3 (Weight in g)
0	177	192	208
3	182	194	210
6	184	200	218
9	186	202	223
12	191	212	224
15	202	217	230
18	207	222	238
21	205	225	241
24	215	232	245
27	222	235	253
30	225	242	257

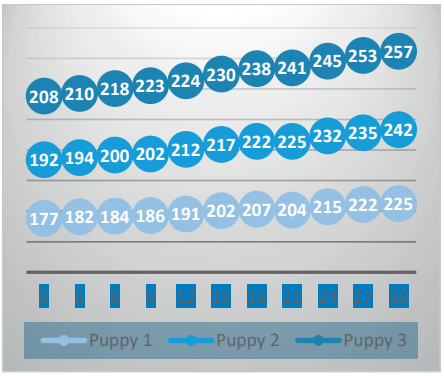

Figure 3. The body weight chart over the course of the 30 days of monitoring

RESULTS AND DISCUSSIONS

Cleft palate, both primary and secondary, requires surgical correction to prevent chronic respiratory infections and to facilitate proper food intake. Numerous authors have suggested that the optimal age for surgery is between 3-5 months of life.

Throughout this period, the puppies require special care and a specific diet to meet their metabolic needs and support necessary physical development. Feeding in a vertical position and the systematic cleaning of food debris are extremely important to ensure quality of life and enable the puppies to reach the appropriate age for corrective surgery.

An extremely important aspect is the physiological characteristics of canine offspring. Canine newborns are born with minimal fat reserves, with most of their energy provided through glycogenolysis. The depletion of glycogen concentration in muscles and the liver after birth occurs very quickly, and hepatic gluconeogenesis is limited due to the immaturity of the liver. At the same time, thermogenesis is absent during the first 7 days after birth. These characteristics make newborn puppies highly sensitive to hypoglycaemia and hypothermia, indicating their dependence on proper feeding capacity and, consequently, an upward trend in their daily body condition score.

CONCLUSIONS

Cleft palate is a huge challenge in veterinary medicine due to the feeding difficulties of the offspring. Respiratory failure with severe cyanosis occurs during or immediately after feeding. Associated complications, such as aspiration pneumonia and malnutrition, lead to exhaustion of the puppies and are one of the main causes of sudden death within the first 30 days postpartum.

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STUDY ON THE EFFICACY OF PHYTOTHERAPY IN THE TREATMENT OF ARTHROSIS IN FIVE DOGS

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Abstract

Phytotherapy is a branch of alternative medicine, based on the healing properties of plants, which humans have used since ancient times. Considering the reduced risks of the bioactive compounds in botanical sources, the introduction of this therapy became of interest to veterinary medicine, especially in correlation with chronic pathologies. This is particularly relevant for managing chronic conditions where long-term pharmacological treatments often lead to adverse reactions. Arthrosis, a degenerative joint disease, is characterized by the progressive degradation of articular cartilage, accompanied by inflammatory processes and resultant pain and treated with a protocol that includes the administration of non-steroidal anti-inflammatory drugs (NSAIDs), analgesics, and adjunctive joint protectors. The present study aims to investigate the efficacy of phytotherapy in five canine patients diagnosed with arthrosis, by introducing a supplement whose composition includes Nettle extract, Boswellia extract, Copper, Turmeric Extract, and Manganese. Before and following the cessation of conventional therapy, each patient underwent a comprehensive pain scale assessment, alongside monitoring of relevant biochemical parameters, to ascertain the impact of the alternative treatment.

Key words*: phytotherapy, alternative medicine, arthrosis, pain management.*

INTRODUCTION

One of the most frequent arthropathies in humans and dogs, arthrosis, is defined as a complex degenerative syndrome, which includes a series of intrinsically interconnected lesions: cartilage degradation, new bone formation organized as osteophytes, and adjacent inflammation of periarticular tissues (Clark, 2015). The aetiology of this condition is multifactorial, but there are six risk factors more frequently attributed to the onset of the disease in scientific literature, respectively genetic factors and factors related to the patient's signalment (conformation, race, weight, age and hormonal status). Studies have shown that the highest chromosomal involvement occurs for hip dysplasia, breeds with a genetic predisposition (such as German Shepherd, Siberian Husky, Labrador retriever, Rottweiler) requiring close monitoring from an early age (Anderson et al., 2020; Beuing et al., 2000; Sallander et al., 2006). A disproportion of joint angles as well as overweight (sometimes occurred after sterilization) are also responsible for triggering degenerative lesions. It is known that arthrosis can evolve in two forms (primary and secondary), mainly affecting the shoulder, elbow, knee and pelvis joints. The process is a gradual one, which takes place in two phases - an inflammatory phase (with insidious evolution) and a destructive phase, in which chemical and mechanical aggression causes changes in the composition of the synovial fluid, leading to a difficult and very painful locomotion (Allegri et al., 2012; Clark, 2015), part of a vicious circle, as shown in Figure 1.

The suspicion diagnosis of arthrosis is established based on signalment data (age, sex, weight, race), in corroboration with the anamnesis and the results of the clinical examination which will highlight the presence of lameness, pain, crepitation and stiffness of the joint. The confirmation of the diagnosis requires the use of imaging techniques - radiography (with the three mandatory diagnostic criteria - the articular pinch, located in the area of maximum pressure, osteochondensation and the appearance of osteophytes) or arthroscopy (which allows direct visualization of intra-articular lesions), as well as arthrocentesis (Pettitt & German, 2015).

The treatment is multimodal, palliative and involves a classical approach and an alternative approach based on phytotherapy (Fox & Mills, 2010). The standard protocol includes the use of drug treatment (non-steroidal anti-inflammatory drugs (AINS) and chondroprotective) and non-drug treatment (obesity control, physical rehabilitation programs, diet rich in Omega 3) (Sallander et al., 2006).

Phytotherapy, part of the CAM (complementary and alternative medicine) concept, is used as an adjuvant therapy for chronic diseases in companion animals, allowing the reduction of doses for allopathic treatments and decreasing the risk of side effects associated with these therapies (Mills & Bone, 2000). Phytotherapy and complementary medicine are often subjects of debate, with some individuals remaining sceptical while others incorporate them into their daily routines (Poppenga, 2007).

The limited scientific evidence in the field of phytotherapy has led to doubt among clinicians regarding its effectiveness and safety. Additionally, existing studies often show results that are only slightly better than those of a placebo (Bone & Mills, 2013). However, in practical application, clinicians who use phytotherapy have observed genuinely positive outcomes. In certain cases, improvements are noted even when conventional treatments have been unsuccessful. It is essential to consider the patient's individual response to treatment, as reactions can vary. This personalized approach plays a crucial role in achieving successful phytotherapeutic treatment (Fox & Mills, 2000).

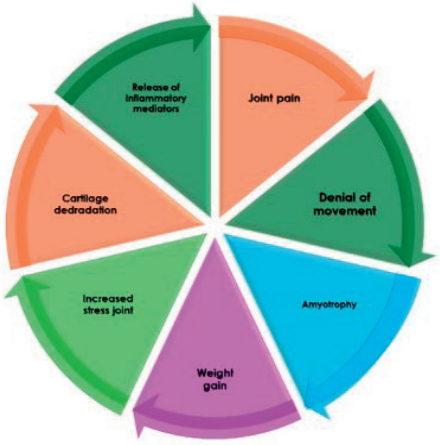

Figure 1. Arthrosis vicious cycle

MATERIALS AND METHODS

This study was carried out during the year 2023 at the University Veterinary Emergency Hospital "Prof. dr. Alin Bîrțoiu" and included five canine patients diagnosed with arthrosis, as shown in Table 1.

Table 1. Signalment data of the five patients included in the study

Species	Breed	Sex	Age (years)
CANINE	Mixed large breed	F	11
	Labrador retriever	M	11
	Bichon Maltese	F	10
	German Shepard	M	7
	Labrador Retriever	M	12

The treatment was carried out exclusively with Doloflamil, a product that contains as active ingredients nettle, curcuma, Boswellia, manganese and copper, for a minimum period of 21 days, the dose administered being 1 tablet per 5 kg. The non-inclusion criteria were - dogs on conventional anti-inflammatory and pain-relieving treatments (the phytotherapeutic product could only be administered 48 hours after stopping them), those diagnosed with polyarthrosis, septic arthritis or other differential diagnoses of arthrosis, and those with very severe pain (a score of more than 55 on the Helsinki Chronic pain index scale).

Pain intensity was measured in a subjective manner, using two scales widely recognized by other researchers - the Helsinki chronic pain index (Hielm-Björkman et al., 2009), and the canine brief pain inventories (Brown et al., 2008), which were completed by the owners before and after the end of treatment. After selecting the five patients and stopping any previous treatment (if necessary), the study protocol involved recording the anamnesis, performing clinical examination, confirming the diagnosis by radiographic technique and taking blood samples. These were necessary to determine whether the dog qualified for participation in the study by evaluating kidney function, liver function, and CBC values. The obtained values, along with additional blood

parameters, were then analysed before and after treatment to assess any potential change and to identify the presence or absence of metabolic side effects.

RESULTS AND DISCUSSIONS

Case no 1, an 11-year-old female mixed-breed dog weighing 30 kg, was diagnosed a year ago with arthrosis affecting both acetabular cavities, the head and neck of the left femur, and the head of the right femur. Her initial treatment included a supplement containing glucosamine, chondroitin, and hyaluronic acid. She was also given Curcumin (1 pill per day), Omega-3 (1000 mg, 1 pill per day), Gabapentin (300 mg), and Silymarin (1 pill per day). However, approximately a year later, she was diagnosed with additional spondylosis in the L3-L4 vertebrae. Blood tests and a cardiological examination revealed no abnormalities. As a result, her previous treatment was discontinued and, 48 hours later, replaced with a Doloflamil. She received five pills per day for 21 days. Pain levels decreased both during and after the treatment. After completing this therapy, Gabapentin was reintroduced, but due to the positive effects of Doloflamil, the dosage was reduced by half, showing significant improvement in her condition.

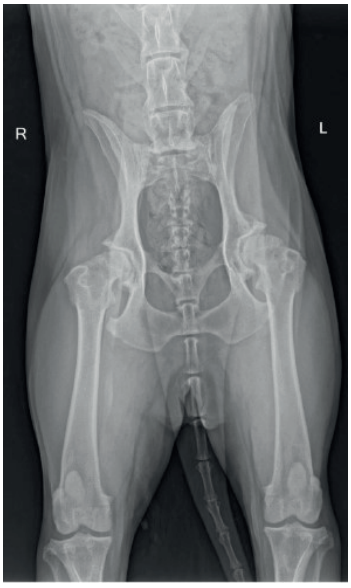

Figure 2. Sclerosis of the femoral head and acetabular cavity, with bilateral mild bone remodeling

Case no 2, an 11-year-old male Labrador weighing 35 kg, retired from police service two years ago. He was diagnosed with arthrosis five years ago, along with spondylosis in the T_3-T_7 region and lameness in his left hindlimb. Blood tests revealed no significant abnormalities. For treatment, he was prescribed Gabapentin 300 mg (one pill per day), WeJoint Plus (one pill per day), Omega 3 (1 g per day), and Hepatiale 550 mg (one pill per day). A year ago, he received monoclonal antibody therapy with Librela 20 mg for two months, administered once per month. This initially provided relief, but after three months, his arthrosis symptoms, pain, and neurological issues returned. He was given Doloflamil for 21 days, starting with five pills per day. Once the Doloflamil treatment was stopped, he resumed his previous Gabapentin treatment, though at a reduced dose, which was sufficient to maintain his comfort.

Case no 3 was represented by a female Bichon Maltese of 10 years and 7 kg, who was diagnosed with bilateral arthrosis of the femoral head, with the left side being more deteriorated than the right. Treatment with Doloftamil was administered for 21 days, at a dose of one pill per day, and the owner noticed a considerable decrease in pain after this period.

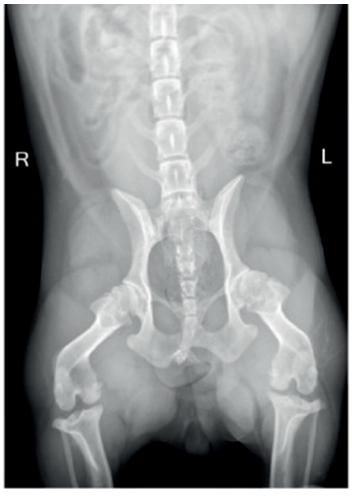

Figure 3 Sclerosis of the femoral head and acetabular cavity, with severe bone remodeling and mineralization on the femoral head with formation of new bone, bilaterally

Case No. 4 was a German Shepherd, 7 years old and weighing 43 kg. He had been diagnosed

with arthrosis 2 years ago, but for about 1 year the symptoms worsened. Previously, he received treatment with We joint. The blood tests showed no abnormalities, so the previous treatment was stopped and the patient started therapy with Doloflamil for 21 days, 8 pills/day. After one day, the pain decreased considerably and remained at this level for a long period, in contrast to the experience with previous therapies.

Patient no 5 was a male Labrador retriever, 41 kg and 12 years old. One year ago, he was diagnosed with bilateral coxo-femoral arthrosis, which was treated with Gabapentin 40 mg (1 tablet/day) and Silymarin 1g (1 tablet/day).

In February 2022, the dog's clinical condition worsened, showing signs of right-side lameness, difficulty lying down and standing up, along with neurological findings of paraparesis and pain upon palpation of the pelvis and thoracolumbar spine. Blood test results were normal. To alleviate severe pain, meloxicam was administered for three days, followed by the introduction of Doloflamil, 24 hours later. The prescribed dosage was 8 pills per day for 21 days. One month later, the dog's gait showed improvement, he was able to run at a light jogging pace. After completing the initial treatment, Gabapentin was reintroduced at half the usual dosage, which was sufficient to manage the residual pain.

The Diagram 1 and 2 are based on two pain assessment scales. The blue columns represent the data recorded before treatment, showing the intensity of pain, the dog experienced prior to receiving Doloflamil. In contrast, the orange columns depict the pain levels recorded after treatment.

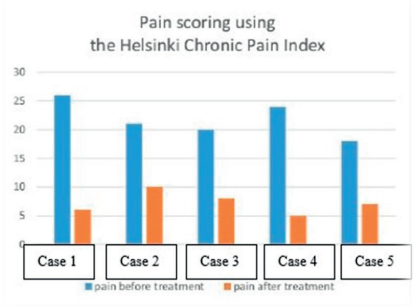

Diagram 1. The results of pain evaluation using Helsinki Chronic Pain Index

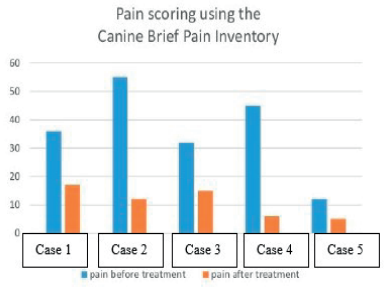

Diagram 2. The results of pain evaluation using Canine Brief Pain Inventory

Pain evaluation considered daily activities such as getting up from decubitus, climbing stairs, and jumping. It also evaluates behavioural changes, like increased vocalization or aggression. The pain is rated on a scale of 0 to 10 in the "Canine Brief Pain Inventory", which includes 10 questions, and on a scale of 0 to 4 in the "Helsinki Chronic Pain Index", which consists of 11 questions, where 0 represents the absence of pain. The total pain score is calculated by summing up the responses from all the questions, providing a numerical value that allows the comparison performed before and after treatment.

Concerning the global results, it can be noticed that regarding the predisposition factors, the research has shown that arthrosis occurs at geriatric age, as the average age of the patients was 10 years, value that has been previously demonstrated by similar studies (Anderson et al., 2020). Related to the breed, out of the five patients included in the study, two of them belonged to breeds whose predisposition has been previously demonstrated – Labrador Retriever and German Shepard (Beuing et al., 2000; Sallander et al., 2006). Regarding the central element of the article, the effect of the tested product in reducing pain, the results show that all patients experienced a significant improvement, regardless of the scale of measurement used. In this study the minimum tested period was as recommended by the manufacturer (21 days) to obtain visible results. However, continuing the treatment over a longer period and combining phytotherapy with allopathic treatment could significantly improve current therapeutic protocols.

It is wide known that the standard pharmacological management of arthrosis in

veterinary medicine frequently involves the administration of nonsteroidal anti-inflammatory drugs (NSAIDs), which exert their therapeutic effect primarily by inhibiting cyclooxygenase (COX) enzymes, thereby reducing the synthesis of pro-inflammatory prostaglandins (Henea et al., 2021). While NSAIDs are effective in alleviating pain and inflammation, their long-term use is associated with significant adverse effects, including gastrointestinal ulceration, renal toxicity, hepatotoxicity, and altered hemostasis, particularly in geriatric or systemically compromised animals (Clark, 2015; Beșchea Chiriac & Solcan, 2024).

In contrast, phytotherapy offers a promising, multi-modal approach to the management of arthrosis in dogs. Botanical extracts such as *Boswellia serrata*, *Curcuma longa* (curcumin), *Harpagophytum procumbens* (devil's claw), and *Urtica dioica* (stinging nettle) have demonstrated anti-inflammatory, antioxidant, and chondroprotective properties in both *in vitro* and *in vivo* studies (Mills & Bone, 2000). These compounds modulate inflammatory pathways without the severe adverse effect profile associated with NSAIDs.

Furthermore, phytotherapeutic agents often exert synergistic effects, targeting multiple biochemical pathways involved in arthrosis pathogenesis. Additionally, polyphenols and flavonoids present in many medicinal plants possess strong antioxidant capabilities, helping to mitigate oxidative stress, a known contributor to chronic joint inflammation and cartilage erosion.

The incorporation of phytotherapy into veterinary practice aligns with the growing emphasis on integrative and multimodal pain management strategies. While phytotherapeutic agents are not without limitations – such as variability in bioavailability, standardization, and regulatory oversight – their favourable safety profile and potential to reduce reliance on NSAIDs support their role as either adjunctive or primary therapeutic options for long-term OA management. Rigorous clinical trials and pharmacokinetic studies are warranted to optimize dosing regimens, evaluate therapeutic efficacy, and ensure quality control.

These arguments emphasise the fact that phytotherapy represents a scientifically credible and clinically relevant alternative to conventional NSAID therapy in canine osteoarthritis. Its ability to reduce inflammation, preserve joint integrity, and minimize systemic toxicity makes it an attractive option in the pursuit of sustainable, long-term management of arthrosis in dogs.

CONCLUSIONS

Arthrosis is a frequently encountered disease in geriatric canine population, with a multifactorial aetiology, which significantly affects the animal's quality of life through the presence of pain. In the present study, the median age was 10 years old, and two patients belong to breeds whose predisposition has been previously proven (Labrador Retriever and German Shepard).

The diagnosis of this condition must include history and clinical examination findings, but confirmation and staging of the disease is performed by radiography, as proceeded with the dogs included in the study

In all five patients, phytotherapy demonstrated a reduction in pain, no matter what pain scale was used.

In patients who needed reintroduction of allopathic medication, the use of phytotherapy led to a decrease in the usual doses. Nevertheless, given the dynamic evolution of arthrosis, neurological and pain reassessment should be performed periodically, no matter which treatment is used.

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THE IMPORTANCE OF VENTRICULAR MEASUREMENTS IN ESTABLISHING THE DIAGNOSIS OF HYDROCEPHALUS IN DOGS

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Abstract

Hydrocephalus is a multifactorial disorder that was properly diagnosed in dogs until the availability of advanced imaging techniques in veterinary practice. The study was conducted on a sample of 15 dogs, aged between 3 months and 12 years, both females and males, of different breeds. To perform the measurements, two ratios were calculated: V/C (width of ventricles/width of cerebral parenchyma), Vh/Ch (height of ventricles/height of cerebral parenchyma) and corpus callosum angle. The average ratio between V/C was 0.68 (values being between 0.52-0.83), the average ratio between Vh/Ch was 0.60 or 60% (values being between 0.88-0.26), and the average of the corpus callosum angle was 69.4° (values being between 56° and 88°). This article evaluates different ventricular measurements based on MRI techniques for establishing hydrocephalus in dogs.

Key words*: dog, hydrocephalus, MRI, ventricular measurements.*

INTRODUCTION

Hydrocephalus represents the distension of the ventricular system due to excessive accumulation of cerebrospinal fluid (CSF), due to an unbalanced situation between production and absorption or an obstructive problem in the flow of CSF into the ventricular system (Estey, 2016).

It is considered to be the most common malformation found in dogs. Thus, changes in ventricular dimensions and associated clinical signs in canine species have been described and documented for over 100 years in pathological studies (Schmidt et al., 2019).

However, before the advent of diagnostic imaging techniques, it was very rarely diagnosed in canine species (Przyborowska et al., 2013).

The purpose of the paper is to approach the diagnosis of hydrocephalus through MRI as completely as possible, to describe the resulting images from an imaging point of view, to apply the technique of making relevant measurements (this method being very rarely practiced in veterinary medicine), to demonstrate whether there is a breed predisposition and whether there is any form of hydrocephalus that is much more frequently encountered than another.

MATERIALS AND METHODS

The study was conducted on a sample of 15 dogs, aged between 3 months and 12 years, both females and males. The breeds that were included are: Pug, Pomeranian, Chihuahua, Yorkshire Terrier, English Bulldog, French Bulldog, Pekingese, Bichon Frise, Miniature Pinscher and mixed breed.

Before anaesthesia, all patients were clinically examined to assess their general health.

General anaesthesia was performed to prevent movement throughout the scan, as any movement can compromise image quality. Premedication was performed depending on the ASA status and their pathologies. Induction was performed with Propofol 3-5 mg/kg, intravenously. Patients were intubated and anaesthesia was maintained with 1.5% Isoflurane and 100% Oxygen; the oxygen flow was set at (500 ml + 10 ml/kg)/min. Spontaneous or intermittent positive airway pressure (IPPV) ventilation was maintained using a volume-cycled ventilator, delivering 12-15 breaths/min to achieve an end-tidal CO_2 of 35-45 mm/Hg. An ocular lubricant was applied to all patients to protect the cornea during the MRI examination (Pavel et al., 2024a).

Patients were perfused with Ringer's solution at a rate of 5-10 ml/kg/h throughout the scan to maintain hydration. All patients had a silicone hot water bottle placed under their abdomen, with their entire body also covered by a polyester cloth blanket to help maintain their temperature within normal values (Pavel et al., 2024b).
Patients during MRI examination were closely monitored (heart rate, respiratory rate, oxygen saturation, blood pressure), which was vital to ensure the safety and stability of anaesthesia, as all of them received during the scan a contrast media agent represented by gadolinium (Tudor et al., 2018).
The MRI protocol consisted of transverse, sagittal, and dorsal planes, using T1, T2, and FLAIR to completely visualize the brain structures (Săvescu et al., 2019).
T2 sequence was performed with a TR: 3000 ms, TE: 108 ms, section thickness: 2.5 mm; the FLAIR sequence used TR: 7000 ms, TE: 114 ms, sequence thickness: 3 mm, and T1 used TR: 500 ms, TE: 15 ms, section thickness: 2.5 mm.
The images obtained were exported in DICOM format and processed using the Syngo MR Workplace software. The selection of cerebral areas of interest was selected to identify the cerebral ventricles and other structures relevant to the study.
For the imaging diagnosis of hydrocephalus, a morphometric analysis was performed to evaluate the exact dimensions and shape of the cerebral ventricles and inter-thalamic adhesion.
Protocol sequences highlighted the cerebrospinal fluid in the ventricular system and adjacent structures. The linear measurements performed with the software were:
• maximum width in mm of the frontal horns of the lateral ventricles in the transverse plane;
• maximum width in mm of the cerebral parenchyma in the same image;
• height of the lateral ventricles in the transverse plane (the average of the height between the two ventricles was taken);
• height of the cerebral parenchyma in the same image.
After the measurements were taken, two ratios were calculated: V/C (width of the ventricles/width of the cerebral parenchyma) and Vh/Ch (height of the ventricles/height of the cerebral parenchyma).
Another relevant measurement for diagnosing hydrocephalus is the value of the corpus callosum angle. This is calculated using software on the T2 sequence, in the transverse plane and is formed between the corpus callosum (which is located between the cerebral hemispheres) and the internal edges of the lateral ventricles.

RESULTS AND DISCUSSIONS

The results from the measurements and calculations for the cases are found below in Table 1.

Table 1. Results obtained from measurements and calculations

No.	Ventricle width (mm)	Brain parenchym width (mm)	V/C	*Corpus callosum* angle (mm)	Ventricle height (mm)	Brain parenchyma height (mm)	Vh/Ch
1	20.43	27.71	0,73	64°	31,72	39.25	0,80
2	26,88	33,65	0,79	58°	32.12	38.52	0,83
3	34.79	48,32	0,71	66°	21,04	27,24	0,77
4	17,90	38,31	0,46	88°	7,97	30,16	0,26
5	23,5	42,3	0,55	79°	29,16	56,98	0,51
6	46,5	58,1	0,83	53°	33	37,2	0,88
7	33,1	47,2	0,70	72°	17,34	31,2	0,55
8	16,9	32	0,52	86°	13,6	37,13	0,36
9	28,6	43,1	0,66	75°	15,1	35,2	0,42
10	31,6	44	0,71	67°	19,4	28,9	0,67
11	40,1	52	0,77	62°	20,3	28,5	0,71
12	22,8	35,6	0,64	78°	12,6	32,45	0,38
13	34,3	42,5	0,80	56°	20,3	28,3	0,71
14	21,6	32,7	0,66	75°	14,53	38,2	0,38
15	44,6	57,2	0,77	62°	22	25,4	0,86

Following the measurements performed for the 15 patients, it was found that on average, the ventricles/brain ratio (V/C) = 0.68 (values between 0.52 and 0.83), this ratio orients the diagnosis of hydrocephalus (Figure 1).

According to Evans (1942), the reference values for this ratio are:

•0.20-0.25 - normal;
•0.25-0.30 - beginning of ventriculomegaly;
•over 0.30 - ventriculomegaly.

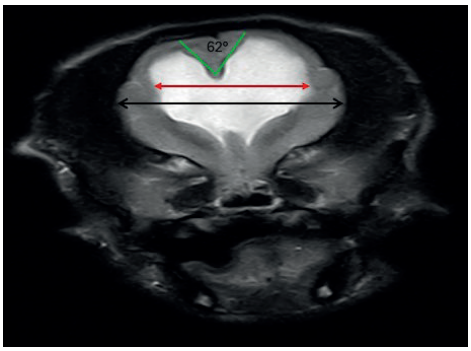

Figure 1. Example of how measurements are performed, T2-weighted transverse image: corpus callosum angle (green lines), width of lateral ventricles (red line), maximum width of cerebral parenchyma (black line). The same idea was used for the ventricle and brain height

The measurements performed revealed an average of corpus callosum angle: 69.4° (values between 56° and 88°). The reference value for the corpus callosum angle is 100-120 and values that are below 90 degrees indicate ventriculomegaly and, respectively, hydrocephalus.

Also, following the measurements, the average ratio between ventricular height/cerebral height (Vh/Ch) was reached = 0.60 or 60% (values obtained between 0.88 and 0.26). The reference values for this ratio, according to Przyborowska (2013) and Woo (2010) are:

• 0-14% - normal;
• 15-25% - moderate ventriculomegaly;
• Over 25% - ventriculomegaly.

In this study regarding the 15 cases of hydrocephalus, most cases were congenital, with a percentage of 79.9% found in brachycephalic and toy breeds and 20.1% of acquired type. Of these, 60% belonged to a brachycephalic breed and 33.3% to a toy breed. These percentages are comparable to the specialty literature that points to similar conclusions. Selby et al. (1979) following a study conducted on a sample of 564 dogs, described the fact that congenital hydrocephalus is most frequently diagnosed in brachycephalic and tiny breeds, such as English Bulldog, Bichon, Pug, Pekingese, and Yorkshire Terrier. One of the reasons for the association of these breeds with ventriculomegaly and hydrocephalus is the shape of the skull (Schmidt et al., 2019).

For the imaging diagnosis of this pathology, a series of measurements were made at the level of the ventricles and the cerebral parenchyma. The first calculation performed is a ratio between the height of the ventricles and the height of the cerebral parenchyma. In a study conducted by Woo et al. (2010) on a sample of 10 healthy Yorkshire Terrier dogs and 10 dogs with hydrocephalus of the same breed, it was concluded that for healthy canine patients, this ratio is below 25%, and for those with hydrocephalus it is well over 20%. Comparing these results with those obtained in my work, namely, the average ratio is 60%, the patients included in the study were correctly diagnosed with hydrocephalus.

Another calculation performed is the ratio between the maximum width of the lateral ventricles and the maximum width of the cerebral parenchyma. This ratio is called the Evans Index in the specialized literature in human medicine. The Evans index is determined by the maximum diameter of the frontal horns of the lateral ventricles divided by the internal diameter of the skull in the same plane. A value greater than 0.3 for the Evans index is the only essential morphological criterion of internal hydrocephalus (Ishii et al., 2008). In contrast, in veterinary medicine, Laubner et al. (2015) performed this measurement in a similar study and resulted in an index of 0.73 (values between 0.58 and 0.92), comparable to the results obtained in our work, which is: the average index of 0.68 (values between 0.52 and 0.83).

Also, the values of the callosal angle are described in human medicine as follows: a normal value is typically between 100 and 120°, in patients with hydrocephalus, that value is lower, generally between 50° and 80° (Gaillard et al., 2015). The value of the callosal angle that resulted from the measurements is 69.4° (values between 56° and 88°). The result obtained is not comparable to studies carried out in veterinary medicine. As far as is known, the callosal angle has not been measured before in canine patients. No significant differences were observed between the callosal angles of dogs with

ventriculomegaly and those with hydrocephalus (Laubner et al., 2015).

Other measurements that could be made by magnetic resonance for future research are: ventricular area and cerebral parenchymal area. Esteve-Rastch et al. (2001) wrote that using these measurements, they tried in a study to differentiate patients with clinical neurological manifestations from those without any symptoms.

Another quantitative measurement is the ventricular volume. This may be useful in the future for studying changes in ventricular volume associated with age, degenerative disease, and ventricular obstruction. When the normal value of the percentage of intracranial volume occupied by the ventricle for different breeds is determined, it is hoped that the diagnosis of hydrocephalus will be made more based on quantitative measurements (Vite et al., 1997).

To continue this study, in addition to the sequences already used (T1, T2, and FLAIR), certain special sequences for this type of pathology can be used. This category includes the DWI (diffusion-weighted imaging) and DTI (diffusion tensor imaging) sequences, which detect changes in the diffusion of water in the brain tissue, useful in detecting cerebral compression and highlighting structural alterations (Yuan et al., 2012). No studies have been carried out in this regard in veterinary medicine at the moment.

Also, to have a much more relevant study on this topic, it would be recommended to carry out a study sample made up of dogs that are of the same breed, as Vullo et al. (1997) did on 17 Beagle dogs from the same kennel. This type of study is more suitable to reduce the variability resulting from: brain anatomical differences, genetic predispositions, physical and biological characteristics specific to each breed, and environmental factors.

CONCLUSIONS

Following this study, it was concluded that MRI is one of the most current and reliable methods for diagnosing hydrocephalus in dogs, especially because it is a pathology that is difficult to diagnose strictly clinically. This is due to the accuracy and attention to detail that can lead not only to an actual diagnosis, but also to finding out the aetiology and mechanism of the pathology.

Finally, it was demonstrated that to have a much clearer and more accurate perspective on the degree of brain damage and, respectively, on the progression of the disease, the use of cerebral calculation and measurement methods is necessary.

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EPIDERMAL GROWTH FACTOR RECEPTOR OVER-EXPRESSION IN CANINE GASTRIC ADENOCARCINOMA IN 36 DOGS

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Abstract

Gastric adenocarcinoma is the most frequent tumour which affects the stomach of dogs, having multiple causes. EGFR (Epidermal Growth Factor Receptor) has an important impact in the development and expansion of gastric tumours, representing the first receptor which provides the connection between overexpression and cancer. The present study included 36 dogs with digestive syndromes, expressed as vomiting and melaena. Routine histopathological examinations on full-thickness gastric biopsies sampled by endoscopy, and on the tissue samples taken during necropsy, were completed by EGFR identification by immunohistochemistry, which could be used to estimate prognosis and select therapy. It has been concluded that 14 cases presented signet-ring cell adenocarcinoma, nine patients with tubular adenocarcinoma, ten with papillary adenocarcinoma, while the rest of three patients had undifferentiated adenocarcinoma. The highest rates of EGFR expression were identified in 38.4% of cases (n=10), highlighting a worse prognosis. EGFR expression was associated with the location of tumoural type, tumour size, cell differentiation, invasion, being positive in 26 cases of gastric cancer tissue (72%), while in the rest of ten cases (28%) were featured by low expression.

Key words*: EGFR, gastric adenocarcinoma, gastritis.*

INTRODUCTION

Gastric adenocarcinoma (GA) is the most frequent tumour with epithelial origin, representing between 50-90% of gastric tumours in dogs (Patnaik A.K., 1977; Swann H.M., 2002). Usually, the aetiology of neoplasia remains uncertain, but in additional instances it may be represented by the animal's diet, polyposis, *Helicobacter pylori* presence and ulcerative colitis. The prognosis of gastrointestinal adenocarcinoma in dogs varies according on the tumour site (Basil M., 1974) Mucous gastric atrophy, which is caused by *Helicobacter pylori* infection and observed at an early age population, contributes to the appearance of gastric cancer (Forman, 1991). In addition, a genetic mutation is considered also responsible in the transition from adenomatous polyposis and gastric adenoma to GA (Yoshiazaki K., 2020). The World Health Organization (WHO) defines stomach carcinomas in dogs based on their development arrangement, categorizing them into five distinct groups: tubular carcinoma, mucinous carcinoma, signet-ring cell carcinoma, undifferentiated carcinoma, and papillary carcinoma (Head K., 2003).

The studies concerning the association between gastric diseases and *Helicobacter pylori* infection have demonstrated that the existence of chronic inflammation accompanied by this bacterial infection cause the major changes of the gastric mucosa, stimulating epithelial cell proliferation, PH secretion, and suppression of cells apoptosis, as well as a development of stem cell niches, enhancing the incidence of stomach tumours (Geyer C., 1993; Hermanns, W.,1995; Yamasaki K., 1998; Asaka M., 2002).

The gastric corpus and the pyloric antrum have different stem cells and stem cells niches which reveals that cancer has two sites as origins, the most common anatomical location being represented by pyloric region (Hayakawa Y., 2017; Saito T., 2020). Certain breeds are more often predisposed to GA, being diagnosed with severe gastric-mucosal atypia, such as: Jack Russel Terrier, Golden Retriever, Australian Terrier, Belgian Tervuren, Cairn Terrier, Collie and Siberian Husky (Candido M.V., 2018) Some studies have demonstrated that a large percentage of Jack Russell Terriers have been diagnosed with gastric adenomas and adenocarcinoma, the most frequent site being the pylorus (Saito T., 2020; Yoshizaki K., 2020)

The significance of EGF in gastric cancer is associated with invasiveness of gastric wall and lymph node metastasis, featuring higher malignant potential. EGF is an essential regulator which generates the gastrointestinal tissue differentiation and proliferation of cells through its receptor, EGFR. EGFR plays a key role in establishing the messenger pathway which determines the proliferation, motility adhesion, survival time, and malignancies of gastric tumours (Tokunaga A., 1995; Woodburn J., 1999; Xia L., 2002)

Clinical signs, complementary blood exams, endoscopic and abdominal ultrasound assessments, macroscopic evaluations, cytological and histopathological investigations, EGFR test were all needed during various stages to distinguish the tumour from the inflammatory process and to minimize inaccurate diagnoses. It was necessary to evaluate the results, determining the essential criteria, correctly interpreting macroscopic, cytological, and histological features, and accurately determining EGFR outcomes.

The methods to diagnose gastric tumours are multiples, but the EGFR, as immunohistochemistry method in diagnosis of GA in dogs, discovered in 1961, is the most used method and it has a major target for gastric cancer diagnostic and therapy (Cohen S., 1962; Mishani E., 2008; Consolaro A., 2010) The prognostic and therapeutically protocols are based on the results of cytopathological and histopathological complementary examinations (Washabau R.J., 2010)

MATERIALS AND METHODS

Dogs' recruitment

A total of 36 dogs of different breeds were included in the present study, as follows: Collie (n=3), French Braque (n=4), Golden Retriever (n=4), Jack Russel Terriers (n=8), Belgian Tervuren (n=6), Swiss White Shepherd (n=6), Labrador (n=2), Fox Terrier (n=3). For all dogs, medical issues were typically identified at patients aged from 9 to 13 years old, independent of gender. A standard diagnostic protocol has been used for all cases, which consisted of: a history, physical examination, blood work tests (hemogram, biochemistry, serum cobalamin concentration, liver enzymes), complete endoscopy, abdominal ultrasound examination, macroscopical analysis of stomach (observed in necropsies and surgeries), cytological and histopathological examinations of all stomach regions collected from endoscopies and surgeries, *Helicobacter pylori* identification method by coloration with Haematoxylin-Eosin statement, immunohistochemistry staining for EGFR expression.

Blood examination

The blood has been collected on EDTA tubes using a differential white blood cell count (WBC) and serum tubes for biochemistry, including examination of serum bile acid concentration, serum vitamin B_{12} and folate, serum albumin concentration, on IDEX system. In 3 out 36 dogs, the lactate dehydrogenase was measured.

Endoscopy, abdominal ultrasound examinations and collection of samples

A preliminary diagnosis was achieved by performing a complete endoscopic examination with an 8 mm diameter frequency range flexible endoscope which allowed inspection of gastric mucosa and collection of upper gastric tract tissue samples, in order to establish histopathological diagnosis. Depending on the lesion's localization, endoscopic ultrasonography procedures used complete width resection and endoscopic submucosal excision. The gastric samples have been collected using an endoscope equipped with a full thickness resection device, a needle cap with 10 mm diameter over the scope clip, and a preloaded 12 mm electrosurgical catch.

Abdominal ultrasonography examination was performed in all patients, using a high-frequency linear transducer (15 MHz).

Standard necropsy procedure was carried out in 14 dogs dead or euthanized animals, and included gross assessment and tissue samples for microscopical investigation.

Gross lesions have been observed and analysed also during surgeries procedures such as gastrotomy (n=16), being followed by sampling for histopathological investigations.

Full thickness gastric biopsies, resections and endoscopic submucosal excision were performed on living animals (n=22) of different

gastric segments. Twelve patients had surgical removal of tumour gastric mass with 2-4 cm surgical safety margins.

Cytological examination

Cytological investigation was associated with histological and macroscopically analyses, having the role in identification of malignancy processes. Following FNA and full thickness gastric biopsies, the samples were air-dried and Diff-Quick stained.

Histological criteria

Histopathological examinations have been performed in all gastric samples (cardia, pyloric segments, fundus), applying a routine procedure of staining and examination. All specimens had 3 mm thick and were immersed in 10% neutral buffered formalin for two days, and finally coloured with haematoxylin and eosin staining.

The incidence of *Helicobacter pylori* has been determined in all 36 cases using the following procedures: ELISA test (by specific serological antibody method), PCR real time, in contrast to immune-histology examination of gastric mucosal samples obtained through endoscopy biopsies of greater, lesser curvature and fundus from living and from necropsied dogs, to relate these diagnostic approaches with pathology significance in dogs with this infection. Following PCR analysis of *Helicobacter* spp., the specific patterns after that 3% of electrophoresis in agarose gel was used, revealed positive results in n=15 from all tissues samples, in an immunoreactive band with approximately 500bp.

EGFR Immunohistochemistry method

The tissue specimens have been screened for EGFR test via an antigen with standardized immunohistochemistry sensitivity. FFPE tissues sections with 5 µm thickness were cleaned, rehydrated, and incubated in 4% hydrogen peroxide in combination with distilled water for 40 minutes.

All samples have been prepared with citrate buffered saline solution at 95°C for 20 min, and incubated with 0.5% hydrogen peroxide solution in methanol for a duration of 15 minutes.

All cases were treated with symptomatic treatments before complementary examinations and register of favourable responses. The symptomatic treatments included: (parasiticides, antibiotics, steroidal anti-inflammatory drug therapy, and gastro-intestinal protectants).

After a classification of responses at the symptomatic treatments, diagnostic results, and general evolution, the patients were treated as presented in Table

Surgery resection was applied in 12 cases, 3 patients had complete tumour resection, and 9 had partial tumour resection.

The potential prognosis was concluded in correlation with the evolution of each patient after the tumour, or partial tumour resection, chemotherapy treatments, localization of the tumour and in function of severity of diagnosis.

RESULTS AND DISCUSSIONS

The most common clinical signs in all patients have included: changes in appetite (n=30), chronical vomiting (n=28), diarrhoea (n=15), melaena (n=10), weight loss (n=25), haematochezia (n=13), hematemesis (n=16), polydipsia (n=3) and abdominal pain, dating from 3 to 6 weeks, with no response to gastro-intestinal dietary, and symptomatic treatments. All patients were treated with symptomatic treatments at the beginning and after complementary examinations and effects, the treatments were completed or changed.

The dogs considered in this study presented ages between 9 and 13 years of age.

A completed blood count test revealed a moderated anaemia (Htc=20-24%) in n=26, and moderate to severe anaemia in the rest of 10 dogs with chronical signs of vomiting, diarrhoea and gastric haemorrhage (19%), a thrombocytosis and leucocytosis at 90000/µl with reference ranges 4000-15000/µl (neutrophilia, eosinophilia, monocytosis and lymphocytosis) in n=30.

A serum biochemical profile revealed hypoalbuminemia at severe to moderate 9-15 g/L in n=15 (reference range 26-35 g/L), an increased valour of alkaline phosphatase 750 U/L (reference range 20-150 U/L) noted in n=19, augmented blood urea nitrogen in n=20 at 50-80 mg/dL (reference range 7-25 mg/dL), and a hypercalcemia in 16 of 36 patients. An augmented valour was remarked for cPL test

(pancreatitis) in liaison with serum bile acid concentration due to hepatic disorders in n=25 in serum cobalamin analysis in n=28 (cPL: 500 µg/L, reference ranges (0-330 µg/L; serum cobalamin: 1200-1400, reference ranges 251-908 ng/L), and high values liver enzyme in n=16 (40-50 UI/L; reference ranges: <40 UI/L), including also higher values of ASAT (57 UI/L, reference ranges:10-50 UI/L) and ALAT (259 UI/L, reference ranges <80 UI/L).

Imaging diagnosis and macroscopic features

Ultrasonographic examination revealed thickening of the gastric wall in pylorus, lesser and greater curvature (hypoechogenic) in 26/36, and the presence of homogenous masses at these levels, measuring 2, 3 and 4 mm, with loss of the normal gastric wall architecture, visible at ultrasound examination in 20/36.

The endoscopic examination revealed the presence of multiple polypoid masses measuring between 8-10 mm diameter, localized in the lesser or greater curvature in n=20. Heavily enlarged folds of gastric mucosa and polypoid mass raised suspicion for malignancy (Figure 1a).

On the pyloric antrum and lesser curvature, multiple diffused and severely oedematous stenosing lesions were observed in n=32 (Figure 1 b and 1d). In the pyloric region it can be noted a haemorrhagic area of a tumour with an irregular surface (Figure 1b).

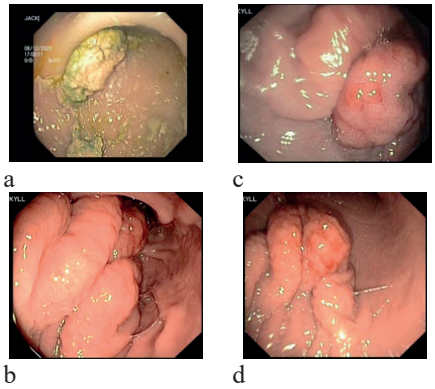


Figure 1. Swiss White Shepherd, 10 years old, polypoid masses (a); Golden Retriever, 13 years old, diffused and oedematous lesions of the pyloric antrum with hypertrophic folds (b); Collie, 8 years old, Irregular and erythematous mass (c); Jack Russel, 12 years old, infiltrating lesion in lesser curvature (d)

At the end of the greater curvature, a highly irregular and erythematous mass focused on several parietal folds was identified (Figure 1c). In the Figure 1d, it was noted eventuated lipped margins around an ulcerative mass. The regional lymph-nodes were involved in n=22.

Macroscopic changes were noticed following necropsy and gastrotomy surgery as oedematous and irregular lesions with severely multifocal haemorrhagic areas visualized at the greater and lesser curvature of the stomach in n=15. The lesions were diffuse and infiltrative, with no marked or unclear ulceration margins, the gastric wall being thickened and indurated (Figure 2 a).

In 9 patients, the structure of the gastric mucosa was granular containing reddened folds as a result of connective tissue hypertrophy, measuring 9-11 cm (Figure 2 b).

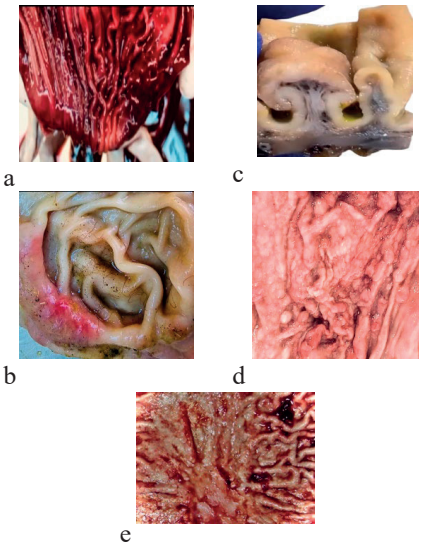


Figure 2. Diffused and increased thickened area, with a prominent ulceration and gastrorrhagia (greater curvature) (a); Localized and high thickening plaque, with reddened folds (greater curvature) (b); Raised polypoid lesion expanded into the lesser curvature (c); Non-ulcerative multiple nodules (d); Ulcerative areas in lesser curvature(e)

The pedunculated masses were observed in a total of 24 cases, measuring more than 10 mm in n=15, and less than 5 mm in n=5 (reference ranges: 2-5 mm), being delimitated by a deep depression from peripheric mucosa. The sessile lesions were presented as nodules being

localized in lesser and greater curvature. Their mucosa was friable and ulcerative (Figure 2 c).
In n=17, multiple giant and exophytic firm nodules adherent to gastric serosa were localized in greater curvature and fundic regions (Figure 2d). The greater and the lesser curvature presented also hypertrophic process (Figure 2d).
In n=25, ulcerative craters have been identified in the stomach wall (lesser curvature) measuring 9 cm. The ulcer margins were elevated, being in contact with gastric folds (Figure 2e). In 18 patients, persistent lesions were identified as non-circumscribed thickened arrows generated by neoplastic cells invasion in the stomach wall, causing erosive craters, ulcerative gastritis, and submucosal fibrosis (Figure 2e).

Cytological examination

In most of the cases (n=28), the cytological examination revealed spherical cells, organized in monolayered adhesive sheets (Figure 3), with an well-defined cytoplasm (Figure 3). Nuclei were round-shaped, being positioned centrally or eccentrically, containing basophilic nucleoli.

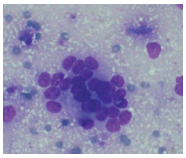


Figure 3. Population of monomorphic epithelial cells (May Grünwald Giemsa staining; x500)

The chromatin was observed as coarse. As malign criteria, the nuclear enlargement, the irregular nuclear membrane, the anizokariosis/ anisonucleosis and the giant nuclei were noted (n=30). The cells were individuals, containing nuclear pleomorphism, and the cytoplasm was well defined. Anisocytosis and anisokaryosis processes were moderate to pronounced, and the N/C proportions were identified as moderate to high (Figure 3).

Histopathological assessment

All samples of stomach, diagnosed with gastric adenocarcinoma diagnosis were classified according to the WHO classification, the results being presented in Table 1.
Neoplastic ducts with various size and dimensions, randomly organized, generating cerebriform formations, specific features in tubular adenocarcinoma, were noted in Figure 4a, in n=9, localized in small and greater curvature. The cells had a polypoid arrangement form, being irregularly distended with atypical and enlarged nuclei. It was noted that sometimes, the cells were as branching tubules with various sizes with mucus and debris (Figure 4a)

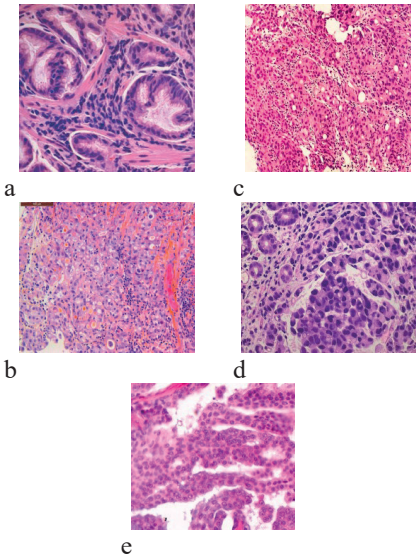


Figure 4. Swiss White Shepherd, 9 years old, Gastric neoplastic ducts with varying size, H.E., x500 (a); Golden Retriever, 12 years old, Signet-ring cell carcinoma, H.E., x300 (b); Border Collie, 8 years old, Undifferentiated adenocarcinoma, H.E., x200 (c); Jack Russel Terrier, 9 years old, Infiltrative mass in pyloric area, H.E., x300 (d); Labrador, 11 years old, Gastric hypertrophy, H.E., x200 (e)

Table 1. Histopathological gastric tumour types

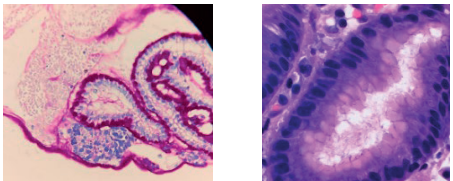
N° cases/36	GA type resulted
14	signet-ring cell adenocarcinoma
9	tubular adenocarcinoma
10	papillary adenocarcinoma
3	undifferentiated adenocarcinoma

A mixture of signet ring adenocarcinoma cells and non-signet ring cells was noted in n=14. It was characterized by a diffuse type and asymmetrical microtrabeculae, associated with significant desmoplasia (Figure 4b).
The mucin-producing cells were identified and the neoplastic cell population showed no indication of glandular or squamous differentiation localized in greater curvature, in n=3 (Figure 4c).

In the Figure 4d, the mass had some tubular glandular differentiation with mucin, but the most of it was composed of undifferentiated tiny cells (poorly differentiated) (Figure 4d).
A high-grade gastric dysplasia, evident hyperplasia and papillary epithelial forms with small fibrous areas, in n=14, suspected to be compatible with papillary adenocarcinoma, located in greater curvature. The cells had a basaloid form, with atypical and enlarged nuclei. The large nucleoli were in mitosis process. In these cases, the regional lymph-nodes were involved (Figure 4e).
In 9 cases diagnosed with signet ring-cell carcinoma, inflammatory cells (neutrophils and eosinophils) were noted in a high quantity, in comparison with tubular and papillary adeno-carcinoma which consisted in lower quantity of inflammatory cells.
Histological analysis of stomach segments, realized in all 36 cases, revealed severe gastritis (ulcerative or non-ulcerative) and gastric cancer, as shown in Table 1.

***Helicobacter pylori* analysis**

ELISA serology test was positive in 20 cases. The Haematoxylin-Eosin coloured smears showed *Helicobacter* spp. as blue-purple colour (Figure 5a) and red (Figure 5b) in 4/36 of cardia samples, in 13/36 of fundus samples, in 6/36 of body samples, and in 4/36 of pylorus samples. It measured between 0.3-0.7µm. *Helicobacter* spp. has been identified in abundance in the mucus that coats the surface gastric epithelium, glandular lamina, and mucosa's surface in the stomach fundus as numerous spiral-shaped organisms (Figure 5).



a b

Figure 5. Positive *Helicobacter pylori* and gastritis (a, b) (Hematoxylin-Eosin, x500)

EGFR analysis

The EGFR test was performed in all 36 patients. Gastric cancer cells expressed EGFR mostly in their membrane and cytoplasm, with brown and yellow particles distributed symmetrically (Figure 6).

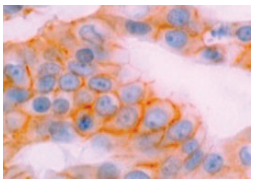


Figure 6. EGFR examination

Table 2. Grades of EGFR expression

Grade of EGFR expression	Breed	Tumour Localization	Histological Diagnosis	EGFR positive/ overexpressed
1	Swiss White Shepherd, French Braque	Fundic and greater curvature	Signet-ring cell adenocarcinoma	EGFR+ in 8
1	Border Collie, Golden Retriever	Pyloric and greater curvature	Poorly differentiated adenocarcinoma	EGFR+in 2
2	Belgian Tervuren, Fox Terrier, French Braque, Golden Retriever	Small and greater curvature	Tubular adenocarcinoma	EGFR+in 10 EGFR severe-expressed in 4
2	Border Collie, Fox Terrier, Golden Retriever,	Greater curvature	Undifferentiated adenocarcinoma	EGFR +severe expressed in 6
3	Labrador, Jack Russel	Pyloric and greater curvature	Papillary adenocarcinoma	EGFR overexpressed n=10

The positive expression of EGFR examination was as graded from 1 to 3 as follows: 1, for the smears with low tumour expression was founded, noted in 10 cases (28%), 2, corresponding to tumour cells staining at positive intensity, in 16 cases (72%) and 3, within the expression of EGFR was overexpressed, in 10 cases (28%) (Table 2).

Therapy and prognostic

The chemo-therapy and surgery protocols were established following clinical evolution and after histopathological analysis results.
In 3 cases, 1 diagnosed with signet-ring cell adenocarcinoma and 2 cases diagnosed with poorly differentiated adenocarcinoma, the chemotherapy treatment (Table 3) was realized in combination with surgical protocols as: partial pilorectomy, for the 1st (<60% of pylori) and partial gastrectomy for another 2 (<50% of stomach removed). No total gastrectomy or gastroduodenal anastomosis were made.
For the rest, the chemotherapy treatment consisted in: intravenous and per-os treatment of: corticotherapy, Toceranib, Chlorambucyl, Cyclophosphamide, Endoxan and Doxorubicine.

Table 3. Treatments and prognostics

Nr. & Breed	Age	Diagnostic & score	Treatment	Prognostic
Swiss White Shepherd (6), French Braque (2)	9-10	Signet-ring cell adenocarcinoma, Grade 1	Vit B12, Corticotherapy, polymixine B, Toceranib	1-4 years
Border Collie (1), Golden Retriever (1)	10-12	Poorly differentiated adenocarcinoma, grade 1	Pylorectomy (1), chlorambucyl	3 months-3 years
Belgian Tervuren (6), Fox Terrier (1), French Braque (1), Golden Retriever (1)	10-11	Tubular adenocarcinoma, grade 2	Chlorambucyl and Cyclophosphamide	16 months-2 years
Border Collie (2), Fox Terrier (2), Golden Retriever (2)	9-12	Undifferentiated adenocarcinoma, grade 2	Endoxan, Cyclophosphamide	10 months
Labrador (2), Jack Russel (8)	10-11	Papillary adenocarcinoma, grade 3	Doxorubicine, Cyclophosphamide	5 weeks to 2-3 months

A chemotherapy prevention with Endoxan, Doxorubicine and Cyclophosphamide was realized in n=10, diagnosed with papillary adenocarcinoma and undifferentiated adenocarcinoma.

In the cases diagnosed with tubular adenocarcinoma and undifferentiated adenocarcinoma, 3 and 4 patients were euthanized 4-5 weeks following the chemotherapy due to resumption of anorexia and regurgitation. The other 7 patients died in the meantime with treatment (2 diagnosed with signet-ring-cell carcinoma and 4 with papillary adenocarcinoma). Median survival time for the patients diagnosed with signet ring cell-adenocarcinoma, with surgical and chemo-therapy treatment was registered between 1-4 years (75%), and one of the other three cases with no responses at treatment, was euthanized after 1 month of therapeutically protocols. For the patients diagnosed with poorly differentiated adenocarcinoma, the median survival was noted between 3 months-3 years, having no metastasis process. The dogs with tubular adenocarcinoma responded to the treatment after 15 days, and the survival time was comprised between 16 months and 2 years, meanwhile survival period for the dogs with tubular adenocarcinoma was

In patients with papillary adenocarcinoma, post-therapy reactions were noted in n=5 as: pulmonary metastasis, and multiple organs dysfunction.

CONCLUSIONS

Signet-ring cell adenocarcinoma, localized in fundic and greater curvature was identified in 6 Swiss White Shepherd dogs and in 2 French Braque (22%), having grade 1 of EGFR expression. In 1 Border Collie and 1 Golden Retriever, the poorly differentiated adenocarcinoma was noted as diagnosis, having grade 1 of EGFR expression. Tubular adenocarcinoma was confirmed in 6 Belgian Tervuren, 1 Fox Terrier, 1 French Braque, 1 Golden Retriever, graded with 2, as EGFR expression. 2 Border Collie, 2 Fox Terrier, and 2 Golden Retriever were diagnosed with Undifferentiated adenocarcinoma, with 2nd grade EGFR expression. 3rd grade of EGFR expression and Papillary adenocarcinoma were identified in 2 Labradors, and 8 Jack Russel.

The survival percentage of signet-ring-cell patients (50%) was significantly distinct from that of patients with poorly differentiated adenocarcinoma (100%), tubular adenocarcinoma (66%), undifferentiated adenocarcinoma (33%), and papillary adenocarcinoma (60%). Poorly differentiated adenocarcinoma and signet-ring-cell patients had a better prognosis than undifferentiated adenocarcinoma. The best prognosis was noted in poorly differentiated adenocarcinoma. In terms of clinicopathological and complementary examinations characteristics, undifferentiated adenocarcinoma patients had a bigger age diversity (9-12). The highest grade of EGFR expression was noted in papillary adenocarcinoma.

The location of tumoral type, tumour size, cell differentiation, and invasion were all correlated with EGFR expression. All complementary examinations and EGFR assessments had an impact in gastric adenocarcinoma tumours progression, promotion and chemotherapy.

Ultrasonography and endoscopy examinations have advantages for diagnosing gastric adenocarcinoma. Endoscopy is far more precise in detecting gastric neoplasia; nevertheless, ultrasounds raise the clinical signs suspicion of gastric neoplasia and may be a less intrusive method of acquiring information before endoscopy (Marolf A., 2015) The subtypes of gastric adenocarcinoma diagnosed after cytopathological and histopathological exams was correlated with necropsy analysis in the meantime when biopsies were done. The results from grade expression of EGFR and severity neoplasia lesions aspects noted during necropsies confirm the theory that the chronicity

neoplastic lesions were related to age, dynamic and progressive process, being a superficial gastritis at the beginning, finishing with an evident gastric atrophy (Rotterdam H., 1981)
Helicobacter pylori infection determined higher grades and intensity of EGFR expression and caused damage gastric epithelial cells.
Canine gastric cancer, similar human stomach cancer, has a very bad prognosis, and current therapy options do not greatly improve survival. The need for new therapies options encourages in-depth research into the role of the complementary examinations as immune-histochemistry (EGFR), in combination with cytology and histopathology (Hugens S., 2017)
Furthermore, there are significant parallels between human and canine gastric cancer in terms of clinical presentation and histological findings, indicating that the dog might serve as a comparison model for human gastric cancer (Hardas A., 2021)
The current study was a complex approach, considering clinical, imaging diagnosis and pathological methods in order to achieve a certain diagnosis and estimate better the prognosis of GA in dogs. Using the WHO principle, this study demonstrated that the most prevalent subtype was papillary carcinoma, tubular adenocarcinoma, and undifferentiated adenocarcinoma. There were no sex variations, and the average age was eleven years of age *Helicobacter* spp. presence was related with elevated chronic inflammation parameters and an expanded chronic inflammatory process, which produced tumour developments (Patnaik A.K., 1980)
Rutine cytology, histopathology, and immune-histochemistry examinations had 78% and 91% accuracy, respectively, in classifying tumour categories in the present cases, being based on biopsy data from surgeries and necropsies.
EGFR is transmembrane tyrosine kinases that increased the accuracy of the diagnostic for gastric neoplasm for the cases considered in this study. The expression of EGFR appeared to be strongly connected, with one or both proteins typically overexpressed in gastric adenocarcinoma cells (Rowinsky, 2004)

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Abbreviations

GA - gastric adenocarcinoma
EGFR - Epidermal Growth Factor Receptor
FFPE - formalin-fixed and paraffin-embedded
FNA - fine needle aspiration
PBS - Phosphate buffered saline
PCR - Polymerase chain reaction
pH - Acid-base production

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UPPER AND LOWER JAW FRACTURES MANAGEMENT IN DOG AND CAT

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Abstract

Car accidents, bites trauma or high-rise syndrome are the main causes of mandible and maxilla fractures in dogs and cats. This article presents the surgical approaches for the treatment of different types of fractures resulting from traumatic maxillofacial and mandibular injuries. Maxillary or mandibular body fractures are the most common oral fractures seen in dogs. In cats, fractures of the mandibular ramus are less common than those of the symphysis. The surgical technique must be adapted to each case according to the complexity of the fracture. The assessment of the integrity of the soft tissues and their eventual surgical restoration is performed intraoperatively. Postoperative recovery must be correlated with the degree of trauma to the adjacent soft tissues. Osteomyelitis and delayed union are common complications in the upper and lower jaw fracture repair process in dogs and cats.

Key words*: maxillary, mandibular, fracture, dog, cat.*

INTRODUCTION

Mandible and maxillary fractures in dogs and cats occur due to trauma caused by blows, road accidents, falls from heights, and aggressive confrontations between animals.

In cats, mandibular fractures represent 16% (Woodbridge, 2013) of the total fractures in this species, and within these, the most frequently affected are the intermandibular symphysis and the hard palate, followed by the mandibular body, condyle and coronoid process caused by falls from height, road accidents, aggressive interaction with other individuals of the same or different species (dogs).

In dogs, head fractures are more common in the cranial vault (Batle et al., 2020) and jaw (De Paolo et al., 2020). Most patients are young, with fractures resulting from road accidents, bites, aggression, and horse strikes (Arzi et al., 2015). Pathological mandibular fractures caused by periodontal disease can occur in older small and toy breed dogs (Harasen, 2008).

Animals with traumatic skull fractures located only in the facial region (mandible and maxilla) did not show nervous signs (Batle et al., 2020)

The maxillofacial complex in the dog is the most prominent part of the skull, making it vulnerable to severe injuries.

The term “maxillofacial” refers to structures involving the incisors, palatine, zygomatic, lacrimal, frontal, and nasal bones, as well as the jawbone itself. This anatomical region is located between strong craniofacial bony structures: the cranial base, mandible, nasal cavities, and paranasal sinuses (Legendre, 2005).

In dogs, in maxillofacial trauma, regardless of aetiology, there are often multiple bone fractures (Verstraete et al., 2020)

Mandibular fractures are more common due to the location exposed to possible mechanical trauma, due to the presence of a thin compact of the mandibular bone as well as the poor coverage with soft tissue (muscular, adipose, subcutaneous, cutaneous) (Stoian, 2009).

This type of fractures is often complicated by dental fractures, lacerations of the oral mucosa, and wounds of the tongue.

The character and temperament of the animal contribute greatly to the occurrence of fractures, with young animals being more exposed to traumatic fractures, and old animals to pathological fractures (Stoian, 2009).

MATERIALS AND METHODS

Medical data of patients presented for surgical consultation in the clinic of the Faculty of

Veterinary Medicine in Bucharest and the "Prof. Dr. Alin Bârțoiu" University Veterinary Emergency Hospital during 2020-2024.
All patients underwent a complete clinical examination, ophthalmological examination, neurological examination, dental examination, radiological examination, computed tomography, biochemical blood examination, CBC, general inhalation anaesthesia, specific surgical protocol for oral and maxillofacial surgery. Postoperatively, the patients remained hospitalized under supervision. Antibiotics, analgesics, anti-inflammatories were administered for 7-10 days. The animals were re-evaluated postoperatively at different time intervals.

RESULTS AND DISCUSSIONS

Most traumatic mandibular and/or maxillary fractures present dramatic clinical symptoms with haemorrhagic hypersalivation, epistaxis (Figure 1), difficulty chewing, pain when opening the oral cavity, oedema, crepitation on palpation, anisognathia (asymmetric occlusion), dislocation or luxation of teeth, skin wounds, oral mucosa or tongue. Halitosis (induced by fermentation and putrefaction phenomena) is a clinical sign of an old fracture. These changes are often discovered only during clinical inspection (under general anaesthesia) of the oral cavity (Stoian, 2009).

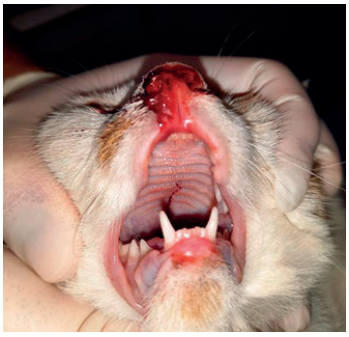

Figure 1. Head trauma: bilateral epistaxis, cleft palate, feline, European breed (original Mocanu)

After the patient is stabilized, an extraoral examination is performed to assess facial symmetry, dental occlusion, the ability to open and close the oral cavity, oral and nasal bleeding, signs of instability and discomfort. The clinical examination involves a complete examination (Clarke & Caiafa, 2014), which consists of inspecting and palpating the entire head. Upon inspection, the presence of oedema, hematomas and lacerations can be detected at the level of the skin adjacent to the mandible or maxilla. Facial asymmetry is a clinical sign usually present in the case of maxillary or mandibular fractures. Palpation is performed with both hands, from the lower edge of the mandible, the maxillary area to the nasal bones, the suborbital region, the temporomandibular joint (Bubenik, 2005). Thus, areas of bone discontinuity, the gap of bone fragments, bone crepitations due to the mobility of bone fragments and painful points are highlighted, due to the pain generated by the traumatic focus (Zacher et al., 2013). In cats that have fallen from the floor, the intermandibular symphysis and the palatal region (cleft palate) are checked. Patients who have suffered head trauma may present multiple maxillofacial lesions; therefore, it is recommended to perform a computed tomography scan. Due to the pain generated by the traumatic focus, the intra -oral examination is recommended to be performed under general anaesthesia. The integrity of the oral mucosa, tongue, teeth, hard palate is monitored. Any changes in the oral occlusion are checked (Kleftouri et al., 2017). In order to accurately establish the diagnosis, the location and extent of the fracture, as well as to choose the technique for immobilization and fixation of the fracture, an initial imaging (radiological) examination is necessary. In polytraumatized patients, in addition to skull radiographs, it is recommended to perform additional radiological images of the rib cage (Woodbridge et al., 2013). The standard views used in radiological imaging for maxillary and mandibular fractures are: latero-lateral, dorso-ventral or ventro-dorsal projection if oblique views at various angles are indicated. The temporomandibular joint is best shown on radiographs when using the lateral, slightly oblique view with the oral cavity partially (slightly) open (Stoian, 2009). Interpretation of radiological films, especially of the caudal cranial region and sinus cavities, is often difficult due to the location and overlapping of bone structures. The use of computed tomography (Figure 2) can be of great use for choosing the most appropriate therapy and

method of stabilizing the fracture, especially in the case of complex fractures (Stoian, 2009). Cats presenting symphyseal (Figure 3) or parasymphyseal fractures usually have other mandibular and/or skull fractures, therefore computed tomography becomes necessary (Tundo et al., 2019). Three-dimensionally printed 3-D models contribute to the understanding of the anatomical particularities of the fracture, serve as an anatomical model for surgical planning and intraoperative guidance, contributing to the predictability and success of complex surgical interventions (Godinho et al., 2024).

The objectives pursued in the restoration of a maxillofacial fracture are: pain management, antibiotic therapy (broad-spectrum antibiotics, cephalosporins possibly associated with metronidazole), restoration of oral occlusion, avoidance/reduction of trauma to the vascularization and innervation of the area during the surgical intervention to reduce the fracture, avoidance of trauma to the teeth located in the fracture focus at the time of fracture reduction, extraction of fractured teeth, restoration of masticatory function (Glyde et al., 2003).

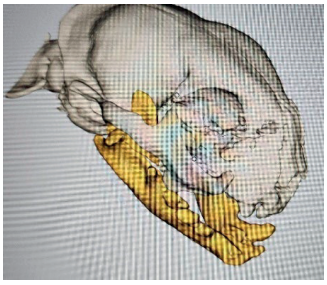

Figure 2. Cat, maxillofacial trauma; straight fracture without displacement of the mandibular ramus (original Uzun)

Figure 3. Intermandibular symphyseal fracture, mental laceration of the skin and subcutis in a cat (original Mocanu)

Figure 4. Dog, radiological image of a mandibular ramus fracture, with displacement (original Ionaşcu)

Complications of mandibular fractures include haemorrhage, nerve damage (Zacher et al., 2013).

The therapeutic approach for fractures of the mandible (Figure 4) or maxilla ranges from conservative, non-invasive management, through "closed" reduction of the fracture, to "open" surgical reduction, with internal fixation. The treatment of fractures is influenced by a number of factors including: the location of the fracture focus, the degree of displacement of the bone fragments, the type of fracture, the stability of the patient and the experience of the surgeon (Fossum, 2019)

The treatment of choice for mandibular or maxillary fractures is represented by surgical treatment - osteosynthesis with: wire or miniplates and titanium screws. The aim of this treatment is to restore the anatomy and function of the mandible and maxilla, by immobilizing, realigning the fractured bones and restoring oral occlusion. To avoid malunion and delayed consolidation, it is necessary that after surgical reduction of the fracture focus there is stability, adequate vascularization and lack of infection. (Freeman et al., 2023)

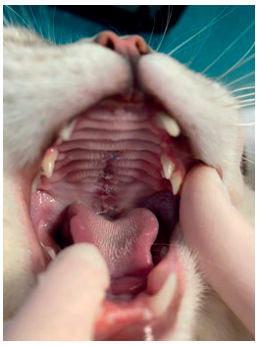

Figure 5. Traumatic cleft palate 14 days after surgery (original Mocanu)

Acute traumatic midline cleft palate defects in cats, commonly associated with feline fall-from-height syndrome, can be successfully repaired using primary appositional closure after debridement, in separate points with absorbable sutures (Figure 5) (Zacher et al., 2013).

The stability of an osteosynthesis is generated by the set of metal implants (wires, screws, plates) that fix the bone fragments, and the delicate manipulation of soft tissues, performing sutures in layers without creating tension preserves the vascularization. Removal of necrotic tissues, intraoperative lavage with sterile saline, postoperative antibiotic therapy, ensuring drainage by installing drain tubes ensures good healing and prevents the development of postoperative infections (Verstaete et al., 2020; Legendre, 2005)

If the patient with a mandibular or maxillary fracture has dental calculus, scaling will be performed first, then the fracture site will be reduced. (Verstaete et al., 2020)

Teeth involved in fractures should not be removed unless they are fractured. This is particularly important in fractures involving the caudal mandibular body because the large premolar and molar teeth occupy a substantial portion of this bone; they are essential contributors to fracture stability.

Unlike long bone fractures where stability is an absolute requirement, in maxillofacial fractures stability can be less rigid in favour of restoring occlusion. In multiple fracture repair, the mandibular fractures are repaired first, the teeth are placed in proper occlusion, and then the maxillofacial fractures are repaired (Stepaniuk, 2014).

Mandibular fractures with minimal displacement and adequate oral occlusion (Fossum 2019) can be treated conservatively, non-surgically, with a muzzle-like bandage made of fabric that allows the mouth to be opened only for drinking water or eating soft food (Fossum, 2019). This type of bandage is kept in place for 6 weeks. A common complication is local dermatitis under the bandage that will heal after the bandage is removed. This type of conservative treatment of mandibular fractures cannot be applied to cats or brachycephalic dog breeds (because there is not enough support from the nasal bones) or when the mandibular fracture is associated with a maxillary fracture, because the pressure exerted on the maxillary fracture causes discomfort (Fossum, 2019).

Postoperatively, the patient's general condition needs to be monitored throughout the convalescence period. In the conditions of a post-traumatic and post-surgical status, nutritional support will be provided by intravenous administration of rehydration solutions, parenteral nutrition solutions, amino acids that will correct any secondary electrolyte deficiencies and will nutritionally support the body. For the management of postoperative inflammation and pain, anti-inflammatory drugs (robexacoxib) and analgesics (tramadol, buprenorphine) will be administered by injection. Postoperative antibiotic therapy by system (injectable) with cephalosporins (possibly associated with metronidazole) for 10-14 days. Per os, the food administered must be soft in the form of a paste. Probiotics can be administered orally (mixed into the food) that favourably influence the digestion processes and increase the body's immunity. If necessary, especially in cats, a feeding tube can be installed (Freeman et al., 2023; Kleftouri et al., 2017).

The animal should be encouraged to eat on its own as soon as possible after surgery; this determines a good evolution and a rapid recovery. The animal owner must be informed about the postoperative complications and follow the recommendations regarding the patient's diet and medication. Thus, it is forbidden for patients with mandibular/maxillary fractures to gnaw bones, toys or to interact intensely or aggressively with other dogs.

Complications of mandibular fractures include haemorrhage, nerve damage, malocclusion, osteomyelitis, malunion, temporomandibular ankylosis, and implant failure (Bubenik, 2005).

Clinical case 1: Male, neutered, long-haired Persian cat was presented for consultation following a facial trauma. The history revealed that the animal had become apathetic, was eating little, and was not grooming itself.

Clinical examination by palpation revealed oedema and tenderness in the left mandibular region. After the initial approach and stabilization of the clinical condition, computed tomography revealed a closed fracture of the mandibular ramus.

Since the patient was brachycephalic, a surgical fixation technique with a 3D printed plate was adopted for open reduction of the fracture and avoidance of complications. The plate was 3D printed according to the patient's anatomical model following the measurements obtained by computed tomography (Figures 6 and 7).

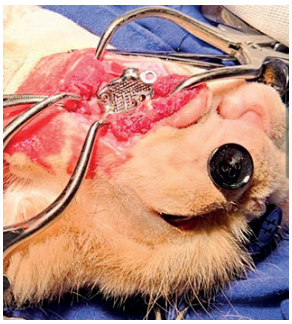

Figure 6. Application of the 3D printed plate in clinical case 1 (original Uzun)

Figure 7. Postoperative radiological examination of clinical case 1 (original Uzun)

Clinical case 2: European cat, 6 months old, fall from the 2nd floor. The patient is stable, alert.
A complete clinical examination was performed, abrasion of the chin region and intermandibular disunion were observed.
The radiological examination confirmed the diagnosis of mandibular symphysis fracture.
The feline patient under general inhalation anaesthesia by endotracheal intubation is placed in the supine position with the head and neck extended.
Tracheostomy for inhalatory anaesthesia is not recommended in cats because it involves risks (Woodbridge et al., 2013d).
The evaluation of dental occlusion after fracture reduction will be performed by detaching the probe tube.
The fracture was examined, necrotic tissues were debrided, lavage was performed with saline, the edges of the intermandibular symphysis were curetted until haemorrhage occurred, and the bone fragments were repositioned in anatomical position.
A small incision is made with a scalpel in the lower mental area that concerns the cutis and subcutaneous connective tissue.
Through the incision, maintaining contact with the mandible, a 14 G syringe needle is inserted and removed at the level of the gingival mucosa in the diastema behind the lower canine. The needle will be slightly curved beforehand in order to be able to follow the curved contour of the mandible.
A 0.3 mm surgical wire will be inserted through the hole at the tip of the needle, then the needle is withdrawn together with the wire, up to the level of the incision, after which we remove the needle. The same procedure is performed on the opposite side.
The wire is inserted by symmetrical traction on both ends of it, so as to apply an equal force. The ends of the wire are shortened and twisted by continuous pulling and rotation. The two ends of the wire are twisted under the mandible, progressively tightening the two mandibular branches, they approach each other, and the intermandibular symphysis is restored; the knot is bent inward.
After stabilizing the fracture focus, the dental occlusion is checked. A perfectly aligned fracture focus is not acceptable if the dental occlusion is not correct (Fossum, 2019).
Extraction of the metal implant is done after 45 days-60 days after surgery.

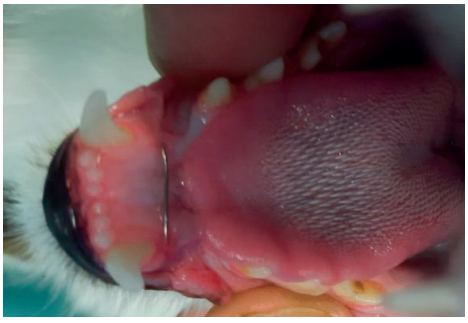

Figure 8. Intermandibular fracture repaired with surgical wire cerclage 60 days after surgery; the gum and adjacent tissues are observed to have grown over the metal implant due to body development as a result of the feline patient's aging: Cat, European breed, 8 months, clinical case 2 (original Mocanu)

Correct occlusion is achieved when, with the oral cavity closed, the mandibular canines are inside the maxillary canines.
Clinical case 3: dog, Mioritic Shepherd breed, male, 2 months old, maxillofacial trauma by bite; rostral fracture of the jaw with displacement, multiple gingival and buccal mucosa lacerations (Figure 9). After stabilization, emergency intervention was performed to avoid haemorrhagic and infectious complications. Skull growth in skeletally immature dogs could be severely restricted and influenced by the use of internal fixation. Because the jaw and other bones of the skull simultaneously undergo growth, elongation, and displacement in the rostrocaudal and lateral directions, rigid fixation with plates that "block" the bone sutures of the skull inhibit the growth of the facial bones (Arzi et al., 2015)

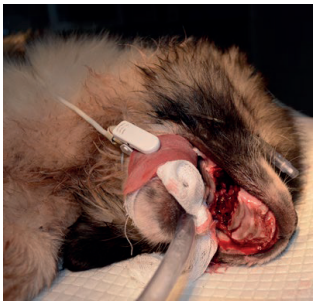

Figure 9. Rostral maxillofacial trauma, open jaw fracture, dog, Mioritic Shepherd, 2 months, clinical case 3 (original Mocanu)

Following these considerations, since it was a paediatric patient, the surgical technique of interfragmentary fixation in separate points (Figure 10) of the bone fragments with surgical wire was applied.

Figure 10. Postoperative radiological image of clinical case 3. The metal implants are visible in the area of the maxillary fracture (original Mocanu)

After decontamination of the area and debridement by abundant lavage with sterile saline, blood clots and necrotic tissues were removed by sharp dissection, haemostasis was performed. The fractured fragments were put back in anatomical position. Holes were made on each side of the fractured maxillary bone into which surgical wire was inserted. Fixation loops were created, the ends of which were intertwined. Oral occlusion was checked. The soft tissues were sutured in layers with absorbable threads in separate points.
Postoperatively, the animal was hospitalized for 10 days, during which treatment with antibiotics, analgesics, anti-inflammatories, and probiotics was applied. From the second day, the animal was able to feed and drink water on its own. Postoperative recovery was favourable, the animal recovered completely (Figure 11).

Figure 11. Appearance of the jaw 4 months after surgery clinical case 3 (original Mocanu)

Due to the normal soft tissue healing process, whereby the fibrous connective tissue that forms during healing contracts as the final scar forms, the patient at the age of 6 months presented with mild mandibular pseudo-prognathism.

CONCLUSIONS

Surgery of maxillofacial trauma in dogs and cats is particularly difficult due to the particularities of the bone structures at this level as well as the complexity of the vascular and nervous structures, but also the existence of peripheral segments of the sense organs (eyes, tongue) that are housed by the viscero-cranium.
The approach to these types of fractures must be multidisciplinary both in terms of establishing the diagnosis through clinical and imaging methods and from a therapeutic point of view. The patient with craniofacial polytrauma must be evaluated by an emergency physician, ophthalmologist, dentist, neurologist, and

maxillofacial surgeon. Imaging examinations (radiography and computed tomography) provide important information that will contribute to understanding the anatomical features of the fracture and will serve to establish the subsequent surgical protocol. The use of the most appropriate surgical techniques established individually for each patient contributes to surgical success and a favourable postoperative outcome.

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RAPID AND RELIABLE REAL-TIME PCR WORKFLOW FOR DETECTION OF AFRICAN SWINE FEVER VIRUS

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Abstract

ASF is a fatal haemorrhagic disease affecting pigs, posing a significant threat to global agriculture as it rapidly spreads across the UE, Asia, and Oceania. Due to the lack of a vaccine or treatment, each outbreak requires pursuit of stringent biosecurity measures and trade restrictions. The disease's emergence is causing significant economic losses due to the stamping out of pig holdings in the affected areas and the pursuit of disease control measures, including trade restrictions to avoid disease spread. Since the onset of the ASF, Romania has been severely affected economically and socially, suffering losses in commercial and backyard farms, unemployment among workers in the pig breeding and meat processing industries, and significant expenditure on disease control and surveillance. ASF remains a major threat with no available vaccine and challenging eradication. Rapid, sensitive diagnostics are crucial. The proposed workflow offers multiple advantages, including the ability to verify extraction, amplification efficiency for each sample through amplification of two internal control systems, ensuring accurate pathogen detection regardless of nucleic acid type presence, and confirming sample cellularity to validate result accuracy and prevent false negatives due to inadequate collection, transport, or storage.

Key words*: ASF, ASFV, cellularity, diagnosis, Real-Time PCR.*

INTRODUCTION

African swine fever (ASF) is a viral disease exclusively affecting domestic and wild pigs. Clinically, it involves a haemorrhagic syndrome; it is quite contagious and has a lethal course in most cases (Sauter-Louis et al., 2021). The emergence of the disease is causing significant economic losses due to the stamping-out procedures in the affected areas, the implementation of disease control measures and the trade restrictions to prevent the spread of the disease (Saatkamp et al., 2000).

African Swine Fever (ASF) through various transmission cycles involving different hosts, such as domestic pigs, wild boar, wild African swine, and possibly other species. The epidemiology of ASF varies significantly between Africa and Europe due to differing environmental factors, with ticks playing a key role in the virus's transmission (Sánchez-Vizcaíno et al., 2015). In regions where these ticks are found, increased surveillance and comprehension of the interactions among different hosts are essential for creating effective control and eradication strategies (Costard et al., 2013).

While the African Swine Fever virus (ASFV) represents a significant threat to livestock, it does not pose a risk to human health (Sánchez-Vizcaíno et al., 2009).

This distinction is important for shaping public health policies. Nevertheless, ASFV is contagious among pigs, requiring strict biosecurity protocols in veterinary environments. A comprehensive understanding of ASF's epidemiology, including its routes of transmission and the roles of various hosts and vectors, is crucial for effectively managing outbreaks and safeguarding pig populations worldwide through targeted research and surveillance initiatives.

The only way to stop the spread of the virus is to slaughter and destroy all pigs on infected

holdings, followed by rigorous disinfection and restrictions on animal movements and trade in the affected area (Busch et al., 2021).

The African swine fever virus was originally grouped within the *Iridoviridae* family but has since been reclassified into the *Asfivirus* genus of the *Asfarviridae* family. While unclassified viruses such as faustoviruses, kaumoebavirus, and Pacmanvirus possess about 30 genes similar to those found in the African swine fever virus, their genomes are substantially larger - approximately 400 kilobase pairs compared to the 170-194 kilobase pairs of the African swine fever virus (Alonso et al., 2018). It is much more resistant and environmentally stable than the classical swine fever virus. This virus can cause extremely high mortality and affects pigs of all ages. Due to the lack of a safe effective vaccine and the common presence of infected wild boars in particular areas, the only method to control the disease is strict biosecurity measures allied to international cooperation on this matter. Knowledge and epidemiological understanding of how the virus may be introduced into susceptible populations of pigs is crucial to provide awareness to prevent the outbreaks and detect and control them immediately and appropriately when they do occur (Mazur-Panasiuk et al., 2019).

Since the onset of the ASF outbreak, Romania has been severely affected economically and socially, suffering losses in commercial and backyard farms, unemployment among workers in the pig and meat processing industries, and significant expenditure on disease control and surveillance.

The correct diagnosis of ASF should include detection of the virus genome or antigen in pig samples and/or the demonstration of a specific antibody response. Those techniques offer high specificity and sensitivity, making it both rapid and suitable for large-scale applications. Among them, PCR is capable of analysing any type of clinical specimen, achieving near-perfect accuracy with almost 100% specificity and sensitivity. PCR is particularly advantageous when working with decomposed tissues, where other methods may falter. It also serves as a valuable tool for analysing *Ornithodoros* soft ticks, which are vectors for several pathogens. The multiplex assay feature enables the simultaneous and differential detection of multiple pathogens, streamlining the diagnostic process. Notably, PCR does not require direct handling of live viruses, though biosecurity precautions are essential until any suspected samples are properly inactivated to ensure safe handling. Additionally, robustness decreases in samples with weak positive results (World Organisation for Animal Health, 2019). Given the similar symptoms of ASF, classical swine fever (CSF), and porcine dermatitis and nephropathy syndrome (PDNS), this method is crucial for swift and reliable diagnosis, enhancing existing molecular diagnostics for identifying ASF in suspected cases (King et al., 2003). A fully validated PCR test with high sensitivity and specificity will detect pigs with low viremia levels, including during early and late infection stages or with attenuated virus strains (Fernández-Pinero et al., 2013). The purpose of this paper is to describe a sensitive and robust workflow for ASF genome detection from various samples, with stringent validation and quality monitoring of the results.

MATERIALS AND METHODS

For this kind of extraction, it is preferred to use 2ml round-bottom tubes (U-shaped bottom) for easy resuspension of magnetic particles and to avoid sedimentation (Potop et al., 2014). Also, for certain sample types, a pretreatment is necessary in order to bring the nucleic acids into solution and make it available for binding to magnetic particles, as described in Table 1.

Table 1. Pre-treatment of dry tissues and pads

Step	Action	Description	Observation
Tissue	Primary lysis	In 2 mL tubes will be pipetting the next reagents in the next order: Approx. 20 mg biological sample 20 µL Proteinase K 300 µL Reagent E0	Incubate at 56°C with occasional shaking or homogenisation by inversion until complete dissolution of the tissue.
Dry swabs	Resuspension biological material	Insert buffer in the tube	Sectioning the buffer rod so that what remains does not go beyond the edge of the tube Energetic vortexing. Spin for liquid collection
		Pipette 500 µL saline into each tube.	

Table 2. Nucleic acid extraction

Step	Action	Description	Observation
Step 1	Lysis of biological samples Nucleic acids binding	In a 2 mL tube pipette the following reagents in the order below: - 20 µL Proteinase K - 200 µL biological sample - 100 µl Reagent E1 - 400 µl Reagent E2 - 20 µl Reagent E3	Homogenisation by pipetting after sample transfer Reagent E1 has a high viscosity, wait 2 seconds after the pipette plunger release
Incubation on shaker at minimum 1200 rpm for 15 minutes, temperature 25°C. Spin centrifugation at the end			
Tubes insertion in the magnetic stand so that they can be opened towards the operator			
Liquid removal by inserting the pipette tip on the opposite side of the pipette, gradually up to the bottom of the tube, with total liquid removal (volume set for the pipette around 900µl)			
Step 2	Nucleic acid wash	1. Pipette 600 µL Reagent E4	-
		2. Incubation on shaker at minimum 1200 rpm for 5 minutes, room temperature	Spin centrifugation at the end
		3. Tubes insertion in the magnetic stand	The tubes are positioned so that they can open towards the operator
		4. Liquid removal	Insert the pipette tip on the opposite side of the pipette, gradually until the bottom of the tube, with total removal of the liquid (volume set for the pipette around 800 µL)
Step 3	Nucleic acid wash	1. Pipette 600 µl Reagent E5	-
		2. Incubation on shaker at minimum 1200 rpm for 5 minutes, room temperature	Spin centrifugation at the end
		3. Tubes insertion in the magnetic stand	The tubes are positioned so that they can open towards the operator
		4. Liquid removal	Insert the pipette tip on the opposite side of the pipette, gradually until the bottom of the tube, with total removal of the liquid (volume set for the pipette around 800 µL)
Step 4	Nucleic acid wash	1. Pipetting 600 µL Reagent E6	-
		2. Incubation on shaker at minimum 1200 rpm for 5 minutes, room temperature	Spin centrifugation at the end
		3. Tubes insertion in the magnetic stand	The tubes are positioned so that they can open towards the operator.
		4. Liquid removal	Insert the pipette tip on the opposite side of the pipette, gradually until the bottom of the tube, with total removal of the liquid (volume set for the pipette around 800 µL)
Step 5	Magnetic particle drying	1. Tubes insertion on thermoblock, 5 minutes at 55°C	Tube caps open!
		2. Tubes insertion in the magnetic stand	The tubes are positioned so that they can open towards the operator.
Step 6	Nucleic acid elution	1. Pipetting 90 µL Reagent E7	Tube caps closure!
		2. Incubation on shaker at minimum 1200 rpm for 5 minutes, room temperature	Stir gently before to resuspend the particles in E7 reagent Spin centrifugation at the end.
		3. Tubes insertion in the magnetic stand	The tubes are positioned with the opening towards the operator
		4. Transfer 80 µl liquid in a new tube	Keep refrigerated for up to one hour or in the freezer until analysed

Pathogen nucleic acid extraction was performed as described previously (Turcitu, 2024), with a custom protocol based on Qiagen chemistry and using magnetic beads for nucleic acids manipulation. Briefly, 0.2 mL of sample was incubated at 56°C with constant shaking along with 20 µL of Proteinase K and lysis buffer, then washed and eluted in 100 µL nuclease-free buffer. Eluates were stored at -80°C until amplification (Table 2).

Amplification/detection was performed using the QuantiNova Pathogen + IC kit (Qiagen, Hilden, Germany), specially designed for pathogen detection, as the name implies, with the internal exogenous control assay provided for inhibition monitoring and a supplementary assay for cellularity evaluation (mammal housekeeping gene). Primers and probe for ASFV were synthesised according to previous recommendations (King et al., 2003). Pathogen assay was marked with FAM fluorophore, exogenous internal control assay was marked with HEX-like fluorophore, and endogenous internal control assay was marked with Cy5 fluorophore; therefore, a triplex protocol was used (Turcitu, 2024). All components were

added according to the manufacturer's recommendations regarding final concentrations. Experiments were conducted on the Q instrument (Quantabio, Beverly, USA).

RESULTS AND DISCUSSIONS

To better evaluate the performance of the designed workflow, serial ten-fold dilution of the original sample was made. Amplification protocol was performed using a singleplex approach (only the pathogen assay was added to the MasterMix) and a triplex approach (all three assays were added to the MasterMix). All reactions were performed simultaneously in replicates, and the results are shown in Figures 1 to 3. Results interpretations were automatically generated using Real Time instrument software features for reaction efficiency and R^2 coefficient calculation (Tables 3 to 5)

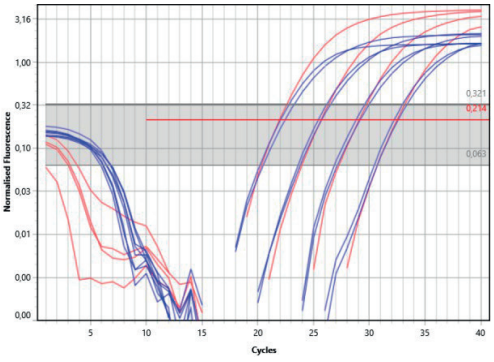

Figure 1. Amplification curves obtained for single-plex assays (red) and multiplex assays (blue) on serial ten-fold dilution of ASF-DNA

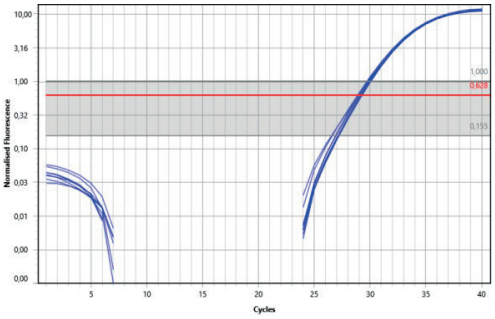

Figure 2. Amplification curves obtained for inhibition (exogenous internal control) assay Signal amplification on the same Ct, showing no inhibitors interference

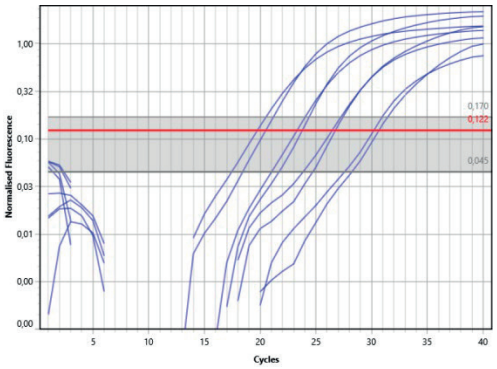

Figure 3. Amplification curves obtained for cellularity evaluation (endogenous internal control) assay on serial ten-fold dilution

Table 3. Results obtained for ASFV target (Green/FAM channel)

Cq	Efficiency	R^2	Result
PPA -1			x=22.10 σ=0.20
21.91	1.04	0.99714	
22.02	0.87	0.99663	
22.38	0.80	0.99735	
PPA -2			x=25.54 σ =0.13
25.60	0.98	0.99900	
25.36	0.88	0.99727	
25.66	0.83	0.99708	
PPA-3			x=28.98 σ =0.16
29.06	0.93	0.99927	
28.78	0.82	0.99853	
29.13	0.78	0.99794	
PPA-4			x=32.37 σ =0.14
32.41	0.93	0.99875	
32.18	0.90	0.99570	
32.51	0.84	0.99686	

Table 4. Results obtained for inhibition control (Yellow/HEX channel)

Cq	Efficiency	R^2	Result
PPA -1			x=28.91 σ=0.08
28.98	0.76	0.99992	
28.83	0.82	0.99952	
PPA -2			x=29.02 σ =0.04
29.06	0.88	0.99922	
28.97	0.91	0.99914	
PPA-3			x=28.98 σ =0.16
29.15	0.93	0.99917	
29.13	0.93	0.99904	
PPA-4			x=29.20 σ =0.03
29.18	0.93	0.99916	
29.23	0.92	0.99929	

Table 5. Results obtained for cellularity (Red/Cy5 channel)

Cq	Efficiency	R^2	Result
PPA cellularity -1			x=20.05 σ=0.37
20.42	0.62	0.99991	
19.68	0.54	0.99988	
PPA cellularity -2			x=23.47 σ =0.23
23.70	0.64	0.99881	
23.24	0.56	0.99992	
PPA cellularity-3			x=26.53 σ =0.165
26.67	0.64	0.99964	
26.38	0.48	0.99909	
PPA cellularity -4			x=30.36 σ =0.22
30.58	0.54	0.99868	
30.14	0.48	0.99661	

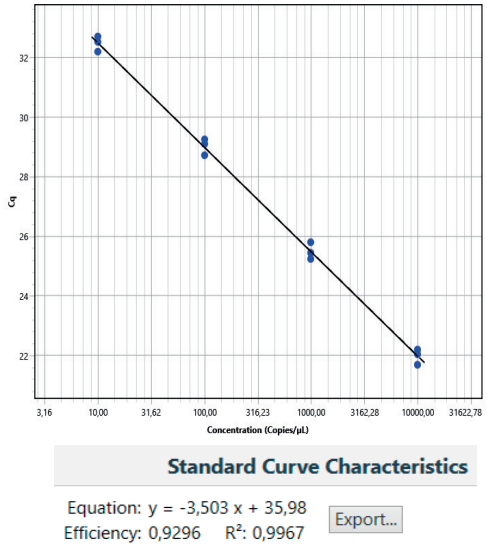

Figure 4. Standard curve for ASFV serial ten-fold dilutions

Results obtained show a good workflow efficiency in terms of removal of sample inhibitors during extraction. as stated by the endogenous internal control amplification curves (Figure 2 - simultaneous amplification of the analysed samples, regardless of ASFV viral load) and Ct results (Figure 4 - comparable values for all samples/dilutions, minimal standard deviation). Moreover, nucleic acid recovery during extraction proved to be adequate for high. medium and low sample viral loads. as stated by the standard curve parameters (slope value -3.5. R^2 coefficient value above 0.99) and replicate values obtained (minimal standard deviation for target as well as cellularity). Besides extraction efficiency. amplification showed a robust pattern. with serial dilution being amplified simultaneously for each dilution and replicate (Figure 1) and standard deviation within accepted range (Figure 4). Moreover, no significant differences were observed when comparing singleplex amplification versus triplex amplification – for each dilution/replicate. the increase of fluorescence starts simultaneously (Figure 1), and the Ct values obtained are comparable (Figure 4); therefore, no loss of sensitivity was observed.

CONCLUSIONS

The workflow described proved to fulfil the requirements for a good, reliable and fast tool for ASF nucleic acid detection. being able to cover all the biological material that can be received for diagnostic and surveillance of the virus. Moreover, by including stringent validation criteria for the obtained results it can give valuable information regarding sample collection, storage and transportation, along with the possibility to detect any inadvertence that might arise during sample processing and nucleic acid detection/amplification.

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EVALUATION OF A TREATMENT PROTOCOL FOR SEVERE EQUINE ASTHMA IN SPORT HORSES: A CLINICAL EXPERIENCE

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Abstract

Severe Equine Asthma (RAO - Recurrent Airway Obstruction) is a chronic respiratory condition commonly encountered in sports horses, significantly affecting athletic performance. This case study evaluates the effectiveness of a complex therapeutic protocol applied to a sports horse diagnosed with severe equine asthma. The protocol included pharmacological therapy (anti-inflammatory drugs, mucolytics, and nebulisations with bronchodilator medications), strict environmental management to reduce allergen exposure, and detailed monitoring of clinical progress. The results indicated a significant improvement in respiratory clinical parameters, a reduction in airway inflammation, demonstrated by the cytological analysis of bronchoalveolar lavage, and a considerable improvement in the cytological profile, leading to a gradual recovery of the horse's exercise capacity. This case highlights the importance of individualized treatment and strict environmental management in optimizing the health of horses diagnosed with severe equine asthma. The study provides valuable information for developing effective therapeutic protocols in current veterinary practice.

Key words*: equine asthma, RAO, environmental management, therapeutic protocol, sport horse.*

INTRODUCTION

Respiratory health is a critical factor in equine performance, particularly in sport horses, where even minor impairments can significantly impact athletic ability. Among the most common chronic respiratory conditions affecting horses is Severe Equine Asthma (SEA), previously known as Recurrent Airway Obstruction (RAO).

This condition is characterized by airway inflammation, mucus accumulation, and bronchoconstriction, leading to exercise intolerance, chronic coughing, and respiratory distress.

Effective management of Severe Equine Asthma requires a comprehensive approach, combining pharmacological therapy, environmental modifications, and long-term monitoring to control symptoms and maintain performance.

This article presents a case study of a sport horse diagnosed with SEA, evaluating the effectiveness of a multimodal therapeutic protocol, including anti-inflammatory treatments, nebulization, and strict environmental management. By analysing clinical progress and treatment outcomes, this study highlights key strategies for optimizing respiratory health in affected horses (Ivester et al., 2018).

MATERIALS AND METHODS

The evaluation began with a review of the horse's medical history and anamnesis, focusing on previous respiratory issues, environmental conditions, and past treatments.

This was followed by a comprehensive clinical examination, including auscultation, respiratory rate assessment, and evaluation of nasal discharge and coughing. To further investigate the condition, an endoscopy of the respiratory tract was performed to visualize mucus accumulation and airway hyperreactivity.

Additionally, lung ultrasonography was used to detect pulmonary changes, while cytological analysis of bronchoalveolar lavage (BAL) provided crucial information on airway inflammation and cellular composition.

RESULTS AND DISCUSSIONS

Patient history and signalment

The affected horse, a 13-year-old castrated male Oldenburg sport horse, presented with clinical signs including coughing, nasal discharge, and

increased respiratory effort, which affected his endurance and performance in show jumping competitions.

Clinical Examination and Diagnostic Work-Up

The first clinical evaluation of the patient was performed on 09.04.2024. During the examination, the horse's vital parameters were within normal limits, but it presented with coughing, laboured breathing, and mucopurulent nasal discharge. Lung fields appeared normal, though subcrepitant rales were detected upon auscultation.

Ultrasonographic examination revealed no significant pleural abnormalities. An endoscopic examination of the respiratory tract showed moderate tracheal changes, with abundant purulent mucus and mild inflammation at the tracheal bifurcation.

Samples from the tracheal and bronchoalveolar lavage were collected for cytological examination, which was performed by a specialized laboratory.

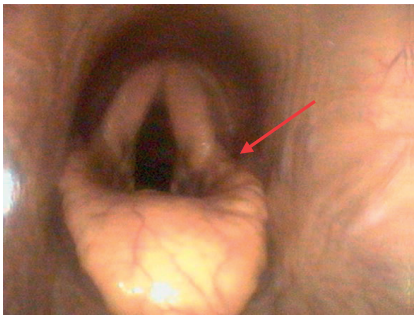

Figure 1. The endoscopic image reveals a moderate inflammatory process affecting the epiglottis

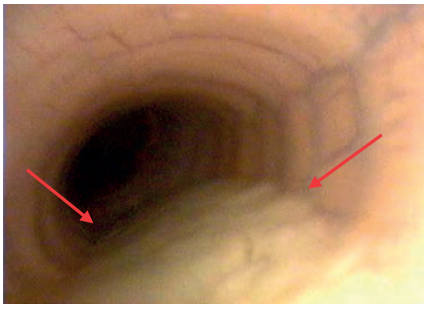

Figure 2. The red arrows indicate the amount of mucus present in the trachea

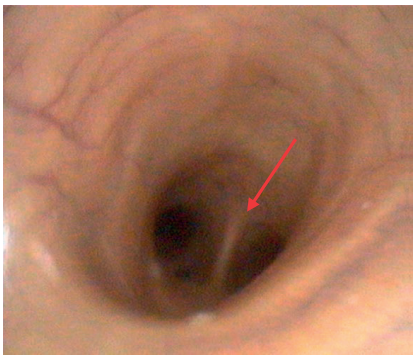

Figure 3. The red arrow indicates moderate inflammation at the carina of the trachea

Cytology & Bacteriology Results

Cytology

Smears reveal an abundant inflammatory population, predominantly composed of moderately to mildly degenerated neutrophils. This indicates a marked neutrophilic inflammation, which may be associated with either an infectious process (bacterial, fungal, parasitic, or other causes) or a non-infectious condition, such as severe equine asthma. In this case, the high proportion of neutrophils, along with the presence of suspect bacterial forms, increases the suspicion of a septic process.

According to scoring system for tracheal mucus: accumulation, localisation, apparent mucus-apparent viscosity and colour, our case is grade 4.

Bacteriology

Aerobic bacteria were identified and found to be sensitive to sulfadoxine-trimethoprim and doxycycline.

Treatment Procedure

The treatment protocol for severe equine asthma involved a multimodal approach to address airway inflammation, mucus accumulation, and bacterial infection.

Inhalation therapy was a key component, with Serroflo 25/250 administered via a Babyhaler inhalation device. The dosage was gradually reduced over three weeks, ensuring effective bronchodilation and anti-inflammatory action.

To manage the suspected bacterial infection, a combination of Borgal (IV/IM) and doxycycline powder was prescribed. Doxycycline was given orally or mixed with feed, depending on the horse's feeding behaviour.

Table 1. The table outlines the treatment protocol implemented based on the laboratory results, focusing on reducing airway inflammation, controlling infection, and improving respiratory function

Category	Medication	Dosage & Administration	Duration
Inhalation Therapy	Salmeterol 25 µg + Fluticasone Propionate 250 µg (Serroflo 25/250)	Salmeterol 200 µg+ proprionat de fluticazonă 2000 µg/ 8 puffs per nostril, 2×/day (2 weeks) → 4 puffs 2×/day (4 days) → 2 puffs 2×/day (3 days)	**3 weeks**
Antibiotics	Trimethoprim and sulfadoxin	15 mg/kg IV/IM, SID	**5 days**
	Doxycycline powder	10 mg/kg PO BID (mixed in feed or orally if needed)	**10 days**
Mucolytics	**Bromhexine powder**	50 mg/kg PO SID	**10 days**
Corticosteroids	Prednisolone acetate Dexamethasone 7.50 mg/ml 2.50 mg/ml	75 mg/horse+25 mg/horse, IM, 1×/week	**4 weeks**
	Flumethasone	0.003 mg/kg IM, 1x/week	**4 weeks**

For mucus clearance, Bromhexine powder was included in the treatment plan to enhance airway function.

Additionally, supportive therapy with Depedin Veyx was administered once weekly to strengthen the immune system and overall recovery.

To further control airway inflammation, Flumethasone, a corticosteroid, was given weekly via intramuscular injection to help reduce clinical signs and improve respiratory function.

This comprehensive treatment strategy aimed to achieve long-term respiratory relief while ensuring the horse could maintain its athletic performance.

In addition to medical treatment, environmental modifications were implemented to minimize allergen exposure and support respiratory health. The horse was kept on dust-free bedding, and stable ventilation was improved to reduce airborne irritants. To further decrease respiratory triggers, the horse was fed high-quality hay, which was either soaked or steamed to eliminate dust and mold spores. These measures played a crucial role in managing severe equine asthma and preventing symptom recurrence.

RESULTS AND DISCUSSIONS

The treatment protocol, combining pharmacological therapy and environmental modifications, significantly improved the horse's respiratory condition. Clinical signs such as coughing, nasal discharge, and increased respiratory effort were notably reduced throughout treatment.

Bronchoalveolar lavage (BAL) cytology showed a marked decrease in neutrophilic inflammation, supporting the effectiveness of the anti-inflammatory and antibiotic therapy. The reduction in purulent mucus, observed via follow-up endoscopy, further confirmed treatment success. Additionally, improvements in airway function and exercise tolerance were noted, allowing the horse to gradually return to training.

Environmental management plays a crucial role in maintaining respiratory health. The use of dust-free bedding, improved ventilation, and soaked or steamed hay minimized allergen exposure, preventing symptom recurrence.

This case highlights the importance of a multimodal approach in managing severe equine asthma, emphasizing the need for individualized treatment, regular monitoring, and strict environmental control to optimize respiratory function and athletic performance.

After completing the treatment, the patient was re-evaluated on 30.10.2024. During the clinical examination, vital parameters remained within normal limits, respiratory sounds were unchanged, and no nasal mucus was present.

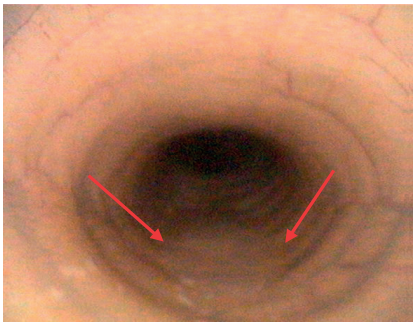

Figure 4. The red arrows indicate a decreased amount of mucus in the trachea following treatment

The horse exhibited only a mild dry cough, and a significant improvement was observed during the endoscopic examination. A very small

amount of mucus was detected in the trachea, with no signs of inflammation in the upper or lower airways.

The cytological examination of tracheal aspirate (TA) and bronchoalveolar lavage (BAL) after treatment showed notable improvement, though some signs of inflammation persisted. In TA, moderate neutrophilic inflammation (73% neutrophils) was still present, along with macrophages and lymphocytes, but no infectious agents were detected. BAL results showed a lower inflammatory cell count, with activated macrophages (74%), a moderate proportion of neutrophils (11%), and lymphocytes (15%), indicating a reduction in airway inflammation. While no pathogens were identified, their presence could not be entirely ruled out. These findings suggest a positive response to treatment, with a significant decrease in neutrophilic inflammation, though mild residual inflammation remains, consistent with mild to moderate equine asthma.

Over the next two weeks, the patient continued a mild maintenance treatment to support recovery and prevent relapse. The horse received Depedin (10 mL IM) and Vecort (4 mL IM) as part of the supportive therapy. Additionally, inhalation therapy was maintained with 8 puffs in the morning and evening daily for two weeks. This follow-up treatment aimed to stabilize respiratory function, sustain the positive response to therapy, and prevent inflammation recurrence.

Long-term environmental management is essential for controlling severe equine asthma and preventing recurrence. To minimize allergen exposure, dry hay should be avoided, with Haygain steaming or soaking for at least 20 minutes recommended to reduce dust and mold. Proper stable ventilation helps decrease airborne irritants, while dust-free bedding such as wood shavings or paper is preferred over straw unless it is of high quality and dust-free. Regular pasture turnout or paddock time is beneficial, but for sensitive to pollen horses, exposure should be adjusted based on seasonal triggers. Training should be conducted in well-ventilated areas, avoiding dusty arenas that could worsen respiratory symptoms. Additionally, routine veterinary monitoring, including bronchoalveolar lavage (BAL) cytology and clinical assessments, is crucial for tracking disease progression and adjusting management strategies. By maintaining these long-term modifications, the horse's respiratory health and athletic performance can be optimized while minimizing the risk of asthma flare-ups.

CONCLUSIONS

This article highlights effective treatment strategies for managing severe equine asthma, emphasizing the importance of a multimodal approach combining pharmacological therapy, inhalation treatments, and environmental management. The significant clinical improvement observed in this case underscores the vital role of allergen control in maintaining long-term respiratory health.

Early diagnosis of equine asthma is crucial for preventing disease progression and preserving respiratory function. The use of advanced imaging techniques such as lung ultrasound and endoscopy, combined with laboratory tests like bronchoalveolar lavage (BAL) cytology and biomarker analysis, allows for a more accurate assessment of airway inflammation and helps tailor individualized treatment plans.

Future research should focus on refining diagnostic methods, developing novel therapeutic approaches, and optimizing environmental management strategies to further enhance equine respiratory care and athletic performance.

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BABESIOSIS AND DIROFILARIASIS - TRIGGERS OR RISK FACTORS FOR CANINE LYMPHOMAS?

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Abstract

Lymphodysplasia is the reversible process by which antigen-stimulated adult B lymphocytes dedifferentiate into peripheral lymphoblasts, then into immunoblasts, plasmablasts and then plasma cells secreting antibodies against the antigen that stimulated them. In lymphoma, malignancy occurs in the intermediate stages of dedifferentiation with monoclonal immunoblastic or plasmablastic proliferation in Plasmacytoma with the secretion of enormous amounts of chimeric antibodies.
The pathogenic mechanism consider in this paper consists of prolonged antigen hyperstimulation with parasitic and cell-destructive proteins that cause an accentuated proliferation of undifferentiated adult lymphoblasts at the level of organized lymphoid structures: the spleen for centroblastic lymphoma and the mediastinal, mesenteric, peripheral submandibular, prescapular, superficial inguinal lymphocenters and polyploidy in multicentric lymphoma.
In oncology, it is a well-known fact that chronic inflammation is an important risk factor on the scale of carcinogenesis and the Babesia spp. and Dirofilaria spp. parasites determine exacerbated inflammatory reactions, their components and toxic excretions being the antigen in the puzzle.
Thus, we ask: canine lymphoma has not been demonstrated to have a clear trigger among the known etiological factors, but should we start looking for proof?

Key words*: canine lymphoma, babesiosis, dirofilariasis, antigen hyperstimulation, inflammatory reaction.*

INTRODUCTION

Canine malignant lymphomas are classified into a group of hemopathies originating in the reticuloendothelial system, which are divided into 2 forms: Hodgkin lymphomas and non-Hodgkin lymphomas.
Malignant non-Hodgkin lymphomas (MNL) represent malignant monoclonal proliferations of B lymphocytes in most cases and in rare cases of T lymphocytes, in various degrees of lymphocyte differentiation and maturation, the most common form being multicentric or generalized lymphoma.
The bacterial etiology with *Helicobacter pylori* in humans has been incriminated in the development of gastric diffuse large B-cell lymphoma (Keikha et al., 2022).
Bridgeford et al., theorized in 2008, that there is a strong connection between "a positive *Helicobacter heilmannii Hhe* status with the presence of feline gastric lymphoma, especially lymphoblastic lymphoma".
Dirofilaria immitis (Heartworm disease) is transmitted by culicids (mosquitoes) and it is a helminthiasis that affects canids, manifested by the presence of the nematode in the heart and in the pulmonary arteries
This parasite's toxins take a toll on the liver, kidneys and secondarily on haematopoiesis and an intensely antigenic action, evidenced by the increase in serum immunoglobulins and the number of lymphocytes, monocytes and eosinophils in the peripheral blood.
"In addition, the simultaneous death of groups of adult worms can trigger an acute disease characterized by the exacerbation of inflammatory reactions and the emergence of serious thromboembolic events" (González et al., 2012).
Babesiosis is caused by unicellular endoglobular parasites that can be found in the erythrocytes.

The vectors are ticks (*Ixodes ricinus*) that transmit the larger *Babesia canis* and the smaller *Babesia gibsoni*. Babesias cause hemolytic anaemia through metabolic toxins and mechanical effects, causing agglutination of dead erythrocytes and adhesion to the capillary endothelium with thrombus formation and disseminated intravascular coagulation (DIC). In addition to the Babesia-induced haemolytic anaemia and hypoxia, some dogs develop a complicated to the babesiosis characterized by immune-mediated haemolytic anaemia (IMHA) and/or signs of inflammatory reactions (Ionita et al., 2023). This is explained by the fact that the structural antigenic components of the babesia are destroyed following specific therapy and this determines an antigenic hyperstimulation effect, which causes hyperplasia of the splenic white pulp and an intense lymphoproliferative and macrophagic reaction in all lymphoid tissues, especially in the lymph nodes - similar to the Macrophage activation syndrome (MAS) - a severe inflammatory systemic abnormality (Mechtoune et al., 2021).

MATERIALS AND METHODS

The evaluation of the 53 patients spanned over 38 months.
The canine patients were both male and female.
The inclusion criteria for the study were:

- current diagnosis of malignant lymphoma (3 cases of centrocytic lymphoma, 23 cases of centroblastic lymphoma and 27 cases of lymphoblastic lymphoma);
- Dirofilariasis - 54% of dogs (chronic Dirofilariasis), 37% of dogs Babesiosis (at least 3 infestations/dog), 9% patients with Dirofilariasis and Babesiosis (Figure 1);
- breed - 48 dogs, breeds with thicker skin (Salmon et al., 1994) and a control group of 5 dogs, breeds with thinner skin (all male, all aged ≈ 6 years, all black);
- coat colour - dogs with black or brindle coats - brown with black and a control group of 5 dogs, breeds with thick skin (Affolter & Moore, 1994), male, all aged ≈ 8 years, all light fur).

Complete anamnesis, history and clinical evaluation were recorded in the study sheet. Most of the time the lymphoma was asymptomatic and was either discovered during the evaluation of the parasitic disease (76% of the cases included in the study) or through the lymph node reaction identified during clinical control (24%) (Figure 2).
Microscopic detection of parasites on blood smears.
Imaging evaluation by both radiography and ultrasound (Esaote Veterinary MyLab Six Crystal Line).
Laboratory determinations were performed with the IDEXX VetTest biochemistry device, the IDEXX BioAnalytics was used for measuring the cytokines and inflammatory markers, the IDEXX Vet Autoread/IDEXX LaserCyte haematology device, IDEXX VetLab Station for proteinogram, SNAP 4DX Plus Tests.
Oncological diagnostic confirmed by cytopathological examination - fine needle aspiration.

RESULTS AND DISCUSSIONS

Autoimmune and chronic inflammatory syndromes are associated with increased risks of Lymphoma.

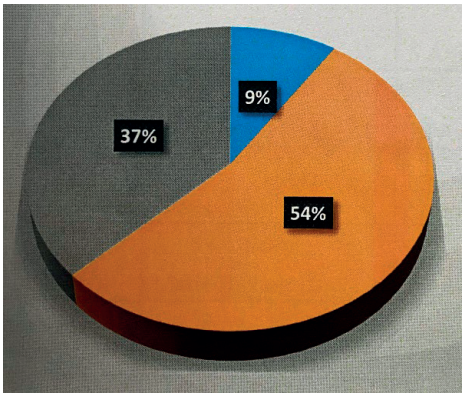

Figure 1. Percentage of lymphoma cases correlated with haemoparasites:
9% patients with Dirofilariasis and Babesiosis
54% patients with Dirofilariasis
37% patients with Babesiosis

As we proceed, our study demonstrates the constant or repetitive effect of the parasites on the immune system of the host and the results that a chronic Dirofilariasis or a repetitive Babesias can have, keeping in mind that chronic inflammation is one of the risk factors of the carcinogenesis.

Because different lymphoma subtypes develop at different stages of lymphocyte differentiation, associations of autoimmune and inflammatory disorders could help oncologists have better understanding of the mechanisms that lead to a lymphoma diagnosis.
This study is an important step to the initiation of a prevention protocol for one of the most frequent types of cancer.

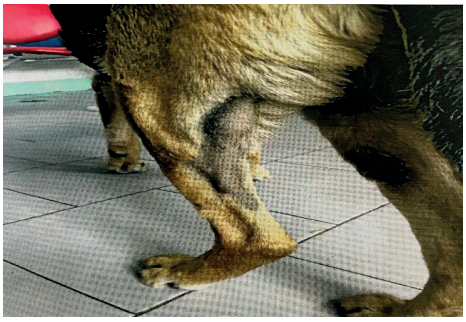

Figure 2. Clinical evaluation - in this case, the popliteal enlargement was the only symptom of centrocytic lymphoma. The owners had brought the dog in for testing for Babesiosis

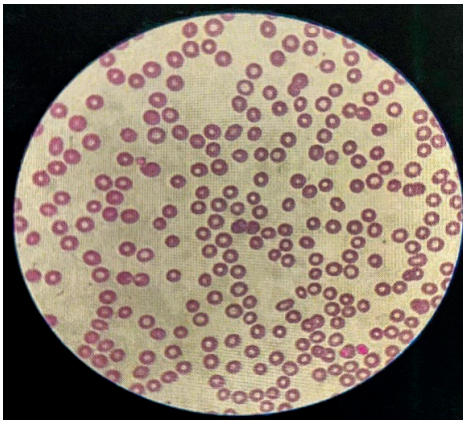

Figure 3. *Babesia* spp.

Microscopic detection of parasites in the blood smears is essential for the inclusion in the study. Pathological values on peripheral blood smears:

- normocytic erythrocytes, polychromasia (++);
- leukocytes: segmented and hypersegmented neutrophils (+++), eosinophils (+++), monocytes and reactive lymphocytes (++);
- platelets: 0-5HPF* (low number), large platelets (+), small platelet aggregates (+).

*High Power Field; (+) low numbers; (++) medium numbers; (+++) high numbers.

The bone marrow releases a larger number of immature red blood cells (reticulocytes) than it physiologically should, without them having reached the last step of the erythropoiesis (Kim et al., 2020).
The need that determines the presence of a larger than normal number of reticulocytes is linked to one cause: a haemolytic anaemia. (Ray et al., 2023). In our study, the aetiology of the haemolytic anaemia is either due to the large number of *Babesia* spp. (Figure 3) parasites that have taken over a large number of red blood cells or have died after specific treatment and destroyed a large number of red blood cells and/or immune-mediated haemolytic anaemia (IMHA) that can be triggered by severe inflammatory reactions (Garden et al., 2019).

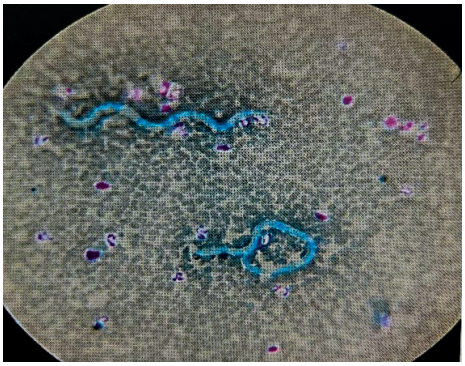

Figure 4. Microfilariae (*D. immitis*)

For patients diagnosed with regenerative anemia due to *Dirofilaria* spp. (Figure 4) the ethiology is based on complicated mechanisms triggered by the toxic proteins that the parasite secrets: intravascular haemolysis and erythrophagocytosis - the macrophage activation by inflammatory cytokines due to chronic inflammatory disease (Nemeth & Ganz, 2014; Mendez et al., 2014).
Regarding the leukocyte values, we correlated the segmented and hypersegmented neutrophils with the presence of macrophages in cytopathological interpretations. Inflammatory cytokines trigger the release of older neutrophils (hypersegmented) from the bone marrow storage pool (Raskin et al., 2004). In this case as well, the demand for a large number of neutrophils is initiated by the severe

inflammatory syndrome that concurrent with the parasitic infestation (Leisewitz et al., 2023).
The immune stimulated lymphocytes that we identified in large numbers on the haematology reports, the ultra-specialized eosinophils and the monocytes are in response to the aggressive antigen stimulation effect in both Dirofilaria and Babesia cases (Wezyk et al., 2023; Milanovic et al., 2017). There have been many studies regarding the implication of platelets in parasitic infestation and the reason for the associated thrombocytopenia (Alonso & Cox, 2015; Cox & McConkey, 2010; Jinna & Khandhar, 2023). The low platelet count is frequent in parasitic infestations and in our study, we associated the thrombocytopenia, the haemolytic anaemia and the high quantity of blood clots to the platelets binding to the ruptured red blood cells or infested erythrocytes, forming micro-aggregates.

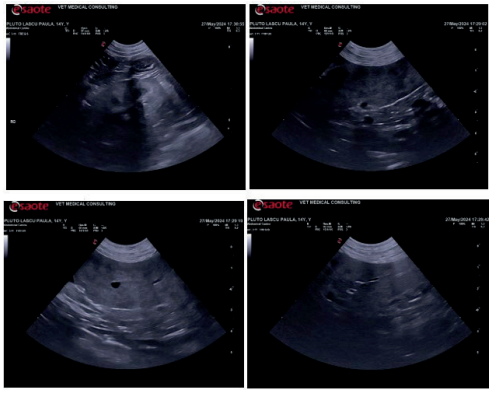

Figure 5. Spline: evident splenomegaly, with regular borders but present of more poorly delimited hypoechogenic infiltrations, disseminated in the mass of the splenic parenchymal; Obvious hepatomegaly, with moderate vein distention and multiple nodular lesions up to 1.5 cm in diameter, disseminated in the hepatic mass

Pathological values on biochemistry analysis:

- liver markers: elevated (+++) ALT, ALKP, Bilirubin, TP, LD;
- renal function markers: elevated (+++) CREA, BUN;
- Calcium levels: elevated (+++);

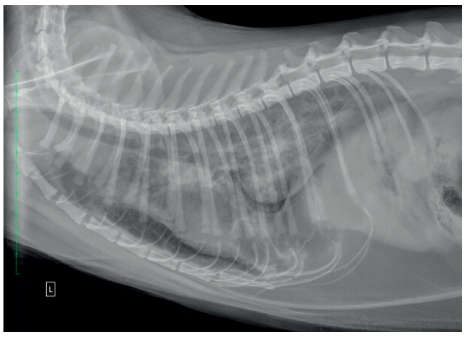

Figure 6. Soft tissue radiopacity in the projection area of the caudal oesophagus pushing the ventral main bronchus. Presence of air in the pleural space. Increased radiopacity in the caudal right lung lobe. Soft tissue radiopacity superimposed on the cardiac silhouette in the caudal mediastinum. Presence of a radiolucent region in the caudal portion of the right cranial lung lobe

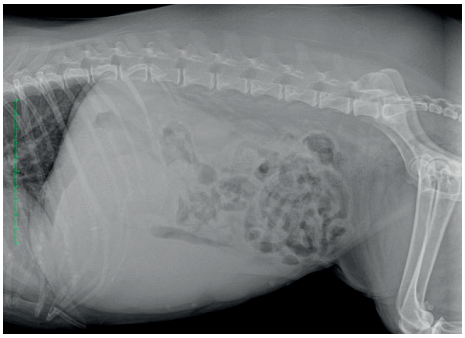

Figure 7. Significant hepatomegaly and splenomegaly

We also registered 2 cases of allergy due to sever infection with Babesia, dogs presenting with pulmonary infiltration with eosinophilia and serum globulins are increased.
Ultrasonography and radiography are vital tools both in parasitic evaluation (Corda et al., 2022) and oncology, giving valuable information in diagnosis, staging and monitoring (splenomegaly, hepatomegaly, glomerular nephropathies) (Sitprija & Boonpucknavig, 1994) cardiac modifications, fluid accumulations in the pericardium, the pleura, the peritoneum etc.). Figure 5 presents changes caused by *Babesia* spp., and Figures 6 and 7 show changes caused by a centroblastic lymphoma.

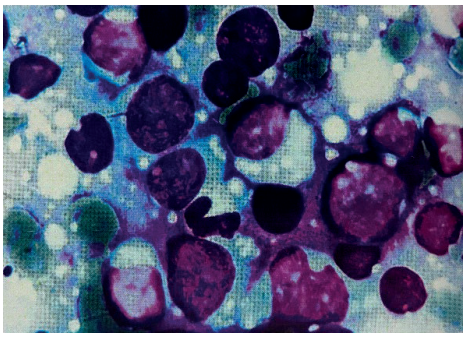

Figure 8. Malignant lymphoblasts

Cytopathological examination through fine needle aspiration offers the oncological pathology confirmation (Figure 8), although it can give false negative or false positive in cases of low-grade lymphoma.

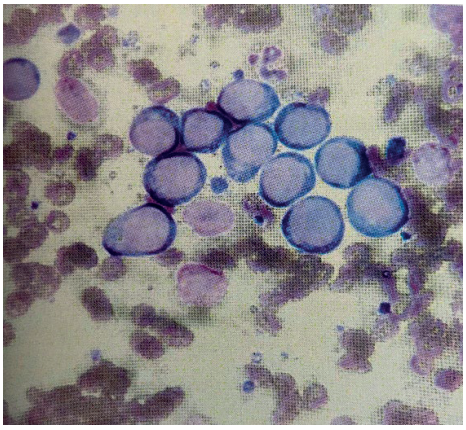

Figure 9. Lymphoblasts

CONCLUSIONS

The increase of immunoglobulins in lymphomas associated with Dirofilariasis and Babesiosis confirms antigenic hyperstimulation along with the associated eosinophilia syndrome.

The parasitic lymphoproliferative and macrophagic reaction in all lymphoid organs that determines a severe systemic inflammatory reaction can be due either to a chronic (in dirofilariasis) or a repetitive (in babesiosis) exposure. In both cases, over time, the inflammation and intense immune reaction can cause DNA damage and lead to carcinogenesis.

The pathogenesis mechanism consists of the hyperstimulation of the antigen with parasitic proteins and cell destruction that causes a pronounced proliferation of undifferentiated adult lymphoblasts at the level of organized lymphoid structures: the spleen for centroblastic lymphoma and the mediastinal, mesenteric, peripheral submandibular, prescapular, superficial inguinal lymph nodes and polyploidy in multicentric lymphoma.

Lymphodysplasia is the reversible process by which antigen-stimulated adult B lymphocytes differentiate into peripheral lymphoblasts (Figure 9), then into immunoblasts, plasmablasts and then plasma cells secreting antibodies against the antigen that stimulated them.

All 53 dogs included in the study has a history of several diagnosis of *Babesia* spp. and/or chronic infestation with *Dirofilaria* spp. In the 38 months of observation, all dogs experienced at some point a severe inflammatory episode with exaggerated symptoms of immune reaction which leads us to concur that pets with chronic or repeat diagnosis of parasitosis will fall under the category of risk factors (chronic inflammation) and of trigger (autoimmune like reaction) for malignant lymphoma.

Lymphoma seems to have a seasonal character, namely early spring starting in March with the appearance of hematophagous insects, that determine either an immune response, a severe systemic inflammatory response or a disease relapse due to organ toxicity; late autumn in October correlated with prolonged exposure to the sun during the summer and the seasonal pattern of decline of the immune system (Wyse et al., 2021).

Skin thickness has proven to be a predictability marker for prognosis and response to treatment. Further study regarding predictability markers is necessary.

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TRANSGENIC ANIMALS: INNOVATIONS, APPLICATIONS, AND ETHICAL CONSIDERATIONS IN MODERN BIOTECHNOLOGY

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Abstract

Transgenic animals represent a key achievement in modern biotechnology, providing novel solutions in industries such as agriculture, medicine, and environmental studies. Techniques such as microinjection, somatic cell nuclear transfer, and CRISPR/Cas9 gene editing have facilitated the development of transgenic animals by allowing precise genetic modifications. These advancements have led to the development of livestock that not only exhibit improved growth rates and disease resistance but also serve as bioreactors to produce human proteins and monoclonal antibodies. However, the application of transgenic technology raises significant ethical considerations, particularly regarding animal welfare and ecological impacts. The potential for unintended consequences, such as insertional mutagenesis and epigenetic silencing of transgenes, needs thorough evaluation of the long-term effects on both the transgenic animals and their ecosystems. Furthermore, public perception and regulatory frameworks around genetic engineering must be addressed to ensure responsible development and application of these technologies. This mini-review aims to synthesise current advances in transgenic animal technology, investigate their various applications, and critically evaluate the ethical concerns of their use.

Key words*: transgenic animals, biotechnology, genetic engineering.*

INTRODUCTION

In the present day, molecular biology is undergoing a remarkable pace of advancement. One of them is the capacity to create transgenic animals. A transgenic animal is an organism whose genome has been altered to incorporate genes from another species or to utilise techniques for animal genome editing to achieve particular traits. The gene (or genes) can be intentionally modified to modify the appearance of an animal (Emmanuel et al., 2018).

In this regard, transpharmers are transgenic animals that are employed in pharmaceutical production. They generate critical human proteins in their milk and eggs as a result of the insertion of human genes. ATIII, α-antitrypsin, and tPA are among the products that have been produced from sheep milk thus far (Gheorghe-Irimia, 2023). Additionally, blood, seminal plasma, urine, silk gland, and insect larvae haemolymph are theoretically viable systems (Wang et al., 2013; Maksimenko et al., 2014; Шепелев et al., 2018). Comparably, transgenic mice have become indispensable models for investigating human disorders since they enable exact gene editing and monitoring of consequent phenotypes (Christapher et al., 2022).

Moreover, transgenesis enables the enhancement of the quality of the entire diet, the quantity of nutrients, and the specific nutritional composition of animal products. Transgenic technology has the potential to facilitate the transfer or enhancement of nutritionally advantageous characteristics (Gheorghe-Irimia, 2024; Șonea et al, 2023a; Șonea et al., 2023b).

Notable techniques include microinjection, which has been routinely employed since the

introduction of transgenic technology and directly delivers DNA into the pronucleus of fertilised eggs (Nagano et al., 2001). Additionally, the incorporation of transformed somatic cells into recipient oocytes is facilitated by procedures like somatic cell nuclear transfer (SCNT), which in turn facilitates the production of transgenic progeny. Recent advancements have introduced targeted genome editing tools, including CRISPR/Cas9, which enables precise modifications at predetermined genomic loci, thereby enhancing the precision of transgene integration and minimising off-target effects (Park et al., 2016). Lentiviral vectors are also effective tools for introducing genes into a variety of animal species, which leads to a high level of transgenic efficiency, particularly in porcine and bovine models (Hofmann et al., 2003). In addition, sperm-mediated gene transfer (SMGT) offers an additional approach to the development of transgenic animals by incorporating genetic material into sperm, which is subsequently utilised for *in vitro* fertilisation (Lavitrano et al., 2002). These developments emphasise the expansion of methods for the development of transgenic animals, which is fostering significant applications in biomedicine and research.

This review aims to investigate the most current advancements in transgenic animal technology, investigate their several applications in biotechnology, and assess their ethical and legal conundrums in use.

TECHNIQUES USED FOR TRANSGENIC ANIMALS' GENERATION

Over the past few decades, a variety of techniques have been employed to obtain transgenic animals. Numerous sequences have been identified as the result of gene sequencing, which has contributed to the understanding of genes and promoters that are relevant to numerous species. The emergence of genomics, proteomics, and a new generation of reproductive biotechnologies all suggest that successful transgenic applications in domestic animals are imminent. The intended purpose of a transgenic animal dictates the procedures and methodologies employed in its development (Wang et al., 2016).

Microinjection is a key technique for generating transgenic animals that involves directly inserting DNA into the pronucleus of fertilised oocytes. This method allows the precise delivery of genetic material, which could contain single or multiple genes meant for expression in the resulting organism. The method begins with the isolation of one-cell fertilised embryos then injects a DNA solution into the pronucleus, which has genetic material derived from sperm (Moreira et al., 2016). The microinjected DNA can integrate into the host genome, but with varying effectiveness; historical evidence indicates that roughly 5% of injection embryos effectively incorporate the transgene (Cho et al., 2009). The intrinsic benefits of microinjection include its simplicity and versatility, which allow for the introduction of numerous types of nucleic acids such as RNA or proteins, increasing its uses beyond DNA (Wudarski et al., 2017). Notably, the effectiveness of transgenic integration frequently depends on cellular repair processes that handle the injected material, resulting in either stable integration or temporary expression (Smirnov et al., 2019). Recent research shows that microinjection continues to support advances in transgenic technologies, such as the use of CRISPR for genome editing, which improves the accuracy of genetic alterations (Zabelina et al., 2022; Harms et al., 2014).

In animal cloning, somatic cell nuclear transfer (SCNT) is the process of converting the genetic material of a somatic cell into an enucleated egg therefore generating a viable embryo. Following the successful cloning of Dolly the sheep in 1996 - a major turning point in reproductive biotechnology - this approach became well-known (Simmet et al., 2020; Ogura et al., 2013). SCNT has applications in genetic modification, particularly in transgenic animals' generation, because it allows researchers to use genetically modified somatic cells as the nuclear donor. This allows for the targeted modification or improvement of traits in the subsequent offspring (Matoba et al., 2014; Bordignon et al., 2013). According to research, SCNT can be used to create clones of genetically enhanced livestock, conserving and transmitting desired phenotypic features while also contributing in species conservation. Moreover, strategies for maximising reprogramming during SCNT,

including raising histone demethylation, have been looked at to raise embryo survival and development (Eun et al., 2017; Simmet et al., 2020).

Another major advancement in the field of genetic engineering is CRISPR/Cas9 gene editing, which offers more accuracy in genome editing. Using a guide RNA (gRNA) component, the method allows the Cas9 nuclease find a particular DNA sequence, thereby enabling site-specific modifications including gene knockouts, insertions, or substitutions (Wang et al., 2016).

When compared to conventional techniques like randomised integration in transgenesis, which is usually linked with off-target effects and unpredictable results, this customised approach boosts the efficacy of gene editing (Carey et al., 2019). Creating transgenic animals by means of CRISPR/Cas9 has several benefits. Particularly in livestock, it provides shorter production cycles and reduced costs; effective gene modifications linked to muscle development and disease resistance in pigs (Whitworth et al., 2016). Furthermore, the ability to co-inject numerous gRNAs at the same time enables multiplex gene editing, allowing precise alterations at multiple loci, increasing its adaptability in both research and agriculture (Wang et al., 2015; Wang et al., 2018). Furthermore, advances in CRISPR/Cas9 technology aim to reduce off-target effects while enhancing specificity and safety in gene-edited species, which are crucial for regulatory approval and public acceptability (Han et al., 2020; Lu et al., 2023).

APPLICATIONS OF TRANSGENIC ANIMALS

Particularly for increasing production efficiency, disease resistance, and the generation of valuable biopharmaceuticals, transgenic animals are becoming ever more significant in agriculture. Some of the practical applications of transgenesis in animal production include increased disease resistance, improved carcass composition, improved milk production and/or compositions, improved feed utilisation and growth rate, and greater prolificacy and reproductive performance. One of the most critical candidate genes utilised in the production of transgenic farm animals to enhance their milk production and growth rate is growth hormone. Another important use is the employment of transgenic animals as bioreactors for the synthesis of recombinant proteins like human butyrylcholinesterase, which can protect against organophosphate poisoning (Huang et al., 2007). Furthermore, transgenic goats can express human lysozyme in their milk, reducing susceptibility to mastitis and increasing overall productivity. Further developments include the development of disease-resistant transgenic pigs, which might greatly reduce reliance on antibiotics while improving animal welfare (Chen et al., 2019). This is aligned with sustainable agriculture principles, as limiting antibiotic use addresses growing concerns about antibiotic resistance among cattle. Furthermore, the regulatory landscape is changing as scientists work to bring transgenic inventions to market and gain public acceptance of genetically altered products (Jagadeesan and Salem, 2015; Murray & Maga, 2016).

Another important fields in transgenic animals production are biomedical research and medicine. Mice, pigs, and rabbits have been genetically engineered to mimic human diseases, allowing for extensive research into pathology and therapeutic efficacy. Because of their anatomical and physiological similarities to humans, transgenic pigs have been used as models for a variety of disorders, including cardiovascular disease, diabetes, and cystic fibrosis (Perleberg et al., 2018). This shared characteristic improves the predictive validity of medication responses and safety assessments in preclinical trials. Furthermore, transgenic rabbits are used in antibody production and are important in pharmaceutical research because they can create specific antibodies against human targets (Flisikowska et al., 2011). The use of transgenic models has also increased since the development of CRISPR/Cas9 gene editing (Tanihara et al., 2021). These developments allow researchers to create models that precisely mimic human disease processes, which is essential for testing new therapy options (Nagaya et al., 2021). Furthermore, as it was presented before, transgenic animals work as bioreactors, producing complex biopharmaceuticals such as

proteins and antibodies at scalable levels (Hryhorowicz et al., 2020).

Moreover, transgenic animals have a wide range of uses in environmental management and research, notably the use of environmental DNA (eDNA) approaches. eDNA is genetic material extracted from environmental samples such as soil, water, or air, which can provide information about biodiversity and ecological health. For example, Xu et al. (2021) highlight how transgenes from genetically modified animals can be detected non-invasively using eDNA techniques, thereby assisting with wildlife monitoring and conservation activities. This feature enables researchers to evaluate the dispersion and ecological impact of transgenic animals without requiring direct observation or capture (Xu et al., 2021). Sundström et al. (2016) show that gene-environment interactions can affect eating and anti-predatory behaviours in both wild and transgenic coho salmon, emphasising the possible ecological effects of releasing transgenic organisms into the wild. Furthermore, the development of transgenic animals raises worries about their escape or release into natural habitats.

BIOSAFETY CONSIDERATION, ETHICS AND CHALLENGES

The ethical and biosafety issues surrounding transgenic animals are complex and important in controlling their development and use. One major issue is the risk of unexpected ecological consequences, as transgenic organisms may interact unpredictably with native species and ecosystems if they escape or are deliberately released. This emphasises the significance of robust biosafety evaluations in properly evaluating risks, particularly gene flow between transgenic and non-transgenic populations (Hoenicka & Fladung, 2006; Heerwaarden et al., 2012). Previous experiences with genetically modified crops have highlighted the difficulties of limiting transgene spread in agricultural contexts, demanding strict control methods (Heerwaarden et al., 2012). Ethically, the welfare of transgenic animals must be prioritised, as genetic changes can cause unexpected health problems (Croney and Millman, 2007). Legislation governing animal welfare is changing; yet, there is frequently a gap between intended ethical norms and actual restrictions, which may prioritise economic or agricultural outcomes over animal well-being (Hedman et al., 2014). Furthermore, novel tools, such as non-invasive monitoring techniques, are being developed to improve animal welfare assessments in transgenic models, perhaps alleviating certain ethical issues (Balafas et al., 2019). From a biosafety standpoint, it is critical that the procedures and technologies used to make transgenic animals undergo extensive risk assessments, taking into account factors such as the possibility of insertional mutagenesis and long-term health consequences. Stringent biosafety regulations, aligned with international protocols such as the Cartagena Protocol on Biosafety, are required to manage the release of genetically modified organisms into the environment while also ensuring the safety of human health and biodiversity (Bannantine et al., 2018).

Regarding challenges, only a limited number of animals can be obtained as a result of the transgenic process's expensive cost. This is the reason why backcrossing is a challenging procedure that must be employed to reintroduce these animals into the existing herd. In order to render this process economically viable, it is imperative to have a comprehensive understanding of the most recent developments in assisted reproduction techniques, including in vitro embryo formation or artificial insemination and embryo transfer. Moreover, the primary environmental concern associated with transgenic animals is the potential for their escape. The risks are substantially different depending on the species and the transgene.

CONCLUSIONS

In conclusion, transgenic animals have considerable potential for scientific research, agriculture, and biomedical applications. Researchers aim to create more exact and efficient transgenic models that precisely reflect real disease and support new treatments as genetic engineering tools - especially CRISPR/Cas9 - increasingly develop. Still, ethical and biosafety issues have to be given the greatest attention if we are to satisfy public concerns about gene flow in wild populations, environmental effects, and animal welfare.

Maintaining transparency and supporting public involvement will be vital as legal systems evolve to guarantee acceptability and cooperation in the application of transgenic technology.

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BACTERIAL AGENTS INVOLVED IN POULTRY MEAT CONTAMINATION: PATHOGEN DIVERSITY, TRANSMISSION ROUTES AND FOOD SAFETY IMPLICATIONS

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Abstract

Poultry meat is an important source of protein and very commonly consumed by the population globally. The bacterial agents involved in its contamination, and which pose a risk to public health, leading to serious diseases that can be transmitted through food, are represented by: Salmonella, Campylobacter, Escherichia coli and Listeria monocytogenes. This study will present the transmission routes of these pathogens, the determinants of their persistence focusing on cross-contamination, poor hygiene of workspaces and environmental factors such as deviations of humidity and temperature. This article explores various strategies to reduce bacterial contamination in poultry, focusing on improving hygiene protocols, refining processing techniques, and enforcing stricter food safety standards. These actions are essential for safeguarding poultry meat and minimizing the public health risks posed by bacterial pathogens in the poultry industry.

Key words*: bacterial contaminants, food safety, poultry meat.*

INTRODUCTION

Poultry meat is a crucial dietary staple and an excellent source of high-quality protein, favoured worldwide for its affordability, versatility, and nutritional value. Recent data show that global consumption has reached an average of 21.9 kilograms per capita annually (Authority, 2016).

The safety of poultry products remains a critical concern due to their vulnerability to bacterial contamination. This contamination poses a significant public health risk, as it can result in the transmission of foodborne illnesses caused by pathogens such as *Salmonella spp*., *Campylobacter* spp., *Escherichia coli*, and *Listeria monocytogenes (*Mor-Mur et al., 2010). The most common *Salmonella* serovars identified in poultry are *Salmonella typhimurium* and *Salmonella enteritidis* (Ricke, 2020).

Contaminated poultry carcasses can also introduce other microorganisms, such as *Clostridium perfringens* and certain *Listeria* species, into the human food chain (Mor-Mur et al., 2010). These bacteria are among the most common agents associated with foodborne outbreaks, with severe health consequences ranging from gastrointestinal illness to systemic infections and, in vulnerable populations, even death (Bintsis, 2017).

The pathways through which these pathogens contaminate poultry meat are complex and multifaceted. Contamination can occur at any point along the production chain, from on-farm conditions to slaughter, processing, distribution, and eventual handling by consumers (Göksoy et al., 2004; Mor-Mur et al., 2010) (Figure 1).

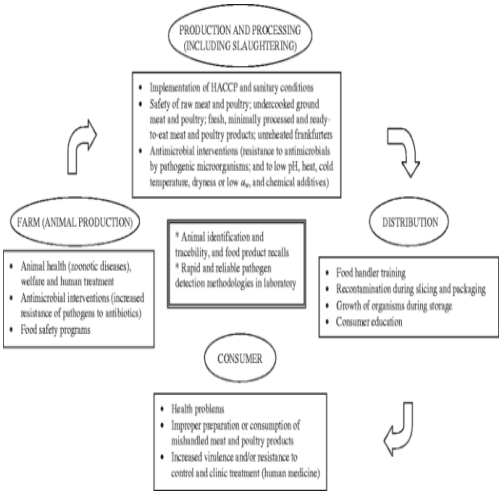

Figure 1. Diagram of potential infection risks in the food chain

Key factors contributing to the persistence and proliferation of these pathogens include inadequate hygiene practices, cross-contamination during processing, and environmental influences such as temperature and humidity fluctuations. Together, these elements create a challenging environment for ensuring poultry meat safety (Alegbeleye et al., 2018).

This study examines the transmission routes of these bacterial pathogens, highlighting critical control points where contamination is most likely to occur. It also explores the determinants that enhance their persistence, with a particular focus on poor workspace hygiene, lapses in sanitation protocols, and the impact of environmental factors. Additionally, the article discusses a range of strategies aimed at reducing bacterial contamination in poultry products.

These strategies include improving hygiene practices throughout the production chain, optimizing processing techniques, and enforcing stricter food safety standards (Martin, et al., 2024).

By addressing these challenges and implementing effective control measures, significant progress can be made toward safeguarding the safety of poultry meat, thus reducing the burden of foodborne illnesses and protecting public health on a global scale (Rouger et al., 2017).

Another significant component in the context of infections is the meat storage practices in commercial units, where refrigeration temperature regulations are not followed. Additionally, another major cause stems from consumer behaviour after purchasing chicken meat, in the context of changes that can occur during transportation, storage, or even from preparation by cooking at improper temperatures (Jackson et al., 2013; Sauter, 1987). These factors play a crucial role in increasing the risk of contamination with the two main bacterial agents associated with chicken meat, namely *Salmonella* and *Campylobacter* (Ovai et al., 2021).

Salmonellosis

Salmonellosis is an infectious disease caused by bacteria of the *Salmonella* genus, which encompasses more than 2,500 serotypes. Among these, two highly pathogenic serotypes primarily affect poultry and are commonly found in certain regions of Central and South America. In most cases, motile *Salmonella* serotypes are not pathogenic to poultry under normal conditions (Lamichhane et al., 2024).

Nevertheless, immunosuppressed birds or very young chicks may be susceptible to infection, particularly when exposed to a high bacterial load (Suzuki, 1994).

A variety of avian species, such as turkeys, quails, geese, ducks, and small domestic birds (e.g., roosters, pheasants, grouse), are susceptible to *Salmonella* infection. These birds may exhibit diverse clinical forms of the disease, ranging from acute to chronic infections, and some individuals may act as asymptomatic carriers (Wibisono et al., 2020) The latter play a significant role in maintaining and spreading the pathogen within avian populations and, potentially, to humans (Wibisono et al., 2020).

Colibacillosis

Colibacillosis is a localized or systemic infection caused by avian pathogenic *Escherichia coli* (APEC). *E. coli* are Gram-negative, facultatively anaerobic bacilli capable of surviving in both aerobic and anaerobic environments, depending on external conditions. Although typically part of the normal intestinal microbiota in birds, these microorganisms can become pathogenic under certain circumstances (Nolan et al., 2015).

The management of colibacillosis in the poultry industry remains a major challenge, primarily due to the complexity of the contributing factors. A critical concern is the emergence and spread of antimicrobial-resistant strains of avian pathogenic *Escherichia coli*. This issue is closely associated with the frequent and often improper use of antibiotics in poultry farms, which promotes the selection of resistant bacterial populations (Redweik et al., 2020).

Furthermore, international regulations particularly those enforced within the European Union impose strict limitations on the use of antimicrobial agents in food-producing animals. While these legislative measures aim to prevent the transfer of resistance genes to human pathogens, they also significantly restrict the therapeutic options available for controlling bacterial infections in poultry (Nolan et al., 2015).

MATERIALS AND METHODS

In this research, a total of 27 relevant bibliographic sources were consulted, addressing bacterial agents involved in poultry meat contamination, exploring pathogen diversity, transmission routes, and food safety implications. The source selection process was carried out according to strict criteria, ensuring the inclusion of the most relevant and up-to-date studies in the field.

The selected studies were published between 1987 and 2024, thus encompassing both foundational research and the latest scientific advancements in the field.

The following academic databases were used to identify sources: *PubMed, ScienceDirect and Google Scholar*. These databases are recognized for the quality and relevance of the scientific publications they index, ensuring access to high-quality, current works.

During the literature search, keywords such as bacterial contamination, poultry meat, *Salmonella*, *Campylobacter*, pathogen diversity, transmission routes, food safety, meat processing, antibiotic resistance, and public health were used. These key words were selected to cover a wide range of topics relevant to the research theme.

Articles published in peer-reviewed scientific journals were selected, analysing aspects related to bacterial contamination of poultry meat, pathogen transmission mechanisms, bacterial diversity, and food safety risks. Relevant studies from the fields of food microbiology, public health, and meat processing technologies were included.

Papers that did not focus directly on bacterial agents or that addressed only pathogens from other food sources were excluded.

A systematic literature review was conducted to identify and analyse studies investigating the same bacterial agents involved in poultry meat contamination. Each source was evaluated based on the relevance and quality of the data presented, and the extracted information was compared to provide a comprehensive overview of the topic.

The information obtained from the selected sources was analysed comparatively to identify patterns and variations in pathogen diversity, their transmission routes, and contamination prevention measures.

The implications for food safety were addressed, including public health risks associated with bacterial contamination and the recommended control measures in the industry and poultry meat handling process.

This selection and analysis methodology allowed for a comprehensive perspective on the bacterial agents involved in poultry meat contamination, their transmission mechanisms, and their impact on food safety.

RESULTS AND DISCUSSIONS

Although infections caused by *Salmonella* and *Campylobacter* have a high incidence within the population, *Campylobacter jejuni* and *Campylobacter coli* are also involved; these bacterial agents cause gastrointestinal pathologies through the consumption of contaminated chicken meat. In intervention strategies for preventing infections, countries adopt different practices as it has been found that the reservoir in urban environments is poultry, which are not necessarily implicated in infections in rural settings (Skarp et al., 2015).

Various intervention strategies have been developed to limit *Campylobacter* colonization in poultry, demonstrating efficacy in both experimental studies and commercial applications. Among these, the administration of probiotics, in particular strains of *Lactobacillus salivarius* and *Lactobacillus reuteri*, has been shown to be effective in inhibiting the growth of *Campylobacter jejuni* and *Campylobacter coli*, most likely through competitive exclusion mechanisms and secretion of antimicrobial compounds (Neijat et al., 2018). Although no commercially available vaccines are currently in widespread use against *Campylobacter* infections, experimental research on immunoprophylaxis, particularly studies focusing on conjugate vaccines utilizing enterobactin as antigen, has demonstrated promising efficacy in reducing intestinal colonization. These findings highlight the considerable potential for developing targeted immunization strategies aimed at the prevention of *Campylobacter* infections (Meunier et al., 2016).

Contamination and transmission routes
Pathogens can localize in various bird organs, such as the skin, lungs, digestive tract, and even the feathers, making contamination possible during the slaughtering process, with a risk of carcass contamination. Although the practices in this slaughtering process can vary depending on the size of the facility, there are common procedures in poultry slaughtering, such as the use of water baths equipped with electrical currents for stunning the birds and the feather removal stage. Due to the small size of the birds, manoeuvrability can sometimes be limited compared to larger animals, which restricts the use of mechanized equipment, increasing the likelihood of cross-contamination (Figure 2) (Rouger et al., 2017).

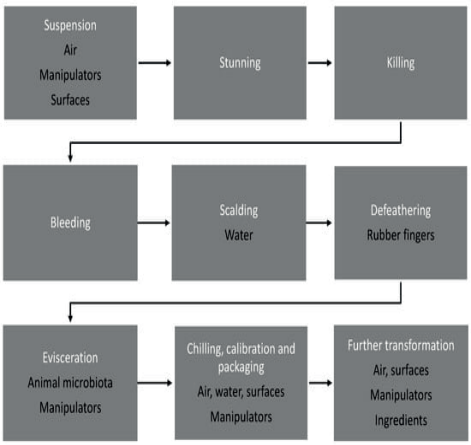

Figure 2. Diagram of the steps in the poultry slaughtering process and associated contamination routes (after, Rouger et al., 2017)

The anatomical and morphological particularities of poultry have necessitated the advancement of dedicated processing technologies capable of accommodating the small size and structural fragility of these animals. In contemporary poultry processing facilities, automation plays a crucial role in enhancing hygienic standards and minimizing microbial hazards. Nevertheless, the efficiency of such automated systems is contingent upon their precise calibration to account for inter-bird variability in size and conformation. Processing steps such as defeathering and evisceration demand high levels of synchronization and mechanical precision to avoid compromising carcass integrity or causing the rupture of visceral organs, events that significantly increase the risk of contamination with enteropathogens such as *Campylobacter* and *Salmonella*.

To address these operational challenges, certain processing facilities have adopted pre-slaughter size sorting mechanisms and employ modular or adjustable equipment systems that can be fine-tuned to match the specific anatomical profiles of poultry carcasses.

Furthermore, the implementation of enhanced sanitation protocols, alongside real-time monitoring of critical control points (CCPs), has become a standardized approach in modern poultry abattoirs. These measures are aimed at mitigating the elevated risk of cross-contamination associated with the automation of processing lines handling small-bodied carcasses (Barbut et al., 2020).

Infection of poultry with *Salmonella* can occur either through direct contact with other carrier birds or through contact with pests, such as rodents. Additionally, another way of transmission can occur indirectly through contaminated bedding, drinking water, or feed

Under favourable environmental conditions, such as room temperature, *Salmonella* spp. has the ability to form a biofilm, giving it increased microbial resistance on work surfaces in slaughterhouses and food processing facilities (Figure 3) (Shaji et al., 2023).

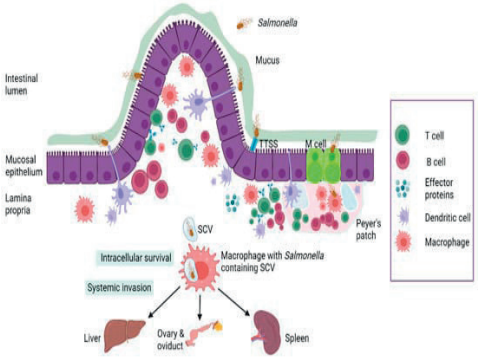

Figure 3. Schematic illustration of *Salmonella* pathogenesis in poultry (after, Shaji et al., 2023)

The transmission of *Campylobacter* spp. to humans occurs through insufficient cooking of chicken meat. It is known that even a small infectious dose can cause human infection, although it is well known that this bacterium

does not multiply in food. Among the species, *Campylobacter jejuni* is the most frequently associated with human gastroenteritis, followed by *Campylobacter coli* (Shaji et al., 2023).

Prevalence and antimicrobial resistance

Listeria monocytogenes represents a significant though sometimes underestimated hazard in poultry products, with recent studies revealing concerning patterns of prevalence and antimicrobial resistance. Research examining various poultry meats, including chicken, duck, quail, and turkey, found *Listeria* spp. in 24.46% of samples, with *L. monocytogenes* specifically detected in 10.32% of samples. *L. monocytogenes* was identified as the predominant *Listeria* species in chicken and quail meat, while other *Listeria* species, such as *Listeria innocua and Listeria selligeri* predominated in duck and turkey products, highlighting the variable ecology of this pathogen across different poultry types. Of particular concern is the high prevalence of antimicrobial resistance observed in *Listeria* isolates from poultry meat. Research has revealed that 55.93% of *Listeria* strains isolated from poultry and pork samples demonstrated multi-resistance, defined as resistance to three or more families of antibiotics. The highest rates of multi-resistance were observed in *L. monocytogenes* (73.68%) and *L. innocua* (70.59%), followed by *L. ivanovii* (50%). This widespread antimicrobial resistance significantly complicates the management of listeriosis in both humans and animals. A particularly troubling aspect is the resistance observed against several key classes of antibiotics, including β-lactams (such as ampicillin and penicillin G), aminoglycosides (such as gentamicin and streptomycin), as well as macrolides and tetracyclines. This pattern of resistance complicates therapeutic approaches, especially in severe cases of listeriosis, where β-lactams and aminoglycosides constitute the cornerstone of antimicrobial treatment in both veterinary and human medicine (Çadirci et al., 2023).

This resistance pattern underscores the need for enhanced control measures throughout the poultry production chain, including improved handling practices, thorough cooking, and effective cleaning and disinfection protocols to prevent cross-contamination (Çadirci et al., 2023).

The observed antimicrobial resistance patterns highlight the pressing necessity for a comprehensive reinforcement of control strategies throughout the poultry production chain. While traditional interventions, such as enhanced hygiene during handling, proper thermal processing, and stringent sanitation measures remain foundational in preventing cross-contamination, the adoption of smart technologies in modern processing environments is becoming increasingly pivotal. These advancements include the deployment of real-time biosensor-based monitoring systems for early microbial detection, analytical software capable of processing large datasets to identify deviations from hygienic norms, and automated control mechanisms that dynamically adjust key processing parameters (e.g., temperature, pressure, or disinfectant dosage) in response to identified risks. The integration of such intelligent, adaptive systems not only fortifies food safety protocols but also enables a shift toward proactive and predictive risk management in poultry processing operations (George & George, 2023).

The emergence of highly resistant *Listeria* strains in poultry products represents an evolving food safety challenge that requires heightened vigilance (Çadirci et al., 2023).

Clostridium perfringens has emerged as a significant concern in poultry production, particularly following changes in antimicrobial use practices. The incidence of *C. perfringens*-associated necrotic enteritis in poultry has notably increased in countries that have discontinued antibiotic growth promoters, representing an unintended consequence of efforts to reduce antimicrobial use in animal production. This trend highlights how changes in production practices can influence the emergence or resurgence of bacterial pathogens (Çadirci et al., 2023).

The mechanisms of *C. perfringens* pathogenicity in poultry involve complex interactions between the bacterium and its host environment. Necrotic enteritis and subclinical infections are primarily caused by *C. perfringens* type A, which produces alpha toxin, and to a lesser extent type C, which produces both alpha and beta toxins. Some strains of *C.*

perfringens type A also produce an enterotoxin during sporulation that is responsible for foodborne illness in humans, establishing a clear link between poultry health and human food safety concerns (Van Immerseel et al., 2004).

Foodborne illness caused by *Clostridium perfringens* in humans is predominantly linked to the consumption of inadequately cooked or mishandled meat products, especially those with high bacterial loads, such as poultry and beef (Dolan et al., 2015).

Symptoms of this condition typically include abdominal cramps and diarrhoea, which manifest within six to 24 hours following the ingestion of contaminated food. The primary causative agent of this illness is the enterotoxin produced by *C. perfringens* during sporulation, which disrupts the epithelial cells of the intestine, resulting in gastroenteritis. *C. perfringens* is capable of proliferating in food that is stored at improper temperatures, particularly in large quantities of food that are inadequately refrigerated or held for extended periods. This creates an optimal environment for bacterial growth and toxin production. Although the illness is generally self-limiting, more severe manifestations can occur, particularly among vulnerable groups such as the elderly or immunocompromised individuals, leading to significant health complications. To mitigate the risks associated with *C. perfringens* contamination, it is crucial to adopt appropriate food handling, cooking, and storage practices to ensure food safety (Gotfried, 2023).

Food safety implications and future directions

The emergence and underestimation of bacterial pathogens in poultry meat present significant challenges for food safety systems worldwide. The high prevalence of antimicrobial resistance, particularly in *Listeria monocytogenes* and APEC (Avian Pathogenic *Escherichia coli*), complicates treatment options for human infections acquired through contaminated poultry products (Martinez-Laorden et al., 2024).

Natural Antimicrobials and Alternative Compounds

The application of bacteriocins (antimicrobial peptides) and biosurfactants derived from lactic acid bacteria for the inhibition of *Salmonella* and *Campylobacter* on the surface of fresh meat. These bio compounds offer an effective alternative to chemical preservatives, contributing to enhanced food safety while minimizing potential health risks associated with synthetic additives.

The integration of silver nanoparticles or zinc oxide into active packaging systems, which release metal ions capable of disrupting bacterial biofilms. This innovative approach offers an additional strategy for controlling microbial contamination in food products, thereby improving the preservation and safety of perishable goods (Chowdhury, Ashrafudoulla, Mevo, Mizan, Park, & Ha, 2023).

Advanced Vaccination

Oral vaccines based on genetically modified strains of *Escherichia coli* and *Salmonella* for stimulating mucosal immunity in poultry and reducing intestinal colonization (Redweik et al., 2020).

Phage Therapy in Meat Processing

The implementation of bacteriophage cocktails specifically targeting *Salmonella* and *Listeria* during the final stages of meat packaging represents a promising biocontrol strategy. This approach enables the selective elimination of pathogenic bacteria without compromising the sensory or nutritional quality of the final product, thereby enhancing food safety through a targeted, residue-free intervention (Abreu et al., 2023).

Intelligent Decontamination Systems Enzymes and Antimicrobial Coatings

The use of lipases and proteases to degrade biofilms on processing equipment, combined with edible films infused with essential oils (e.g., oregano oil) (Chowdhury et al., 2023).

CONCLUSIONS

The significant public health burden associated with bacterial contamination of poultry meat necessitates continued research and implementation of effective control strategies across the entire production chain.

While substantial progress has been achieved in understanding the epidemiology and transmission dynamics of major pathogens like

Campylobacter and *Salmonella*, emerging and underestimated pathogens such as *Listeria monocytogenes* and *Clostridium perfringens* warrant increased attention.

The complexity of contamination sources and transmission routes underscores the importance of integrated approaches combining interventions at farm, processing, retail, and consumer levels.

Future research directions should focus on developing improved detection methods for rapid identification of contaminated products, advancing our understanding of pathogen ecology throughout the production chain, and evaluating the efficacy and cost-effectiveness of various intervention strategies in different production contexts.

Additionally, the increasing global poultry meat trade necessitates harmonized approaches to food safety standards and surveillance systems to effectively monitor and control bacterial pathogens across international boundaries.

As poultry consumption continues to increase worldwide, effective control of bacterial contamination remains essential for protecting public health and ensuring consumer confidence in poultry products.

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TREND OF ANTIBIOTIC RESISTANCE IN *STAPHYLOCOCCUS* CHROMOGENES ISOLATED FROM RAW MILK SAMPLES OF DAIRY COWS IN ROMANIA BETWEEN 2018 AND 2023

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Abstract

Staphylococcus chromogenes (St. chromogenes) continues to be one of the primary causative agents of mastitis in dairy cattle. This retrospective study aimed to present the trends of antimicrobial resistance in St. chromogenes isolated from raw milk samples of dairy cows with subclinical and clinical mastitis over a six-year period. From January 2018 to December 2023, a total of 79 St. chromogenes isolates were evaluated for antimicrobial resistance against 22 antibiotics. The current study revealed a significant increase in resistance to various antimicrobial agents. For example, amoxicillin resistance increased from 23.33% to 50.00%, marbofloxacin from 12.50% to 25.00%, doxycycline from 13.33% to 75.00%, oxytetracycline from 13.33% to 50.00%, and streptomycin from 16.67% to 50.00%. The increasing trend of antimicrobial resistance underscores the need for robust infection control strategies and judicious antibiotic use in dairy farms. To summarise, this study can serve as an essential resource for evaluating treatment protocols and mitigating the further spread of resistance.

Key words*: cow, mastitis, trends, drug resistance, Romania.*

INTRODUCTION

Bovine milk production plays a significant role in the global economy, serving as a crucial income source for producers of all sizes, including small, medium, and large-scale operations (Bojovic & McGregor, 2023; Crippa et al., 2024; Jafri et al., 2024).

Nevertheless, infections of the mammary gland, primarily caused by bacteria, pose a significant challenge to animal welfare, productivity, and the economy, particularly within dairy farming, resulting in substantial losses for the dairy sector (Antók et al., 2019; Crippa et al., 2024; Dhital et al., 2023).

Bovine mastitis and intramammary infections have been associated with more than twenty types of Non-aureus staphylococci (NAS), including *St. chromogenes* (Beuckelaere et al., 2021; Persson Waller et al., 2023; Phophi et al, 2019). Many studies have reported that *St. chromogenes* was isolated from animals exhibiting mild, moderate, or severe symptoms of subclinical and clinical mastitis as well as chronic infections (Getahun et al., 2024; Király et al., 2024; Persson Waller et al., 2023; Zigo et al., 2022). *St. chromogenes* is considered a host-adapted species, capable of overcoming the udder's physical defences and forming biofilms (Bochniarz et al., 2016; Crippa et al., 2024). Additionally, this microorganism is highly adapted to the bovine mammary gland, where it may serve as a microbial reservoir and a potential source of infections (Crippa et al., 2024). The highest representation of virulence factors (production of hemolysis, gelatinase, and biofilm; the ability to hydrolyse DNA; resistance to antibiotics) was found in NAS, such as *St. chromogenes* (Antók et al., 2019; Bochniarz et al., 2016; Zigo et al., 2022). Resistance to antibiotics represents a significant risk to both animal and human health (Naranjo-Lucena & Slowey, 2023).

The escalating issue of antibiotic resistance is already a critical challenge, reducing the effectiveness of antimicrobial agents and hindering treatment options for both veterinary and human medicine (Naranjo-Lucena & Slowey, 2023; Sipahi et al., 2023). Therefore, the main objective of this study is to evaluate the

antibiotic resistance of *St. chromogenes* isolated from Romanian dairy cattle. This analysis aims to elucidate local antimicrobial resistance trends, thus contributing valuable information on the evolutionary dynamics of antibiotic resistance in the context of bovine intramammary infections.

MATERIALS AND METHODS

The present study was conducted on 79 *St. chromogenes* bacterial isolates, obtained from raw milk samples of dairy cows with subclinical and clinical mastitis, collected over a six-year period. This investigation was carried out at the Synevovet Laboratory in Bucharest, Romania, with the analysis period spanning from January 2018 to December 2023.

The samples were cultured on Columbia agar and Cled agar and then incubated at 35-37°C for 20-24 h in aerobic conditions. After incubation, *Staphylococcus* species were selected based on their colony morphology and then were identified using MALDI-TOF MS (matrix assisted laser desorption/ionization time-of-flight mass spectrometry) technology.

The isolates' antibiotic resistance profiles were determined by a disk diffusion method on Mueller-Hinton agar plates. The sizes of the inhibition zones around the antibiotic discs were measured using the ADAGIO automated system (Bio-Rad). The resistance profiles were interpreted based on the standards set by the Clinical and Laboratory Standards Institute (CLSI) for the following antimicrobials: amoxicillin, ampicillin, amoxicillin-clavulanic acid, erythromycin, streptomycin, ceftiofur, doxycycline, oxytetracycline, tetracycline, penicillin, penicillin-novobiocin, gentamycin, pirlymicin, trimethoprim-sulfamethoxazole, kanamycin-cephalexin, penicillin G-framycetin, cefquinome, neomycin, cefoperazone, enrofloxacin, marbofloxacin, and cloxacillin.

Then, the strains were categorized as resistant (R), intermediate (I), and susceptible (S). Data collection and processing were performed using Microsoft Office Excel (Microsoft, USA). Figures were also generated using Microsoft Office. Statistical analyses for this study were conducted using a linear regression (parametric approach) from SAS with *p*-values less than 0.05 considered statistically significant.

RESULTS AND DISCUSSIONS

In our study, the 79 strains of *St. chromogenes* showed a varied overall resistance to the tested antibiotics as seen in Table 1.

Table 1. Antimicrobial susceptibility of *St. chromogenes* isolates tested between 2018 and 2023

Antimicrobials	Total	Susceptible No. (%)	Intermediate No. (%)	Resistant No. (%)
β-lactamase				
Amoxicillin (A)	79	57 (72.15)	0 (0)	22 (27.85)
Ampicillin (AM)	79	58 (73.42)	0 (0)	21 (26.58)
Amoxicillin-clavulanic acid (AMC)	79	79 (100%)	0 (0)	0 (0)
Cefoperazone (CFP)	79	73 (92.41)	2 (2.53)	4(5.06)
Cefquinome (CEF)	79	74 (93.67)	0 (0)	5 (6.33)
Ceftiofur (EFT)	79	79 (100)	0 (0)	0 (0)
Penicillin (P)	79	58 (73.42)	0 (0)	21 (26.58)
Penicillin G-framycetin (PFY)	79	79 (100%)	0 (0)	0 (0)
Penicillin-novobiocin (PNV)	79	78 (97.73)	0 (0)	1 (1.27)
Cloxacillin	79	77 (97.47)	0 (0)	2 (2.53)
Quinolones				
Enrofloxacin (ENR)	79	79 (100%)	0 (0)	0 (0)
Marbofloxacin (MAR)	79	77 (97.47)	0 (0)	2 (2.53)
Macrolide				
Erythromycin	74	64 (86.49)	0 (0)	10 (13.51)
Tetracyclines				
Doxycycline (DO)	79	62(78.48)	0(0)	17(21.52)
Tetracycline (TE)	79	49 (66.22)	2(2.70)	23(31.08)
Oxytetracycline (OT)	79	60 (75.95)	0 (0)	19 (24.05)
Aminoglycosides				
Gentamicin (CM)	79	78 (98.73)	0 (0)	1 (1.27)
Streptomycin (S)	79	57 (72.15)	5 (6.33)	17 (21.52)
Neomycin (N)	79	79 (100)	0 (0)	0 (0)
Kanamycin-cephalexin (KCL)	79	79 (100%)	0 (0)	0 (0)
Sulphonamides				
Sulphamethoxazole-Trimethoprim (SxT)	79	78 (98.73)	0 (0)	1 (1.27)
Lincosamide				
Pirlimycin	77	72 (93.51)	0 (0)	5 (6.49)

These results demonstrate a concerning level of antimicrobial resistance, with tetracycline exhibiting the highest resistance rate at 31.08%, followed by amoxicillin (27.85%), then penicillin, and ampicillin, both with the same level of resistance (26.58%). Additionally, significant resistance was observed against oxytetracycline (24.05%), doxycycline (21.52%), and streptomycin (21.52%). The observed antimicrobial resistance in *St. chromogenes* isolates from bovine milk with mastitis may result from a combination of factors, including the overuse or misuse of antibiotics, inadequate treatment regimens, and poor farm hygiene practices (Antók et al., 2019). Our findings regarding the resistance of *St. chromogenes* are consistent with those reported in a similar study conducted globally, further

supporting the reliability and reproducibility of these results. Los Santos et. al presented the penicillin resistance percentages of *Staphylococcus* spp. isolates in South America. In their study, the penicillin resistance was detected at 25% for *St. chromogenes*, this value being similar to our results (De Los Santos et al., 2022). A total of 142 confirmed coagulase-negative staphylococcal isolates were used in a study conducted in South Africa. Ninety-three percent (93/100) of *St. chromogenes* were resistant to at least one antimicrobial. *St. chromogenes* exhibited a high prevalence of resistance to penicillin (87%), ampicillin (87%), erythromycin (69%), and streptomycin (54%) compared to our study, where the resistance rates to these antibiotics were much lower, specifically 26.58%, 26.58%, 13.51%, and 21.52%, respectively (Phophi et al., 2019). Getahunet al. also studied the antibioresistance of coagulase-negative staphylococci from bovine milk in Gondar City and found that all the *St. chromogenes* exhibited 100% resistance to amoxicillin, and ampicillin. In contrast, our study shows a much lower resistance to these antibiotics, with amoxicillin resistance at 27.85% and ampicillin resistance at 26.58%. The stark contrast in resistance rates between the two studies may be attributed to differences in geographic location, sample characteristics, testing methodologies, or antibiotic usage patterns (Getahun et al., 2024).

Regarding susceptibility, according to Bochniarz et al. who examined 38 isolates of *St. chromogenes* from 335 samples of milk from cows with subclinical coagulase-negative staphylococci mastitis, 84.2% of the isolates were susceptible to amoxicillin with clavulanic acid, 81.6% to ampicillin, and 73.7% to penicillin. Our study shows higher susceptibility to amoxicillin with clavulanic acid (100%) compared to their study (84.2%), suggesting that *St. chromogenes* in our region may be more sensitive to this combination antibiotic. However, the susceptibility to ampicillin in our study (73.42%) is slightly lower than in their study (81.6%), indicating that our isolates might have a higher level of ampicillin resistance. The results for penicillin susceptibility are almost identical (73.7% vs. 73.42%), indicating similar resistance levels in both regions (Bochniarz et al., 2016).

The presence of antibiotic-resistant bacteria in bovine mastitis and the potential transmission to humans through unpasteurized dairy products pose significant public health risks (Ghimpețeanu et al., 2022). The frequent use of antibiotics by farmers to treat mastitis contributes to the development of resistant strains, leading to financial losses and diminishing the effectiveness of prevention and management strategies (Khasapane et al., 2024; Neculai-Valeanu et al., 2024).

Moreover, this study highlighted concerning trends in antimicrobial resistance among *St. chromogenes* isolated from bovine with intramammary infections by analysing milk samples collected over a six-year period. Our research indicates a notable level of resistance among *St. chromogenes* strains against tetracyclines, with doxycycline resistance frequencies recorded as 37.50% in 2022, and 75.00% in 2023. Similarly, for oxytetracycline, the resistance rose from 13.33% in 2018 to 50.00% in 2023. The resistance rates for tetracycline displayed variations over the study investigation. Specifically, in 2019, the resistance percentage for tetracycline was recorded as 84.62%, higher than the rates of 14.29% in 2021, 42.86% in 2022 and 75.00% in 2023. Among aminoglycosides, streptomycin resistance increased from 12.50% in 2020 to 50.00% in 2022. Resistance to β-lactamase, particularly amoxicillin, increased from 23.33% in 2018 to 37.50 % in 2022, and 50.00% in 2023. The resistance percentages for ampicillin peaked in 2022, with 37.50% compared to 14.29% in 2021, and 25.00% in 2022, 2023, respectively. For cefoperazone, the maximum resistance percentage was observed in 2018 at 10.00%, followed by a decrease to 6.67% in 2019. Examination of milk samples indicated a rise in resistance for quinolones, notably marbofloxacin, from 12.50% in 2022 to 25.00% in 2023. Additionally, our results indicated a rise in resistance rates for pirlimycin between 2018 and 2019, from 6.67% to 15.38%, followed by a decrease to 12.50% in 2022 as presented in Table 2.

The analysis of antibiotic resistance trends for *St. chromogenes* between 2018 and 2023 revealed statistically significant findings as seen in Figure 1. The resistance of this pathogen to oxytetracycline rose significantly, with a

p-value of 0.0028, suggesting a notable and significant trend. Similarly, resistance to amoxicillin also exhibited significant upward trends, with a p-value of 0.0177, indicating statistical significance. Additionally, resistance to marbofloxacin, doxycycline, and streptomycin demonstrated significant increasing trends, with p-values of 0.0406, 0.0481 and 0.0443, respectively, confirming statistical significance. In contrast, cefoperazone and penicillin resistance in this pathogen showed a significant decrease, with p-values of 0.0344 and 0.0464, respectively, suggesting that the reduction in resistance was statistically significant and unlikely to be due to random variation.

Our results, which reflect trends in antimicrobial resistance of *St. chromogenes* strains, indicate an increase in resistance rates by the end of 2023.

Future strategies should focus on strengthening surveillance of antimicrobial resistance, ensuring rigorous adaptation of control measures to prevent intramammary infections on dairy farms, and directing research efforts toward developing innovative treatment protocols to maintain the efficacy of existing antibiotic therapies.

Table 2. Antibiotic resistance profile of *St. chromogenes* isolates from raw milk (n=79)

Antimicrobials	***St. chromogenes* % of resistant isolates (no. of tested isolates)**						
	2018	**2019**	**2020**	**2021**	**2022**	**2023**	***p*-value**
β-lactamase							
Amoxicillin (A)	23.33 (30)	26.67 (15)	25.00 (8)	28.57 (14)	37.50 (8)	50.00 (4)	0.0177*
Ampicillin (AM)	26.67 (30)	33.33(15)	37.50(8)	14.29 (14)	25.00 (8)	25.00 (4)	0.4602
Amoxicillin-clavulanic acid (AMC)	0 (30)	0 (15)	0 (8)	0 (14)	0 (8)	0 (4)	0
Cefoperazone (CFP)	10.00 (30)	6.67 (15)	0 (8)	0 (14)	0 (8)	0 (4)	0.0344*
Cefquinome (CEF)	6.67 (30)	13.33 (15)	0 (8)	0 (14)	0 (8)	25.00(4)	0.6000
Ceftiofur (EFT)	0 (30)	0 (15)	0 (8)	0 (14)	0 (8)	0 (4)	0
Penicillin (P)	26.67 (30)	40.00 (15)	37.50(8)	21.43(14)	0 (8)	0 (4)	0.0464*
Penicillin G-framycetin (PFY)	0 (30)	0 (15)	0 (8)	0 (14)	0 (8)	0 (4)	0
Penicillin-novobiocin (PNV)	0 (30)	6.67 (15)	0 (8)	0 (14)	0 (8)	0 (4)	0.4411
Cloxacillin	6.67 (30)	0 (15)	0 (8)	0 (14)	0 (8)	0 (4)	0.1583
Quinolones							
Enrofloxacin (ENR)	0 (30)	0 (15)	0 (8)	0 (14)	0 (8)	0 (4)	0
Marbofloxacin (MAR)	0 (30)	0 (15)	0 (8)	0 (14)	12.50 (8)	25.00 (4)	0.0406*
Macrolide							
Erythromycin	19.23 (26)	14.29 (14)	0 (8)	0 (14)	37.50 (8)	0 (4)	0.8596
Tetracyclines							
Doxycycline (DO)	13.33 (30)	13.33 (15)	0 (8)	35.71 (14)	37.50 (8)	75.00 (4)	0.0481*
Tetracycline (TE)	14.29 (28)	84.62 (13)	0 (8)	14.29 (14)	42.86 (70	75.00 (4)	0.5719
Oxytetracycline (OT)	13.33 (30)	20.00 (15)	25.00 (8)	28.57 (14)	50.00 (8)	50.00 (4)	0.0028*
Aminoglycosides							
Gentamicin (CM)	3.33 (30)	0 (15)	0 (8)	0 (14)	0 (8)	0 (4)	0.1583
Streptomycin (S)	16.67 (30)	20.00 (15)	12.50 (8)	21.43 (14)	37.50 (8)	50.00 (4)	0.0443*
Neomycin (N)	0 (30)	0 (15)	0 (8)	0 (14)	0 (8)	0 (4)	0
Kanamicină-cefalexin (KCL)	0 (30)	0 (15)	0 (8)	0 (14)	0 (8)	0 (4)	0
Sulphonamides							
Sulphamethoxazole-Trimethoprim (SxT)	3.33 (30)	0 (15)	0 (8)	0 (14)	0 (8)	0 (4)	0.1583
Lincosamide							
Pirlimycin	6.67 (30)	15.38 (13)	0 (8)	0 (14)	12.50 (8)	0 (4)	0.6000

*statistically significant differences.

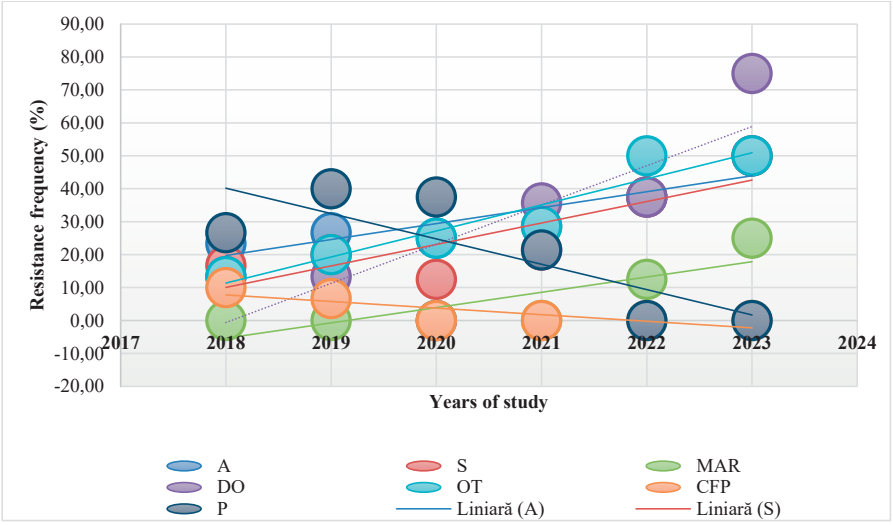

Figure 1. Antibiotic resistance of *St. chromogenes* across 2018 to 2023. The trend of increasing resistance to oxytetracycline, amoxicillin, marbofloxacin, doxycycline, and streptomycin is noticeable. On the other hand, the trend of decreasing resistance to cefoperazone and penicillin is visible

CONCLUSIONS

The observed resistance of *St. chromogenes* to multiple commonly used antibiotics, including tetracycline (31.08%), amoxicillin (27.85%), penicillin (26.58%), ampicillin (26.58%), and others, underscores the growing challenge of managing mastitis infections. A concerning increase in antimicrobial resistance has been noted, particularly for oxytetracycline. This trend emphasizes the need for ongoing surveillance, prudent antibiotic use, and the exploration of alternative treatment options to effectively address resistant strains and improve herd health management. In order to accurately monitor and address the changing dynamics of bacterial resistance, it is crucial to conduct thorough and continuous surveillance of antimicrobial sensitivity and resistance trends over time, as intended in the current study.

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MONITORING OF VETERINARY DRUG RESIDUES IN ANIMAL-DERIVED FOODS

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Abstract

The presence of veterinary drug residues in food raises significant public health concerns. Exposure to these residues, especially at high levels, can lead to adverse health effects for consumers, including allergic reactions, antibiotic resistance and other toxicological risks. The indiscriminate use of drugs in animal husbandry to promote growth or prevent disease leads to the accumulation of residues in animal tissues and products of drug residues including antibiotics, hormones, and anti-inflammatory substances. A total of 1605 animal-derived samples were analysed, including 209 beef, 453 pig, 61 sheep and goat, 11 horse meat, 700 poultry, 68 milk, 82 egg, 1 rabbit, 2 wild game and 18 fish samples. The detected antibiotics were penicillins, tetracyclines, sulphonamides, aminoglycosides and cephalosporins, in the following proportions depending on the type of sample: beef (13%), pigs (28%), sheep/goats (4%), horses (1%), poultry (44%), milk (4%), eggs (5%) and fish (1 %). Tetracycline residues were the most prevalent in poultry meat, detected in 64% of the analysed samples.

Key words*: monitoring, residues, veterinary drugs, food.*

INTRODUCTION

The presence of veterinary drug residues in food has become a growing concern in recent years. While drugs are essential for maintaining animal health and welfare, their residues in food can pose risks to human health, animal welfare and environmental sustainability. Constant exposure to residues can produce adverse effects in human consumers including allergic reactions, antibiotic resistance and other toxicological and carcinogenic risks (Ciocîrlie et al., 2024; Goran et al., 2016). The extensive use of veterinary drugs in animal husbandry - antibiotics, antiparasitics, hormones, non-steroidal anti-inflammatories, etc. - with the aim of promoting growth or preventing diseases, will ultimately lead to the accumulation of residues in animal tissues and implicitly in the products and by-products derived from them (Dobre, 2019).

The beneficial effects of NSAIDs, antibiotics, and antiparasitics on digestive and respiratory conditions in farm animals are well documented, with obvious benefits in favour of combinations of substances (Anderson, 1988; Dobre et al., 2019).

From this perspective, ensuring the safety and quality of food products requires strong, well-established monitoring and enforcement mechanisms to detect and limit the presence of veterinary drug residues (Muaz K. et al., 2018). To avoid contamination incidents and to ensure compliance with maximum residue limits, proactive measures for better surveillance, risk communication and transparency are needed to maintain consumer confidence and market stability (Ciocîrlie et al., 2024).

In this context, this paper presents an analysis of antibiotic contamination in food of animal origin, detected in samples analysed by the Competent Authority in Romania, during 2022-2023.

MATERIALS AND METHODS

The residue analysis methods were liquid chromatography and gas chromatography suitable for the detection of trace levels of drug residues including antibiotics, hormones, anti-inflammatory substances, etc.

High-performance liquid chromatography is one of the most widely used methods for the analysis of chemicals, separating compounds based on their interaction with the mobile and stationary phases. Gas chromatography has been used to separate volatile and semi-volatile compounds,

with samples sometimes needing to be derivatized to increase their volatility.
The samples analysed in the I.I.S.P.V. laboratories were represented by muscle tissue from cattle, pigs, sheep/goats, horses, poultry, rabbits, farmed game; milk, egg and fish samples were also monitored.
Food samples were analysed for antibiotic residues, and the proportion of each antibiotic type present in the studied samples was recorded.
The period analysed in this study is between January 2022 and the end of December 2023.

RESULTS AND DISCUSSIONS

Food residue analysis is done to verify compliance with the regulatory efforts of the Competent Authority with the stated goal of protecting consumers from health risks - antibiotic resistance and allergic reactions.
At the same time, a comparative analysis was also desired compared to the situation in previous years in which the number of samples analysed positive for antibiotics remains at a constant level, quite high in pigs and poultry in 2022.
During 2023, a number of 1605 positive samples were identified in terms of antibiotic content, compared to 1817 samples in the previous year, a decrease of 12% as shown in Table 1.

Table 1. Number of positive samples of products of animal origin, analysed in 2023 compared to 2022

Year	2023	2022
Totally positive evidence	1605	1817
Beef	209	137
Pork	453	584
Seep/Goats	61	79
Horses	11	27
Poultry	700	661
Milk	68	138
Eggs	82	150
Rabits	1	3
Wild Game	2	22
Fish	18	16

In 2023, antibiotic residues were detected in poultry meat in 700 samples, which represents 44% of the food samples on which determinations were made (Figure 1). Among the antibiotics identified in these samples, tetracyclines, penicillins, aminoglycosides, sulphonamides/fluoroquinolones and cephalosporins were found. Compared to 2022, during which 661 samples containing antibiotics were recorded, it can be said that we are dealing with an increase in the incidence of antibiotic contamination from 36% (Figure 3) to 44% (Figure 2).

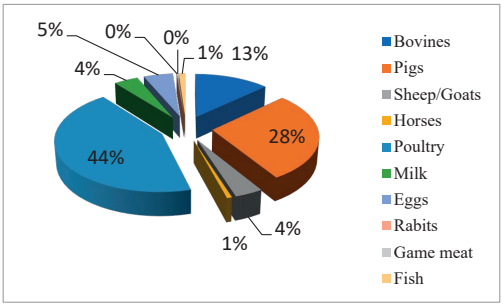

Figure 1. Proportion of samples containing antibiotics detected in animal products in 2023

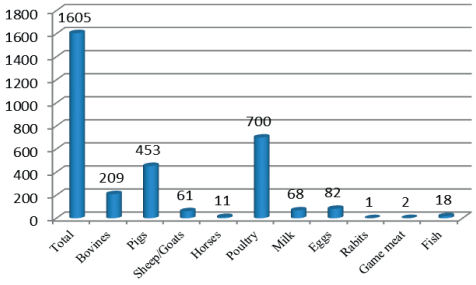

Figure 2. Number of samples containing antibiotics detected in animal products in 2023

This trend of increasing antibiotic residues in poultry meat can be attributed to the use of feed medicated with broad-spectrum antimicrobials and/or failure to comply with the withdrawal/ waiting time before slaughter.
A significant percentage of positive samples was also observed in pork: 28% in 2023 (453 samples), slightly down from 32% (584 samples) in 2022 (Figures 3 and 4).
The analysis of positive samples from poultry meat in 2023 showed the presence of several types of antibiotics in different proportions as follows: tetracyclines 64%, sulphonamides/quinolones 25%, lactamines 8%, aminoglycosides 3% as shown in Figure 6.

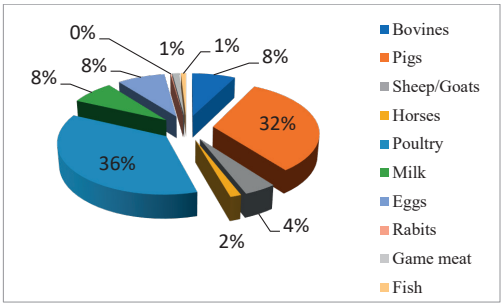

Figure 3. Proportion of samples containing antibiotics detected in animal products in 2022

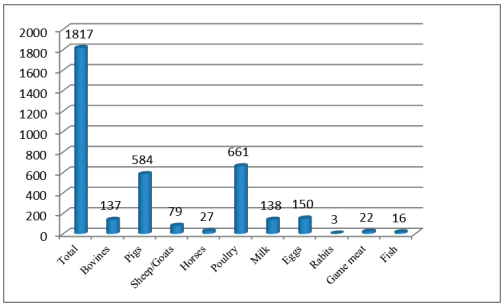

Figure 4. Number of samples containing antibiotics detected in animal products in 2022

In real numbers, out of a total of 700 positive samples, 448 showed traces of tetracycline, 175 sulphonamides/quinolones, 56 lactamine residues and in 21 aminoglycosides were present (Figure 5).

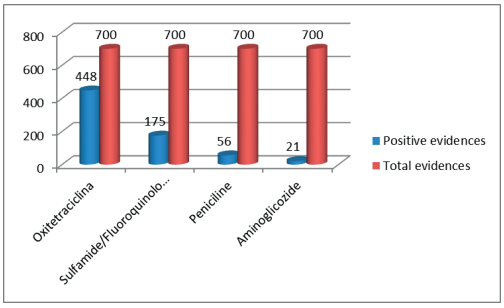

Figure 5. Number of samples containing antibiotics detected in poultry meat in 2023

Regarding the analysis of positive pork samples detected in 2023, the presence of several types of antibiotics in different proportions is observed. Ampicillin was the most frequently detected antibiotic, present in 240 of the 453 positive samples, which represents 53% of the total. Chemotherapeutics were identified in 118 samples. Tetracyclines were present in 95 samples, representing 26% of the total. Overall, 21% of the pork samples contained antibiotic residues and 26% of the total, that is 21% of the samples contain antibiotics (Figures 7 and 8).

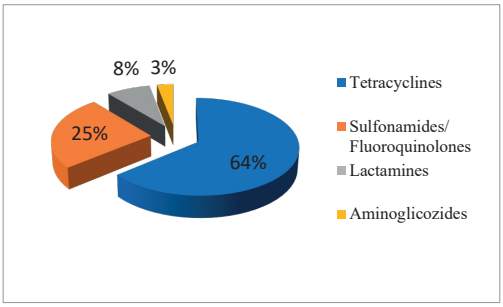

Figure 6. Proportion of different antibiotics present in poultry meat in 2023

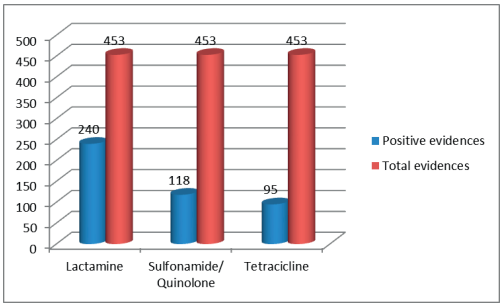

Figure 7. Number of samples containing antibiotics detected in pork meat in 2023

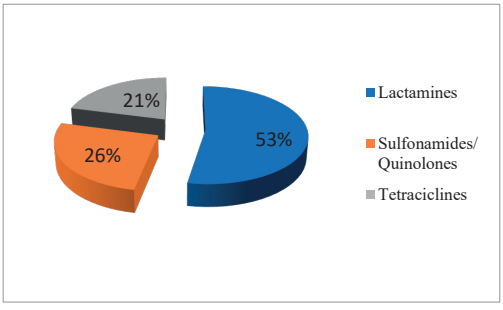

Figure 8. Proportion of different antibiotics present in pork meat in 2023

The food samples analysed in our study show the same trend of persistence of veterinary drug residues in final products intended for public consumption.

Whether they are used as growth stimulants, for prophylactic purposes or to treat diseases

(digestive, respiratory or parasitic) (Crivineanu et al. 2002) the various drugs are still found in the samples analysed by the competent authority as part of the monitoring process.

Whether determined globally or separately, antibiotics occupy the central position. In our case, in birds, tetracyclines were in the first position as in other studies (Yang Y. et al., 2020), along with sulphonamides and quinolones which were also very often identified (Er et al., 2013).

In pigs, the most common drug was ampicillin, probably due to its ease of administration and high degree of bioavailability via the digestive tract, as has also been observed in other studies (Paz-González et al., 2024).

CONCLUSIONS

Oxytetracycline was the most frequently detected antibiotic, present in 64% of the samples analysed.

Poultry samples had the highest proportion of antibiotic residues, with 44% testing positive in 2023, compared to 36% in the previous year, followed by pork samples, with a positivity rate of 28% in 2023, and a slight decrease from 32% in 2022.

Among the detected antibiotics, oxytetracycline was the most prevalent, found in 64% of the positive samples, followed by chemotherapeutics such as sulphonamides and quinolones, present in 25% of cases.

In pork, ampicillin was the most frequently identified antibiotic, detected in 53% of the positive samples.

A significant gap remains between the goal of eliminating drug residues from animal-derived food and the current reality.

Sustained efforts are needed to ensure the presence on the markets of foods free of veterinary drug residues.

Further efforts are required to enhance regulatory compliance, raise awareness among livestock producers, and implement more robust surveillance systems to reduce public health risks associated with veterinary drug residues.

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THE INFLUENCE OF THE PHYSICOCHEMICAL QUALITY OF SOME ASSORTMENTS OF TRADITIONAL CHEESES FROM DÂMBOVIȚA COUNTY DURING A YEAR

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Abstract

The physicochemical quality of traditional cheeses depends on the properties of the raw milk. Key parameters analysed include fat percentage, moisture, and dry matter. This study assesses these parameters in traditional cheeses from Dâmbovița County and tracks their evolution throughout the year. The analysed varieties telemea cheese, burduf cheese, and urda show variations, with fat percentage increasing over time. In cow`s milk telemea cheese, fat rose from 47.43% (June) to 51.87% (November), while in burduf cheese, it increased from 41.28% (January) to 54.10% (November). Sheep`s milk telemea cheese (61.02%) and sheep urda (84.41%) had the highest fat values, influenced by milk origin. Moisture and dry matter fluctuated, with alternating rising and falling trends. Fat percentage was the most influenced by seasonal factors, primarily due to the animal's physiological stage: early lactation, peak production, gestation onset, and late lactation. The findings highlight significant seasonal variations in cheese composition, underlining the importance of monitoring these parameters to ensure product quality.

Key words: *burduf cheese, telemea cheese, physicochemical quality, urda.*

INTRODUCTION

Cheese is a food product based on fermented milk. Cheese making involves a complex process involving interdependent chemical, biochemical, and microbiological changes (Cheung and Mehta, 2015). Cheese is a highly valued food in most human cultures, being present over time in people's daily lives. It is a food rich in nutritional components, being a source of proteins, fatty acids, minerals and vitamins, ranking among the most consumed foods globally due to its biologically active substances (Diana et al., 2014).

The diversity of cheeses available on the market is great, as there have been numerous technological advances in their manufacture and maturation over time (Pereira et al., 2009). The acceptance of an assortment of cheese by consumers depends in particular on its appearance, aroma and texture, which are in turn influenced by a combination of microbiological, biochemical and technological factors that modify the microstructure directly or indirectly (Pereira et al., 2009).

Cheese production in Romania has increased in the last 10 years from 66,290 tons to 96,400 tons, ranking 18th in the EU (Eurostat, 2018). One of the fundamental factors driving this growth was the development of small local factories, new varieties of cheese, and the growing market demand for traditional cheeses (Mureșan et al., 2021).

Artisanal food products are gaining more and more popularity, as they are perceived as local and authentic products (Cirne et al., 2019). These products usually benefit from a remarkable richness of taste, offering valuable organoleptic properties that are appreciated by consumers (Pasquali et al., 2022).

The exceptional quality of traditional cheeses is closely related to the fat content of the milk and its fatty acid profile. Milk fatty acids play an important role in the development of cheese flavours and tastes (Pop et al., 2013). During the ripening process, the breakdown of these fats leads to the formation of volatile compounds, which contribute to the unique taste and aroma characteristics of cheese (Mierlita et al., 2011).

The chemical composition of milk is one of the main factors influencing cheese production. Since milk fat and protein are the main constituents of cheeses, the quality of the product is influenced by their concentrations in

milk (Bojanić Rašović et al., 2013). The chemical composition and physical properties of milk can vary, being influenced by different factors, which are reflected on the properties of the cheese. Variations in the amount and composition of milk are due to genetic factors (55%) or paragenetic factors, the most important of which is nutrition.

Other relevant paragenetic factors include: breed, feed type, lactation stage, age, milking quality, health status, environmental conditions (temperature, humidity, air circulation), mode of maintenance, etc. (Bojanić Rašović et al., 2017). Most cheese varieties can be classified by fat content, water content, and how maturation is achieved (Varnam and Sutherland, 2001). The physicochemical determinations that are the subject of the study are: determination of fat content, determination of moisture content and determination of dry matter content.

Each of these determinations reveals whether the traditional cheeses analysed are of a higher quality or not. For example, determining the percentage of fat is important because fat has numerous functions in cheeses, influencing the firmness, mouthfeel and flavour of cheese. It also contributes to the nutritional properties of cheese, as most cheeses contain significant amounts of fat (Fox et al., 2004).

Dry matter content determination provides information about the total amount of solids in cheeses, which includes fats, proteins, carbohydrates, minerals, and other nutrients. It can be used to evaluate the quality and consistency of cheeses. The water content of cheese is essential for its stability and shelf life.

This study aims to qualitatively analyse the physicochemical properties, especially the percentage of fat, the percentage of moisture and the percentage of dry matter during a year and the influence of the seasons on these determinations.

MATERIALS AND METHODS

The materials used in this study are traditional cheeses (telemea cheese, burduf cheese and urda), which come from local producers, as well as from distribution markets, thus having access to a wide variety of products. It is worth mentioning that the origin of milk is not always known. The samples were collected from batches of varying sizes, depending on the cheese assortment analysed. The batches ranged from about 15 kg, to large lots, about 250 kg.

The samples were collected in Dâmbovița County, Moroieni commune, being a region famous for its traditions in the production of traditional cheeses, but also other artisanal products. This selection of the study area was supported by its reputation, locally, in terms of the quality and diversity of traditional products. The sample collection process started in January 2024 and was completed in November of the same year, thus ensuring relevant coverage of seasonal diversity and possible fluctuations in milk composition throughout the seasons.

The samples collected consisted of the selection of the following assortments within which several varieties are distinguished:

Telemea cheese - comprising a total of 60 samples analysed; this assortment presents the following varieties: cow's milk cheese (19 samples collected from batches between 50 and 100 kg), sheep's milk cheese (10 samples collected from batches between 8 and 30 kg), aged cow's milk cheese (10 samples collected from batches between 83 and 150 kg), aged sheep's milk cheese (5 samples collected from batches of 20 and 47 kg), fresh cheese from cow's milk (10 samples collected from batches between 14 and 20 kg), fresh sheep's milk cheese (7 samples collected from batches between 19 and 27 kg).

Burduf cheese - comprising a total of 60 samples analysed and collected from batches between 20 and 250 kg.

Urda - comprising a total of 40 samples analysed; this assortment presents the following varieties: sweet urda (15 samples collected from batches between 14 and 26 kg), salted urda (5 samples collected from batches between 25 and 34 kg), cow's milk urda (10 samples collected from parrots between 12 and 30 kg), sheep's milk urda (3 samples collected from batches of 13, 14 and 15 kg).

The methods used to determine the fat content of cheeses were two in number, namely:

The Van Gulik method or ISO 3433/2008: it was developed by the Dutch chemist Cornelis van Gulik in the 1930s and is still used today in the food industry. This method is based on the principle of solubility of fats in ethyl ether. The process involves extracting fats from a sample

of cheese using ethyl ether as a solvent. The extracted fats are weighed and expressed as a percentage of the total mass of the cheese. The Van Gulik method is one of the standard methods used in lactic analysis, being recognized for its accuracy and for the fact that it can be widely applied in the cheese industry (ISO 3433, 2008).

SR EN ISO 1211/2010 is an international standard that establishes a method for determining the fat content of dairy products and cheeses. This standard is issued by the International Organization for Standardization (ISO) and is adopted in Romania, under the SR (Romanian Standard) logo. The method involves extracting fat from a sample of dairy product or cheese using an organic solvent, usually ethyl ether or heptane. After extraction, the fats are separated from the solvent, and their weight is determined by evaporation or other drying methods. The fat content is then expressed as a percentage of the total mass of the product. The use of this standard helps to ensure the quality and safety of food, as well as to the protection of consumers' interests (ASRO, 2010).

The method used to determine the dry matter content of cheeses was:

ISO 5534/2004 I standard: is an international standard issued by the International Organization for Standardization (ISO) and refers to the determination of dry matter in milk and dairy products at 102°C by the gravimetric method. This standard describes a method for determining the dry matter content in milk and dairy products by drying samples at 102°C and measuring their weight. This is considered a reference method for determining the dry matter content and is used to ensure the accuracy of the analysed results (ISO 5534, 2004).

The determination of the moisture content was carried out by the extraction method, according to the ISO 5534/2004 I Standard, using the values obtained for the analysis of the dry matter. The moisture calculation was made by difference, subtracting the percentage of dry matter from 100%.

RESULTS AND DISCUSSION

Within the telemea cheese varieties, the values for each parameter analysed are presented in the following table (Table 1). As can be seen, the highest (average) percentage of fat in cheese belongs to the sheep cheese variety (62.56%), a percentage due to the large amount of fat in sheep's milk. The lowest (average) percentage is recorded for fresh cow's cheese (49.86%), a percentage similar to that of the cow's cheese variety (49.79%).

Tabel 1. Physicochemical parameters of the varieties of telemea cheese

Assortment	The physicochemical parameters investigated (average)		
	Fat content (%)	Moisture content (%)	Dry matter content (%)
Cow's milk telemea	49.79	49.58	50.42
Sheep's milk telemea	62.56	48.37	51.63
Aged cow's milk telemea	52.82	48.69	51.31
Aged sheep's milk telemea	60.17	51.02	48.98
Fresh cow's milk telemea	49.86	51.47	48.53
Fresh sheep's milk telemea	58.88	49.27	50.73

The highest values of the average moisture percentage were recorded in the varieties of fresh cow's milk telemea (51.47%) and aged sheep's milk telemea (51.02%). The lowest values were recorded for sheep milk telemea cheese (48.37%). The values of the dry matter are similar, but qualitatively different from those of the percentage of moisture. Radulović et al. (2011) demonstrated by analysing cow cheese that the dry matter values did not exceed 47.14%, which is a relatively low percentage. On the other hand, the values of the fat percentage were on average 60.00%, values significantly higher than those obtained in the present study. Angheloiu et al. (2016) state that matured telemea cheese has a higher amount of dry matter, which is also observed in this study if we compare the fresh and old varieties of telemea cheese where differences of 2-3% can be observed. Teneva-Angelova et al. (2018) state that following research in the Balkan Peninsula, the average moisture percentage of telemea cheese was 52.5%, a value that exceeds the results obtained in the present study. Neagu

et al. (2013) concluded that the average fat percentage was 50.54%, and the dry matter percentage was 44.16%. The distribution by seasons is difficult to interpret considering the multiple varieties of this assortment, however we will present the evolution of the analysed parameters of the cow cheese variety in the following table (Table 2) and schematically average (Figure 1 and Figure 2), because 10 samples belonging to both the summer and autumn seasons were collected.

Tabel 2. The evolution of the physicochemical parameters of the cow's telemea cheese variety

Assortment	Month of the year	The physicochemical parameters investigated (average)		
		Fat content (%)	Moisture content (%)	Dry matter content (%)
Cow's milk telemea	6	47.43	48.91	51.09
Cow's milk telemea	7	48.71	49.51	50.49
Cow's milk telemea	8	49.77	49.38	50.62
Cow's milk telemea	9	50.11	47.93	52.07
Cow's milk telemea	10	50.96	48.98	51.02
Cow's milk telemea	11	51.75	52.57	47.43

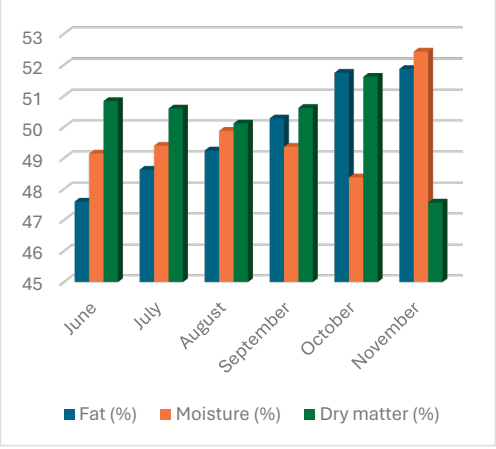

Figure 1. Changes in the physicochemical characteristics of cow's milk telemea cheese

As can be seen in the figures and table presented above, the fat percentage has a continuous increase starting from 47.43% in June and reaching 51.75% in November.

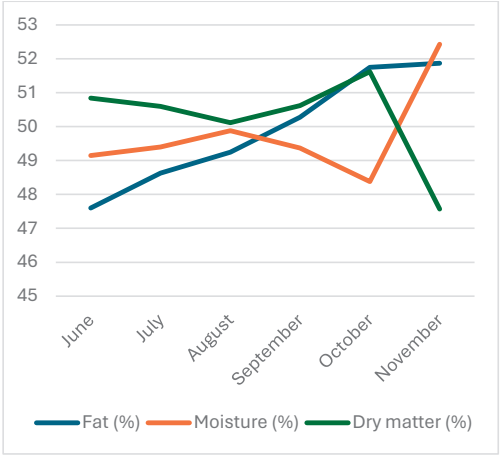

Figure 2. Evolution of the physicochemical properties of cow's milk telemea cheese

These variations are normal considering the physiological stage in which the animals are, namely the end of lactation and the onset of gestation. The other parameters vary from one month to another, with upward and downward trends in the summer months, and at the end of autumn they will be inversely proportional.

The following table (Table 3) shows the variations in the physicochemical parameters of burduf cheese depending on the month in which they were collected and analysed.

As can be seen, the values of physicochemical parameters differ from month to month. The fat percentage is the one that follows the most obvious upward course, having minimum (average) values in January (42.68%) and reaching (average) values of 53.89% in October and November.

In the table, three values appear for each month, representing the average of the samples collected closer to the beginning, middle, or end of the month. The lowest value of fat percentage is recorded in January (41.28%), and the highest value in November (54.10%). The values of the other parameters undergo changes during the year, not being very significant. The average values of moisture percentage and dry matter percentage in January were 47.84% and 51.41%, respectively, compared to 52.3% and 47.70% respectively in the last two months. Variations in fat percentage can also be due to the physiological stage of the animals, but also to the feeding conditions.

Tabel 3. Variations in the parameters of burduf cheese depending on the season

Assortment	Month of the year	The physicochemical parameters investigated		
		Fat content (%)	Moisture content (%)	Dry matter content (%)
Burduf cheese	1	41.25	46.36	53,64
Burduf cheese	1	45.53	46.55	53.45
Burduf cheese	1	41.28	50.62	49.38
Burduf cheese	2	42.79	51.48	48.52
Burduf cheese	2	41.59	50.61	49.39
Burduf cheese	2	41.63	46.47	53.53
Burduf cheese	3	43.47	54.87	45.13
Burduf cheese	3	46.28	52.32	47.68
Burduf cheese	3	47.21	49.95	50.05
Burduf cheese	4	46.28	56.95	43.05
Burduf cheese	4	47.58	55.02	44.98
Burduf cheese	4	48.74	48.63	51.37
Burduf cheese	5	49.85	46.68	53.32
Burduf cheese	5	49.57	48.46	51.54
Burduf cheese	5	49.72	47.14	52.86
Burduf cheese	6	49.33	54.79	45.21
Burduf cheese	6	47.48	45.15	54.85
Burduf cheese	6	48.74	50.17	49.83
Burduf cheese	7	49.42	48.14	51.86
Burduf cheese	7	50.59	49.25	50.75
Burduf cheese	7	51.42	50.14	49.86
Burduf cheese	8	51.64	49.14	50.86
Burduf cheese	8	52.24	55.21	44.79
Burduf cheese	8	52.50	48.48	51.52
Burduf cheese	9	51.63	49.89	50.11
Burduf cheese	9	51.98	48.85	51.15
Burduf cheese	9	52.26	55.78	44.22
Burduf cheese	10	53.78	53.34	46.66
Burduf cheese	10	53.57	53.27	46.73
Burduf cheese	10	53.79	47.32	52.68
Burduf cheese	11	54.01	50.91	49.09
Burduf cheese	11	53.98	52.72	47.28
Burduf cheese	11	54.10	56.24	43.76

Kondyli et al. (2012) state that there are seasonal differences in the amount of fat in milk. Animals towards the end of the lactation period secrete less milk, but with a greater amount of fat.

Pop et al. (2013), state that following an analysis on burduf cheese, values of 19.50% fat and 46.00% dry matter were recorded. Values that are lower than those presented in this study. Neagu et al. (2013) concluded that the average fat percentage was 45.75%, and the average dry matter percentage was 47.62%. For a better visualization of the results, below we will present two graphical representations.

The first figure (Figure 3) represents the average values of the physicochemical parameters analysed in the first half of the year, while the second figure (Figure 4) presents the data for the second half of the year.

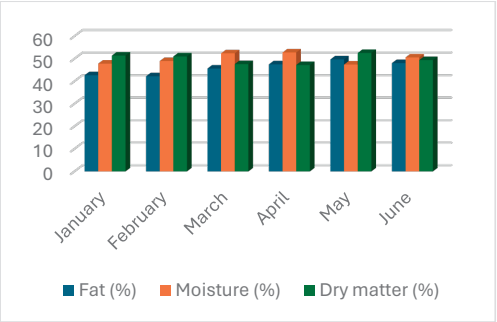

Figure 3. Average physicochemical properties of burduf cheese in the first half of the year

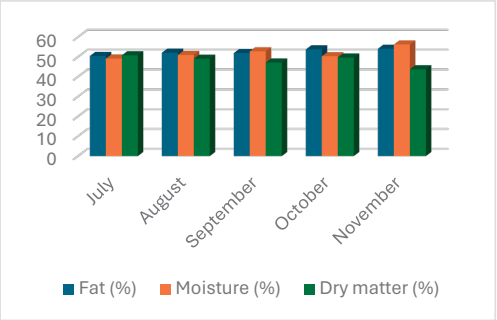

Figure 4. Average physicochemical properties of burduf cheese in the second half of the year

The following graphic representation (Figure 5) represents the variation of the fat percentage during the year, being the parameter at which a significant and continuous increase is observed.

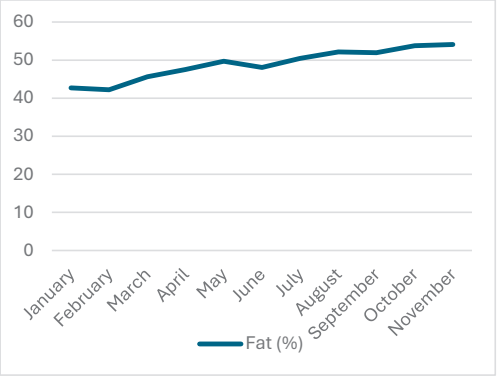

Figure 5. Variations in the fat percentage of burduf cheese throughout the year

The physicochemical properties of urda have been presented (Table 4) as follows. In the following table, four varieties of urda are represented. As can be seen, the sweet urda

variety showed high values of fat percentage (85.56%) in July, representing the highest value recorded. High values of fat percentage were also obtained by the varieties salted urda (84.24%), sheep's milk urda (84.41%) and cow's milk urda (70.10%). The lowest values were recorded for cow's milk urda varieties (52.35% and 55.51%). The percentage of humidity varied during the summer and autumn months, registering the lowest value within the sheep's milk urda assortment (57.11%), and the highest cow's milk urda (71.18%). The percentage of dry matter varied during the study, registering low values within the cow's milk urda assortment (28.82%), and the high values were recorded by the salted urda variety (43.09%). Bojanic Rasovic et al., (2017) state that analysing the samples of urda from Montenegro they concluded that the percentage of fat did not exceed 35.65%, with a percentage of moisture 63.86% and a percentage of dry matter that did not exceed the value of 52.25%. The fat percentage is much lower than in the present study, the other determinations are relatively similar.

Table 4. The average values of the physicochemical properties of urda cheese

Assortment	Month of the year (average)	The physicochemical parameters investigated		
		Fat content (%)	Moisture content (%)	Dry matter content (%)
Sweet urda	5	79.50	66.05	33.95
Sweet urda	6	62.55	60.49	39.51
Sweet urda	7	85.56	59.47	40.53
Salted urda	6	59.06	64.67	35.33
Salted urda	7	60.32	57.05	42.95
Salted urda	8	68.30	56.91	43.09
Salted urda	9	83.82	56.98	43.02
Salted urda	10	84.24	57.97	42.03
Cow's milk urda	8	52.35	60.74	39.26
Cow's milk urda	9	55.51	71.18	28.82
Cow's milk urda	10	58.97	60.39	39.61
Cow's milk urda	11	70.10	66.33	33.67
Sheep's milk urda	10	80.21	57.11	42.89
Sheep's milk urda	11	84.41	58.28	41.72

Pappa et al., (2016) state that the percentage of moisture in the urda variety of sheep's milk was 56.97%, and the percentage of fat was 28.28%, a much lower value than the results obtained in the present study. Snežana Paskaš et al., (2019) determined an average moisture percentage of 79.59% with an average fat percentage of 5.63%.

If we analyse the distribution of the fat percentage according to the season, we can say that a slight but continuous increase is observed, from May to November. Of course, it is difficult to make a statistic due to the multiple varieties of its assortment. Considering the results obtained by other researchers, we can say that the analysed urda varieties meet all the criteria of a superior, nutritious and healthy product from a physicochemical point of view.

CONCLUSIONS

The collection and analysis of the samples took place over a period of 11 months, starting in January 2024 and ending in November 2024. During this period, a total of 160 samples were collected and analysed. The assortments of traditional cheeses analysed, as they were presented, were: telemea cheese, burduf cheese and urda. Within these assortments, several varieties are distinguished, mainly depending on the origin of the raw material and the method of preservation. The study was carried out in Dâmbovița County, Moroieni commune, with the aim of establishing the physicochemical quality of the assortments of traditional cheeses analysed.

The physicochemical quality was established following the determination of the main parameters that attest to the superior quality of cheeses, namely: the percentage of fat, the percentage of moisture and the percentage of dry matter.

Within the telemea cheese assortment, the values of the main physicochemical parameters are closely related to the raw material from which it is obtained. Thus, the values of the fat percentage were reduced in the varieties of cow's milk telemea cheese (49.79%) and fresh cow's milk telemea cheese (49.86%), the highest values were recorded in the varieties of sheep's milk telemea cheese (62.56%) and aged sheep's milk telemea cheese (60.17%). The percentage

of moisture and the percentage of dry matter varied slightly from one variety to another; the lowest values were 48.37% (sheep's milk telemea cheese - percentage of moisture) and 48.98% (aged sheep's milk telemea cheese - percentage of dry matter. The highest values were for the percentage of moisture 51.47% (fresh cow's milk telemea cheese) and for the percentage of dry matter 51.63% (sheep's milk telemea cheese). Separately, the cow's milk telemea cheese was analysed where the trend of increasing the fat percentage over the analysed period was observed, starting from values of 47.43% in June and reaching 51.87% in November.

In burduf cheese, due to the large number of samples analysed and the uniformity of the distribution on lines, the increase in the percentage of fat is the most obvious parameter observed. The lowest value was recorded in January (41.28%), and the highest in November (54.10%), with an increase of 12.82% over the 11 months. The other parameters analysed did not vary much during the year, so the average for the first half of the year was 49.93% moisture percentage and 49.84% dry matter percentage. For the second half of the year, the values were 51.17% for the percentage of humidity and 48.82% for the percentage of dry matter.

Within the urda varieties, an increase in the percentage of fat can be observed from one month to the next. Thus, the sweet urda variety varied between 62.55% and 85.56%, the salted urda variety between 59.06 and 84.24%, the cow's milk urda variety between 52.35% and 58.97%, and the sheep's milk urda variety between 70.10% and 84.41%. In the other determinations, very wide variations were not observed, with slight growth trends followed by slight decreases depending on the parameter.

In conclusion, the results obtained demonstrate that the analysed cheese varieties are of superior quality from a physicochemical perspective and the percentage of fat is most influenced by the succession of seasons, being low in winter and spring, increasing in summer and reaching the highest levels in autumn, in general, due to the physiological stage of the animals, as well as the temperature, grazing conditions, and the quality of green forage.

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PROFESSIONALISM COMPETENCIES IN VETERINARY MEDICINE: A NECESSITY FOR THE FUTURE

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Abstract

The rapid evolution of veterinary medicine and the implementation of the 2023 Standard Operating Procedures (SOP) by EAEVE highlight the importance of integrating professionalism competencies into the education of future veterinarians. These competencies - effective communication, professional ethics, teamwork, and critical thinking - complement technical knowledge and are essential for delivering excellent veterinary education. Professionalism competencies are mandatory as part of the Day One Competences required of veterinarians, ensuring they are prepared to meet professional standards from the very beginning of their careers. They foster adaptable and resilient professionals capable of addressing complex challenges, promoting interdisciplinary collaboration, and adhering to the highest ethical standards. Furthermore, they enhance the quality of veterinary services and strengthen public trust while supporting holistic medical care. To meet the EAEVE 2023 standards, aligning the veterinary curriculum with these competencies is crucial. Investing in their development ensures a sustainable future for veterinary medicine, animal welfare, and the profession's long-term relevance. Continuous improvement of these skills throughout one's career is a professional and social responsibility, ensuring veterinarians remain competitive and effective in a dynamic field.

Key words*: professionalism competencies, veterinary education, EAEVE standards, Day One Competences, interdisciplinary collaboration.*

INTRODUCTION

Veterinary medicine is a complex and multifaceted profession that requires a comprehensive skill set to address the diverse challenges encountered in the care of animals. While technical proficiency in diagnosing, treating, and preventing diseases is crucial, effective veterinary practice also demands a range of interpersonal, ethical, and leadership skills. These competencies, often referred to collectively as professionalism competencies, are increasingly recognized as essential to the practice of veterinary medicine. In the past, professionalism competencies were often referred to by other terms, such as: soft skills, non-technical skills, transversal skills or competences, key skills, core skills or life skills, transversal competencies, employability skills (MENTORTEC et al., 2017).

These terms were used interchangeably to describe the set of competencies that are essential for effective professional behaviour but are not directly related to technical or academic knowledge.

Professionalism encompasses not just the clinical aspects of veterinary care but also the ethical standards, communication strategies, emotional intelligence, teamwork, and leadership abilities that contribute to the overall effectiveness of veterinary professionals.

The foundation of veterinary practice lies in technical expertise - veterinarians must be adept at applying scientific knowledge and clinical skills to diagnose and treat a wide variety of animal conditions. However, as the field continues to evolve, there is a growing recognition that professionalism competencies are equally important. These non-technical competencies are vital for ensuring that veterinarians provide not only effective but also compassionate and ethical care to animals. As such, they play a significant role in enhancing patient care, improving the veterinarian-client relationship, and maintaining public trust in the profession (Bok, 2015; Gordon et al., 2023).

Communication, for instance, is one of the most critical components of professionalism in veterinary medicine. Veterinarians must be able to explain complex medical information clearly

and empathetically to pet owners, facilitating informed decision-making and fostering positive relationships. Empathy and emotional intelligence also enable veterinarians to navigate emotionally charged situations, such as delivering bad news or dealing with the grief of pet owners. These skills help veterinarians create an environment where clients feel heard and supported, contributing to greater client satisfaction and improved adherence to treatment plans (Bok, 2015; Gordon et al., 2023).

Teamwork and leadership are also integral aspects of professionalism in veterinary medicine. Effective teamwork ensures that all members of the veterinary care team - veterinarians, veterinary technicians, nurses, and support staff - work collaboratively toward the common goal of delivering high-quality care. Leadership, on the other hand, is essential for guiding the team, managing complex cases, and making ethical decisions, particularly when working under pressure (Bok, 2015; Gordon et al., 2021).

Ethical decision-making is another critical area of professionalism. Veterinarians regularly face difficult ethical dilemmas, such as determining the best course of action in life-threatening situations, navigating the balance between animal welfare and client resources, or handling end-of-life care decisions. Professionalism competencies help veterinarians navigate these dilemmas while considering the welfare of animals, the needs of their clients, and the broader ethical guidelines of the veterinary profession (Epstein & Hundert, 2002).

This review examines the essential role of professionalism in veterinary medicine, highlighting its increasing importance in veterinary practice. It explores how professionalism competencies - such as communication, empathy, emotional intelligence, teamwork, and ethical decision-making - are integral to maintaining the integrity of the profession and ensuring optimal care for animals. Additionally, this review will address how veterinary education can integrate these competencies into its training programs to better prepare students for the complexities of modern and global veterinary practice. By understanding and emphasizing the importance of professionalism, veterinary schools can help shape well-rounded professionals who can provide not only excellent medical care but also compassionate and ethically responsible service to both animals and their owners.

1. PROFESSIONALISM COMPETENCIES' IMPORTANCE IN VETERINARY MEDICINE

1.1 Defining Professionalism in Veterinary Medicine

Professionalism in veterinary medicine refers to a set of values, behaviours, and attitudes that veterinarians must consistently demonstrate in their practice. While technical skills and clinical expertise are undeniably critical to effective veterinary care, professionalism extends beyond these competencies and encompasses qualities such as integrity, empathy, responsibility, communication, and a commitment to lifelong learning (Epstein & Hundert, 2002). These qualities enable veterinarians to make informed decisions, demonstrate compassion, and collaborate effectively with others, thus promoting the overall well-being of both animals and their owners.

According to Epstein & Hundert (2002), professionalism involves the judicious application of knowledge, technical skills, and emotional intelligence to ensure the welfare of patients and the satisfaction of clients. It is a guiding principle for veterinarians' decision-making processes, clinical practices, and interpersonal interactions, ensuring that the care provided is not only clinically effective but also ethically sound. Professionalism encompasses both the technical aspects of veterinary medicine, such as diagnostic proficiency and treatment, and the non-technical aspects, such as the ethical, communicative, and emotional intelligence skills required to work effectively with animal owners and other healthcare professionals.

Veterinarians must demonstrate professionalism in all aspects of their work - not just in the clinic but also in their interactions with colleagues, clients, and the broader community. The Royal College of Veterinary Surgeons (RCVS, 2012) emphasizes that professionalism should guide not only clinical decisions but also interpersonal interactions and ethical reasoning. This guidance ensures that trust in the veterinary profession is maintained and that veterinary

professionals serve as role models for ethical and responsible behaviour within their communities.

1.2. Impact on Stakeholders

The development of professionalism competencies significantly impacts multiple stakeholders involved in veterinary practice. These stakeholders include the patients (animals), their owners, veterinary professionals, and society as a whole. By examining the effects on each of these groups, it becomes clear that professionalism is fundamental to maintaining high standards in veterinary care and fostering public confidence in the veterinary profession.

1.2.1. Impact on Patients

Professionalism directly influences the quality of veterinary care. Veterinarians who demonstrate integrity and empathy are more likely to provide accurate diagnoses, implement effective treatment plans, and deliver appropriate follow-up care. Numerous studies have shown that professionalism in veterinary practice contributes to improved patient outcomes, reduced error rates, and fewer malpractice claims (Rose, 2021). For example, in high-pressure situations such as emergency care or decisions regarding euthanasia, professionalism enables veterinarians to make ethically sound decisions that prioritize patient welfare while minimizing stress for both the animal and the owner.

In emergencies or when dealing with difficult ethical decisions, professionalism ensures that veterinarians approach situations with a calm demeanour and make decisions that align with the ethical standards of the profession. By adhering to these principles, veterinarians ensure that animals are treated with the dignity and care they deserve, and that treatment plans are designed with both animal welfare and client interests in mind (Florian et al., 2024). This level of professionalism promotes better clinical outcomes and reduces the risk of errors or oversights in patient care.

1.2.2 Impact on Animal Owners

The relationship between veterinarians and animal owners is built on trust, effective communication, and empathy. Clear communication ensures that pet owners understand their pets' diagnoses, the treatment options available, and the associated costs. This understanding increases the likelihood of pet owners adhering to treatment protocols, which, in turn, improves treatment outcomes and enhances client satisfaction (Shaw et al., 2016). In veterinary practice, professionalism means providing clients with information in a way that is both clear and sensitive to the emotional context of the situation, especially when discussing serious diagnoses or difficult decisions such as euthanasia (Pun, 2020).

Veterinarians who demonstrate empathy and emotional intelligence foster strong, collaborative relationships with pet owners. Empathy allows veterinarians to acknowledge the emotional stress and distress that clients often feel, providing them with the support and comfort needed during difficult times (Mustățea et al., 2024). By managing these emotional interactions with professionalism, veterinarians not only improve the client experience but also encourage long-term relationships based on trust and mutual respect (Shaw et al., 2016).

A lack of professionalism, however, can lead to misunderstandings, dissatisfaction, and even legal disputes. Miscommunication, unaddressed concerns, or a lack of empathy can cause clients to feel dissatisfied with the care their animals receive, leading to negative outcomes for both the patient and the veterinary practice. On the other hand, demonstrating professionalism fosters a positive atmosphere that encourages compliance with treatment plans and results in higher client retention rates (Pun, 2020).

1.2.3. Impact on Society

Professionalism in veterinary medicine extends beyond the individual relationships between veterinarians and their patients or clients and plays a critical role in the wider society. Veterinarians contribute to public health by preventing the spread of zoonotic diseases, promoting food safety, and advocating for animal welfare. Their professional integrity ensures that veterinary practices adhere to ethical standards, which contributes to the overall well-being of society. For example, professionalism in the use of antibiotics is crucial for combating the global issue of antimicrobial resistance. Veterinarians who

adhere to ethical guidelines regarding the use of antibiotics in animals help prevent the overuse of these critical medications, thus protecting public health (Mossop, 2012; Mossop & Cobb, 2013).

Furthermore, professionalism enhances the reputation of the veterinary profession and helps to maintain public trust in its practitioners. Ethical decision-making, effective communication, and the consistent demonstration of professional behaviour reinforce the credibility of veterinary medicine in society. When the public trusts veterinarians to act in the best interest of both animals and humans, this trust supports the role of veterinarians in public health initiatives, such as disease prevention and control (Epstein & Hundert, 2002).

Veterinarians also contribute to global health efforts through One Health initiatives, which emphasize the interconnectedness of human, animal, and environmental health. By maintaining high ethical standards and engaging in effective collaboration with other healthcare providers, veterinarians play an essential role in addressing global challenges such as zoonotic disease outbreaks and environmental sustainability. The professionalism displayed by veterinarians in these efforts helps to safeguard both animal and human health, benefiting society as a whole (Mossop & Cobb, 2013).

Professionalism competencies in veterinary medicine are integral to the success of individual practitioners, the satisfaction of clients, and the well-being of patients. These competencies go beyond clinical expertise and encompass ethical decision-making, communication, empathy, and teamwork - skills that ensure veterinarians can navigate the complexities of modern practice. The impact of professionalism extends across various stakeholders: animals' benefit from better care and ethical treatment, owners experience improved satisfaction and trust, and society as a whole gain from the contribution of veterinarians to public health and animal welfare. Therefore, the development of professionalism in veterinary education is essential for preparing veterinarians to meet the demands of the profession and to uphold the integrity and reputation of the veterinary field.

2. CORE PROFESSIONALISM COMPETENCIES IN VETERINARY MEDICINE

Professionalism in veterinary medicine encompasses both technical (hard) and non-technical (soft) competencies. While technical skills are essential to effective veterinary practice, non-technical competencies are equally crucial for providing holistic care and fostering strong relationships with patients, clients, colleagues, and authorities. These non-technical competencies enable veterinarians to navigate the emotional, ethical, and interpersonal challenges inherent in the profession. The following core professionalism competencies are fundamental for veterinarians to ensure that they are not only skilled clinicians but also compassionate, ethical, and collaborative professionals.

2.1. Communication Skills

Communication is one of the most critical competencies for veterinarians, as it directly impacts the quality of care provided to both patients and clients. Effective communication encompasses the ability to convey complex medical information in a manner that is clear, concise, and understandable to clients. Veterinarians must be able to explain diagnoses, treatment options, and prognoses in a way that clients can easily grasp, which ultimately promotes informed decision-making and adherence to treatment protocols (Shaw et al., 2016).

Communication also plays a vital role in managing difficult conversations, such as explaining terminal diagnoses or recommending euthanasia. It is important that veterinarians communicate with sensitivity and empathy in these emotionally charged situations, ensuring that clients feel supported and understood. Effective communication skills are not limited to interactions with clients; they are also crucial for fostering collaboration within the veterinary team. Clear and open communication between colleagues, technicians, and support staff ensures that care is coordinated, patient outcomes are optimized, and misunderstandings are minimized (Armitage-Chan, 2020; McMurray & Boysen, 2017). Communication with authorities requires more than just

empathy; it involves clear, precise, and efficient language that is accessible to all parties, especially when dealing with non-specialist authorities. This ensures that the information is understood correctly, meets the specific requirements of the authorities, and is conveyed with respect, ultimately fostering effective collaboration and decision-making (WHO, 2015).

In addition to verbal communication, non-verbal cues such as body language, tone of voice, and facial expressions also play an essential role in conveying empathy and professionalism. Veterinary schools must integrate communication training into their curricula to ensure that students are equipped to handle a wide range of communicative challenges in practice (EAEVE, 2023; Militaru et al., 2020; van Gelderen Mabin & Taylor, 2023).

2.2. Empathy and Emotional Intelligence

Empathy and emotional intelligence are key components of professionalism in numerous professions, including veterinary medicine. Empathy involves the ability to understand and share the feelings of others, which is essential for building trust with clients and creating a compassionate environment for both animals and their owners. Veterinarians often work with clients in emotionally difficult situations, such as dealing with sick or injured pets, and empathy allows them to offer support while maintaining professionalism (Bell, 2024; Stoewen, 2024).

Emotional intelligence refers to the ability to recognize, understand, and manage one's own emotions as well as the emotions of others. It involves skills such as emotional awareness, self-regulation, motivation, and social skills, all of which are necessary for managing challenging interactions with clients and colleagues. For example, a veterinarian with high emotional intelligence is better able to remain calm and composed in stressful situations, such as performing emergency surgeries or managing difficult client expectations. Additionally, emotional intelligence is critical for maintaining positive working relationships within the veterinary team, as it helps individuals navigate conflicts, manage stress, and provide constructive feedback (Bell, 2024; Laura et al., 2024).

Empathy and emotional intelligence are particularly important in fostering a positive work environment. A veterinarian who demonstrates empathy not only helps clients feel supported but also creates a workplace culture where team members feel valued and understood. The ability to handle difficult emotional situations with professionalism contributes to a healthy and productive work atmosphere, which ultimately benefits both patients and clients.

2.3. Ethical Decision-Making

Ethical decision-making is another cornerstone of professionalism in veterinary medicine. Veterinarians frequently encounter ethical dilemmas, such as whether to recommend euthanasia for a terminally ill animal or how to balance the cost of treatment with the client's financial resources. Ethical decision-making involves carefully weighing the welfare of the animal, the needs and wishes of the client, and the ethical guidelines of the profession (Epstein & Hundert, 2002).

Veterinarians must navigate these complex decisions while maintaining a commitment to animal welfare and ensuring that they act in the best interest of their patients. They must also consider the legal and moral implications of their decisions, taking into account the ethical principles of autonomy, beneficence, non-maleficence, and justice. For example, in situations where an animal's prognosis is poor, a veterinarian must decide whether to pursue aggressive treatment or to recommend euthanasia, all while considering the animal's quality of life, the emotional impact on the owner, and the resources available (Mullan & Main, 2001).

Veterinary schools must teach students how to approach ethical dilemmas in a structured and thoughtful way. This includes exposing students to ethical frameworks, case-based learning, and discussions about the moral implications of veterinary practice. Additionally, professional codes of ethics, such as those provided by the American Veterinary Medical Association (AVMA) and the Royal College of Veterinary Surgeons (RCVS), offer guidelines for navigating ethical challenges and ensuring that decisions align with the profession's ethical standards (AVMA, 2025b; RCVS, 2012).

2.4. Teamwork and Leadership

Veterinary practice is inherently collaborative, with veterinarians frequently working alongside technicians, nurses, office staff, and other healthcare professionals. Effective teamwork is essential to ensure that all members of the veterinary team contribute to patient care and that the care provided is coordinated and efficient. Veterinarians must possess the ability to work well within teams, delegating tasks appropriately, providing support, and fostering a collaborative work environment (Mellanby et al., 2011; Mills, 2019).

In addition to teamwork, leadership skills are crucial for veterinarians who are responsible for managing a clinic or leading a team of veterinary professionals. Veterinarians must be able to guide their teams through complex medical cases, inspire trust and respect, and create a positive workplace culture. Leadership involves the ability to make informed decisions, manage conflicts, and provide direction and motivation to colleagues (Mills, 2019; Nicol, 2024). Leadership is particularly important in emergency situations, where quick decision-making and clear communication are necessary to ensure optimal patient outcomes.

Veterinary schools should provide students with opportunities to develop both teamwork and leadership skills. This can be achieved through group projects, clinical rotations that involve multidisciplinary teams, and mentorship programs that allow students to observe and practice leadership within the veterinary setting. Effective leadership also involves the ability to mentor junior colleagues, fostering professional development and ensuring that team members have the support they need to excel.

The core professionalism competencies in veterinary medicine - communication skills, empathy and emotional intelligence, ethical decision-making, and teamwork and leadership - are essential for ensuring that veterinarians are not only technically proficient but also capable of navigating the interpersonal, emotional, and ethical challenges that arise in practice. These competencies are vital for fostering trust with clients, maintaining a positive work environment, and ensuring that the welfare of animals remains the central focus of veterinary care. Veterinary education must integrate these competencies into the curriculum through case-based learning, mentorship programs, and leadership training, ensuring that students are fully equipped to meet the demands of the profession.

3. STRATEGIES FOR INTEGRATING PROFESSIONALISM INTO VETERINARY EDUCATION

Integrating professionalism into veterinary education is essential to ensure that students graduate with the necessary competencies to handle the challenges of the veterinary profession. Professionalism is not only about technical skills; it encompasses the ability to communicate effectively, make ethical decisions, work collaboratively in teams, and uphold high ethical standards. As such, veterinary education programs must prioritize the development of professionalism through various teaching strategies and assessment methods. Below, we explore several key strategies for integrating professionalism into veterinary curricula.

3.1. Case-Based Learning

Case-based learning is an educational approach that involves students working through real-world scenarios to develop problem-solving skills and apply theoretical knowledge to practical situations. This method is particularly valuable in teaching professionalism because it encourages students to consider the ethical, legal, and interpersonal dimensions of veterinary practice. By using real or simulated case studies, students can explore the complexities of clinical decision-making, client communication, and teamwork, all while considering the impact of their decisions on patients, clients, and society.

Through case-based learning, students are prompted to reflect on the moral and ethical issues inherent in veterinary practice. For example, a case might present a difficult decision regarding euthanasia, where students are required to navigate the emotional and ethical challenges associated with such decisions, all while adhering to professional standards and communicating effectively with clients. This approach fosters critical thinking, ethical reasoning, and the development of

professional behaviour, as students learn to balance technical expertise with compassion and integrity (Gates et al., 2021).

Studies have shown that case-based learning enhances students' ability to analyse complex issues and consider multiple perspectives, which is vital in veterinary medicine, where professionals often face ethical dilemmas and multifaceted challenges (Kaufman & Mann, 2010). Furthermore, case-based learning promotes the development of communication skills, as students must articulate their reasoning, justify their decisions, and collaborate with others in resolving the case.

3.2. Mentorship Programs

Mentorship is a critical strategy for the development of professionalism in veterinary students. By pairing students with experienced veterinarians, mentorship programs provide opportunities for role modelling and professional identity formation. Mentors serve as guides, offering valuable insights into the realities of veterinary practice, including how to navigate ethical challenges, communicate effectively with clients, and manage stress and burnout.

Mentorship is particularly effective in developing professionalism competencies because it allows students to observe and engage with professionals who exemplify the standards of behaviour and ethics they are expected to uphold. Mentors provide personalized feedback and guidance, helping students reflect on their own practice and identify areas for improvement. In addition to technical expertise, mentors can offer advice on handling difficult client interactions, managing conflicts, and maintaining a professional demeanour in challenging situations (Eby et al., 2008). Through these interactions, students develop a deeper understanding of what it means to be a professional and how to navigate the complexities of veterinary practice.

Furthermore, mentorship programs allow students to explore the human aspects of veterinary care, including emotional intelligence and empathy, which are essential for building strong relationships with clients and colleagues. By witnessing the behaviour of professional role models, students learn not only clinical skills but also how to embody professionalism in their daily practice (Gates et al., 2021).

3.3. Simulation-Based Training

Simulation-based training is another effective strategy for integrating professionalism into veterinary education. This approach involves role-playing exercises and simulated scenarios where students practice their communication, ethical decision-making, and teamwork skills in a controlled, supportive environment. Simulation-based training offers a safe space for students to experience real-world situations without the risks associated with live practice.

For example, veterinary students can engage in role-playing exercises where they must communicate with clients about difficult decisions, such as treatment options for terminally ill animals or dealing with emotional owners after an accident. These exercises allow students to practice empathy, active listening, and clear communication, while also considering the ethical implications of their decisions (Issenberg et al., 2005). Simulation-based training can also be used to teach conflict resolution and teamwork skills, helping students work collaboratively in high-pressure situations, such as during emergency care.

The use of simulation enhances students' preparedness for real-world practice by reinforcing professionalism in situations that may be emotionally challenging or morally complex. By allowing students to repeatedly practice these skills in a low-risk environment, simulation-based training builds confidence, reduces anxiety, and enhances their ability to handle such situations when they arise in real practice (Kaufman & Mann, 2010).

Moreover, simulation exercises provide an opportunity for feedback, allowing students to assess their communication and decision-making skills and identify areas for improvement. These exercises also help instructors assess professionalism competencies in a structured and standardized way, offering a consistent evaluation framework for all students (Gates et al., 2021).

3.4. Structured Assessments

Structured assessments are a critical tool for evaluating professionalism competencies in veterinary education. Unlike technical

knowledge, which can be assessed through exams and clinical skills evaluations, professionalism competencies require a more nuanced approach to assessment. Tools such as Objective Structured Clinical Examinations (OSCEs) and multi-source feedback mechanisms have been widely adopted to provide structured evaluations of students' professionalism.

Objective Structured Clinical Examinations (OSCEs): OSCEs are a well-established method for assessing clinical skills, but they can also be adapted to assess professionalism. In OSCEs, students rotate through a series of stations where they must demonstrate their abilities in various clinical scenarios, including those that require professional behavior such as communicating with clients or handling ethical dilemmas. Each station is designed to evaluate specific competencies, such as communication, decision-making, and emotional intelligence, allowing for a comprehensive assessment of professionalism in practice. OSCEs offer a standardized approach to evaluating professionalism, ensuring that all students are assessed on the same criteria, regardless of their clinical rotations or instructors (Norcini & Burch, 2007).

Multi-Source Feedback: Multi-source feedback, also known as 360-degree feedback, is another effective assessment tool. It involves gathering feedback from multiple sources – such as peers, mentors, faculty, and clients – regarding a student's professionalism. This approach provides a more holistic view of a student's professional behaviour, capturing different perspectives and providing valuable insights into areas for improvement. Multi-source feedback is particularly effective in assessing interpersonal competencies like communication and teamwork, which may not be adequately captured through traditional exams or OSCEs (Eby et al., 2008).

These structured assessments provide students with clear, actionable feedback on their professionalism competencies and allow educators to identify students who may need additional support or guidance. By using consistent, evidence-based assessment tools, veterinary schools can ensure that professionalism is being effectively taught and evaluated, and that students are developing the necessary skills to be successful in their future careers.

Integrating professionalism into veterinary education is a multifaceted process that requires the use of diverse strategies to ensure that students develop the full range of competencies needed for effective practice. Case-based learning, mentorship programs, simulation-based training, and structured assessments all contribute to fostering professionalism in veterinary students. These strategies not only teach technical skills but also promote critical thinking, ethical decision-making, and emotional intelligence, which are essential for navigating the complexities of veterinary practice (Kleinsorgen et al., 2021; MENTORTEC et al., 2017).

As veterinary education continues to evolve, it is crucial that professionalism remains a central component of the curriculum. By adopting innovative teaching strategies and assessment tools, veterinary schools can better prepare students to meet the ethical, interpersonal, and technical challenges of modern practice (EAEVE, 2023).

4. CHALLENGES IN INTEGRATING PROFESSIONALISM INTO VETERINARY CURRICULA

Integrating professionalism into veterinary curricula presents multiple challenges that can hinder the effective development of professionalism competencies in veterinary students. While technical competencies such as diagnostic skills, medical knowledge, and surgical techniques can be clearly defined, assessed, and integrated into educational programs, professionalism competencies present unique difficulties due to their subjective nature. These challenges range from a lack of consensus on how professionalism should be defined and measured to logistical constraints, such as the dense nature of veterinary curricula and the resources required to deliver comprehensive professionalism education. Addressing these challenges is crucial to ensuring that future veterinarians possess both the technical and interpersonal skills necessary to meet the complex demands of modern practice.

4.1 Lack of Consensus on What Constitutes Professionalism

One of the fundamental challenges in integrating professionalism into veterinary education is the lack of a universally accepted definition of professionalism. Professionalism encompasses a broad range of attributes, including ethical decision-making, communication skills, emotional intelligence, empathy, teamwork, and cultural sensitivity. However, what exactly constitutes professionalism can vary depending on cultural context, institutional priorities, and personal perspectives. This lack of consensus complicates the process of designing curricula that adequately address all facets of professionalism, and it makes it difficult for accreditation bodies, educators, and students to agree on the core competencies that should be developed (Vreuls et al., 2022).

Unlike technical competencies, which can be evaluated through concrete assessments such as exams, practical demonstrations, and clinical performance, professionalism is inherently more subjective. Attributes such as empathy, communication, and ethical decision-making are not easily quantifiable. While behavioural assessments, peer reviews, and self-reflection exercises are often used to gauge professionalism, these methods are less standardized and can vary widely in their effectiveness (Eby et al., 2008). The subjective nature of professionalism makes it harder to establish clear benchmarks for student progress and success, posing a significant barrier to consistent integration into veterinary curricula.

4.2. Assessment Challenges

In addition to difficulties in defining professionalism, assessing these competencies presents a considerable challenge. Traditional forms of assessment, such as written exams and clinical skills evaluations, are designed to measure technical knowledge and clinical abilities. However, professionalism competencies cannot be easily measured with objective tests. Some schools have adopted tools such as Objective Structured Clinical Examinations (OSCEs), multi-source feedback, and portfolios to assess professionalism. These tools, while effective, still rely heavily on subjective interpretation and can be influenced by factors such as individual biases, varying levels of student engagement, and inconsistent feedback from assessors (Gordon et al., 2023).

Moreover, the effectiveness of these assessment tools can vary significantly across different educational contexts. For example, in large veterinary schools, OSCEs may be logistically challenging and resource-intensive to implement. Furthermore, there is no universally agreed-upon methodology for assessing professionalism across veterinary programs globally. While some institutions may incorporate structured assessments, others may rely on informal observations, making it difficult to ensure consistency and comparability across educational systems (Armitage-Chan & Whiting, 2016).

4.3. Resource Constraints

Another significant barrier to integrating professionalism into veterinary curricula is the lack of resources. Developing and implementing a comprehensive professionalism curriculum requires investment in teaching materials, faculty development, and assessment tools. Veterinary schools with limited funding or staff may struggle to integrate these elements into their existing programs. Moreover, faculty members may not always possess the necessary expertise in teaching and assessing professionalism, particularly in areas such as emotional intelligence or cultural competence. While many veterinary faculty members are experts in clinical and technical areas, they may not have received formal training in pedagogy or the teaching of soft skills, which makes it more difficult to effectively integrate professionalism into their courses (Gates et al., 2021).

Additionally, the relatively high cost and time investment associated with the development and delivery of professionalism education can deter veterinary schools from prioritizing it. For example, mentorship programs, which are an effective way to instil professionalism in students, require faculty time and commitment, making them resource-intensive (Niehoff et al., 2005). Schools that are already facing challenges in meeting the demands of their clinical and technical curricula may find it difficult to allocate sufficient time and resources to the teaching of professionalism.

4.4. Balancing Technical Training and Professionalism

Veterinary curricula are often densely packed with technical subjects, from anatomy and pharmacology to clinical skills training. As a result, finding space for the development of professionalism competencies within an already crowded curriculum can be challenging. In many veterinary schools, the primary focus remains on technical training, which is essential for ensuring that students are proficient in diagnosing and treating animal diseases. While these technical skills are critical, they are insufficient by themselves for the well-rounded veterinarian.

Professionalism competencies are often seen as secondary or supplementary to technical education, and there may be a perception that focusing too much on professionalism detracts from the essential clinical training. However, this view can undermine the importance of developing interpersonal skills, ethical reasoning, and communication abilities. In order to meet the expectations of the veterinary profession and the broader society, veterinary educators must find a way to balance the teaching of technical skills with the cultivation of professionalism competencies (Gordon et al., 2021). This requires innovative curriculum design that integrates professionalism into both clinical and preclinical training, ensuring that it is taught not only as a stand-alone subject but also in the context of real-world veterinary practice.

4.5. Time Constraints and Student Readiness

Time constraints represent another challenge in the integration of professionalism into veterinary curricula. Veterinary students often face intense workloads, with long hours spent on clinical rotations, labs, and exams. The demands of the program may leave little time for students to reflect on or engage with professionalism in a meaningful way. While many veterinary schools offer elective courses in professionalism or related topics, these may be overlooked or deprioritized by students who feel they must focus on core technical subjects to meet academic requirements and prepare for exams (EAEVE, 2023; Pohl et al., 2021).

Moreover, students may not always be ready to engage with professionalism competencies, particularly if they view these skills as less important than technical knowledge. Early in their education, students may not fully appreciate the significance of professionalism, and they may not recognize how these skills will impact their careers. As a result, students may be less motivated to develop these competencies unless they are convinced of their importance and relevance to their future practice (Kaufman & Mann, 2010).

The integration of professionalism into veterinary curricula presents a range of challenges, from the lack of consensus on its definition and assessment to the constraints of time, resources, and curriculum design. However, these challenges are not insurmountable. By refining the assessment tools used to evaluate professionalism, investing in faculty development, and designing curricula that integrate professionalism into all aspects of veterinary training, veterinary schools can overcome these barriers. It is essential that professionalism education becomes an integral part of veterinary training to ensure that future veterinarians are equipped with the full spectrum of skills - technical, ethical, and interpersonal - needed for effective practice (EAEVE, 2023; Kleinsorgen et al., 2021).

5. THE ROLE OF ACCREDITATION BODIES IN PROMOTING PROFESSIONALISM

Accreditation bodies are central to ensuring that veterinary education programs meet specific standards of excellence, not only in technical education but also in the integration of professionalism competencies. Organizations like the European Association of Establishments for Veterinary Education (EAEVE) and the American Veterinary Medical Association (AVMA) provide accreditation guidelines that emphasize the importance of professionalism as a core component of veterinary curricula (AVMA, 2021; EAEVE, 2023). These bodies set the expectations for veterinary schools worldwide, ensuring that their graduates are well-rounded professionals capable of addressing the ethical, interpersonal, and technical challenges they will face in practice.

5.1. Accreditation as a Framework for Professionalism

Accreditation serves as a vital mechanism for ensuring that veterinary programs provide high-quality education, and its role extends beyond technical skills. Both the EAEVE and AVMA require that veterinary programs include professionalism competencies as part of their curriculum in order to achieve accreditation. The inclusion of these competencies reflects the recognition that veterinarians must possess not only strong technical skills but also the ethical, deontological, and interpersonal qualities necessary for success in the profession. By mandating professionalism as part of their accreditation criteria, these organizations underscore the importance of ethical conduct, communication, and teamwork in veterinary practice (AVMA, 2021; EAEVE, 2023).

For example, EAEVE's accreditation standards outline that veterinary programs must integrate professionalism competencies throughout the curriculum, including aspects such as communication, teamwork, ethical reasoning, and patient advocacy. These competencies are assessed through both direct observation in clinical settings and formal examinations (EAEVE, 2023). The AVMA similarly emphasizes the development of core professional attributes such as empathy, ethical decision-making, and emotional intelligence in their accreditation standards for veterinary schools in the United States (AVMA, 2021).

5.2. Ensuring Competency-Based Education and Continuous Improvement

Accreditation bodies not only provide a framework for initial education but also promote a continuous cycle of improvement within veterinary schools. By setting clear expectations for professionalism, accreditation bodies encourage schools to regularly evaluate and update their curricula to align with evolving standards and societal needs (EAEVE, 2023; Mossop & Cobb, 2013). This includes the integration of new methodologies in teaching and assessment, such as the use of simulation-based learning, reflective practice, and mentorship programs that foster the development of professionalism in students (Armitage-Chan & Whiting, 2016).

One of the key aspects of the accreditation process is the emphasis on competency-based education. This approach ensures that graduates are not only exposed to theoretical knowledge but also to practical experiences where they can demonstrate their professionalism competencies. Accreditation bodies require veterinary schools to implement systematic assessment processes, such as Objective Structured Clinical Examinations (OSCEs) and multi-source feedback, to evaluate the professionalism of students in real-world settings (Eby et al., 2008; Gordon et al., 2023). These assessments measure students' abilities in areas such as patient communication, ethical decision-making, and teamwork, which are essential to their future practice as veterinarians.

5.3. Aligning with International Standards and Best Practices

The role of accreditation bodies extends to ensuring that veterinary education aligns with global standards and best practices. For instance, the EAEVE collaborates with the World Health Organization (WHO) and the World Organisation for Animal Health (OIE) to ensure that veterinary education programs meet international expectations for competency and professionalism. This collaboration ensures that veterinary professionals are equipped to contribute to global health issues, including zoonotic disease control, food security, and animal welfare (EAEVE, 2023).

The AVMA also participates in international discussions on veterinary education, influencing global standards through its involvement in international veterinary organizations such as the Federation of Veterinarians of Europe (FVE) and the World Veterinary Association (WVA). Through these partnerships, the AVMA helps shape a shared understanding of what constitutes professionalism in veterinary medicine, facilitating the exchange of best practices and fostering mutual recognition of veterinary qualifications (AVMA, 2021).

5.4. Fostering Public Trust and Professional Integrity

One of the primary goals of accreditation is to uphold the reputation of the veterinary profession by ensuring that graduates possess the necessary competencies to meet the expectations of society. Professionalism, in this

context, is critical to maintaining public trust in veterinary services. Accreditation bodies hold veterinary schools accountable for producing graduates who not only excel in technical skills but also act with integrity, compassion, and ethical responsibility in their daily practice.

Professionalism competencies, such as the ability to communicate effectively with clients, demonstrate empathy, and make ethical decisions, are crucial for fostering trust and building strong relationships between veterinarians and their clients. For example, the AVMA's guidelines on professionalism stress the importance of veterinarians being advocates for animal welfare, acting in the best interest of their patients, and engaging in transparent communication with clients (AVMA, 2021). Accreditation bodies thus serve as gatekeepers, ensuring that veterinarians adhere to high standards of professionalism that benefit both animals and society.

5.5. Impact of Accreditation on Curriculum Design and Pedagogical Approaches

Accreditation bodies influence the design and delivery of veterinary curricula by ensuring that professionalism is not relegated to isolated courses but is woven throughout the educational experience. Both the AVMA and EAEVE advocate for the integration of professionalism training into all aspects of the veterinary curriculum, from preclinical courses to clinical rotations. This approach ensures that students develop professionalism competencies gradually and continuously throughout their training, rather than in a piecemeal fashion.

Moreover, accreditation encourages veterinary schools to adopt a range of pedagogical methods to teach professionalism. These include experiential learning, where students engage in real-world scenarios, as well as reflective practice, which helps students critically evaluate their own professional behaviour and development. Research has shown that these methods are effective in fostering the development of professionalism competencies in veterinary students, preparing them to handle the complexities and challenges of veterinary practice (Ho & Lau, 2025).

5.6. Challenges in Implementing Professionalism Standards

While accreditation bodies have made significant strides in promoting professionalism, challenges remain in implementing these standards across veterinary education programs. One challenge is the variability in the resources and commitment of veterinary schools to meet these standards. Schools with fewer resources may struggle to implement innovative teaching methods, such as simulation-based learning or mentorship programs, which are critical for teaching professionalism.

Furthermore, there is ongoing debate about the best ways to assess professionalism competencies. While tools like OSCEs and multi-source feedback are widely used, there is no universal agreement on the most effective methods for evaluating attributes like empathy, emotional intelligence, and ethical decision-making. As a result, accreditation bodies must continue to refine their assessment methods to ensure that professionalism is accurately measured and adequately taught (AVMA, 2025a; Kareskoski, 2023).

Accreditation bodies such as the EAEVE and AVMA play a pivotal role in shaping the future of veterinary medicine by ensuring that professionalism competencies are embedded in veterinary education (AVMA, 2021; EAEVE, 2023). Through their rigorous accreditation standards, these organizations help to ensure that veterinary schools produce graduates who are not only technically competent but also ethically responsible, empathetic, and capable of working effectively within interdisciplinary teams. By continuously evolving accreditation standards and promoting best practices in education, these bodies contribute to the development of a highly skilled, professional, and ethical veterinary workforce that can meet the complex challenges of modern practice.

CONCLUSIONS

Professionalism competencies are not merely supplementary to veterinary training—they are fundamental to the success and credibility of the profession. While technical knowledge remains the foundation of veterinary medicine, communication, ethical reasoning, empathy, and teamwork are equally crucial in delivering high-quality patient care and maintaining trust with clients and society.

Veterinary education must emphasize the continuous development of professionalism by integrating real-world case studies, mentorship

programs, and structured assessments into curricula. By prioritizing professionalism alongside clinical expertise, veterinary schools can develop diverse systems to ensure that students receive comprehensive training in these competencies. Emphasis should also be placed on the continuous professional development of faculty members to enhance their ability to teach soft skills effectively, fostering the necessary environment for students to navigate ethical dilemmas, improve client relationships, and collaborate within multidisciplinary teams.

Future research should focus on identifying best practices for assessing professionalism in veterinary students and examining its long-term impact on clinical effectiveness, veterinarian well-being, and client satisfaction. Additionally, accreditation bodies such as EAEVE and AVMA must continue to refine their standards, ensuring that professionalism remains a core component of veterinary education worldwide.

By embedding professionalism competencies into veterinary training, the profession will continue to evolve, ensuring that veterinarians remain not only skilled veterinarians but also ethical, compassionate, and respected members of the healthcare community.

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A SURVEY-BASED EVALUATION OF THE IMPACT OF VARIOUS TEACHING METHODS ON DAY-ONE COMPETENCIES IN VETERINARY ANAESTHESIA

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Abstract

For veterinary medicine, day one competencies such as performing first aid in emergencies and safely administering general anaesthesia demand both theoretical understanding and practical expertise. Endotracheal intubation has been identified as a challenging clinical skill for students. This study aimed to assess veterinary students' confidence levels in performing endotracheal intubation in anaesthesia, document their previous experiences with the procedure, and describe their emotional states during the training. Students completed a survey evaluating the usefulness of high-fidelity models and cadavers in acquiring intubation skills. The training curriculum progressed from lectures and non-animal practice to hands-on work with high-fidelity models and cadavers, culminating in performing anaesthesia on dogs and cats in a university clinic. The feedback related to the necessary practical teaching training is crucial for tailoring the teaching process to ensure students develop the required competence before progressing to the next stage of training on live animals.

Key words*: competencies, anaesthesia, intubation, students.*

INTRODUCTION

The principles guiding the use of animals in research and education are rooted in the three Rs: reduction, replacement, and refinement. In veterinary medical training, these principles have significantly decreased the frequency of multiple-survival surgeries. Instead, students are increasingly practicing on cadavers and different models.

Key anaesthesia skills, recognized as essential for entry-level veterinarians, include general anaesthesia induction, endotracheal intubation, aesthetic depth assessment, pain evaluation, and expertise in local aesthetic techniques.

Ethical and financial concerns have been raised about using live animals solely for practicing these skills (Burzumato et al., 2020).

In response, significant advancements have been made in recent years to develop non-animal teaching models, with numerous innovations highlighting this progress (Gee et. al., 2025).

Utilizing skill lab mannequins and cadavers is essential for developing day-one competencies in veterinary anaesthesiology. The present study aimed to determine to what extent third-year students, who take both theoretical and practical courses in veterinary anaesthesiology, acquire the necessary day-one competencies by the end of their studies. Additionally, it sought to identify the most effective methods for achieving the desired outcomes.

MATERIALS AND METHODS

A total of 190 undergraduate veterinary students from the same academic year (third-year were invited to complete a series of two surveys over a 4-month period, corresponding to one teaching semester, beginning in February 2024. The first survey was conducted at the start of the second semester of the third year, coinciding with the anaesthesia theory course. The second survey was conducted at the conclusion of the teaching period, covering both theoretical and practical components. Of the 190 students, 141 participated, representing 74.21% of the third-year students in the Romanian section of the veterinary medicine program, University of Agronomic Sciences and Veterinary Medicine of Bucharest.

RESULTS AND DISCUSSIONS

The demographic profile of the responding students showed that 83.3% were women, 14.9% were men, and 1.8% identified as another gender. The participants' ages were distributed as follows: 66.7% were aged 20-22, 26.3% were aged 22-25, and 7% were over 25 years old. The evaluation of students' educational potential based on their prior academic performance revealed that the majority (83.3%) demonstrated moderate achievement, with general averages between 7 and 9. A smaller proportion (12.3%) showed weaker performance with averages below 6, and only a minority (4.4%) excelled with averages between 9 and 10. These findings highlight the diverse academic backgrounds within the cohort and suggest the need for tailored teaching strategies to address varying levels of preparedness, ensuring all students have the opportunity to build on their existing knowledge and achieve competency in the subject matter. Given that this group of students experienced disruptions to their education during the COVID-19 pandemic, when restrictions overlapped with their first year of university. the study also investigated how their learning experiences were affected (figure 1). Analysis revealed that 85.35% of students faced significant challenges due to pandemic-related measures, and 17.5% reported they were still working to recover from the impacts on their education similar with other data for veterinary medicine profession (Muresan et al., 2020).

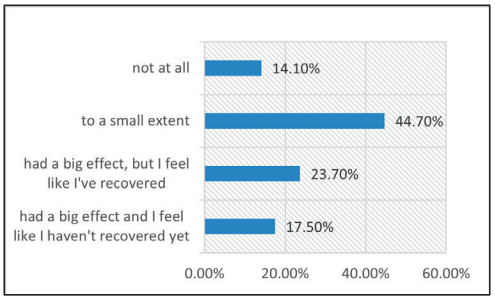

Figure 1. Do you think that in the context of the measures taken during the COVID19 pandemic, you're learning experience in previous semesters was affected?

These findings opened the way for further research to assess the competencies of students who did not experience disruptions to their education during the COVID-19 period. To explore students' perspectives, we asked about their ability to understand the anaesthesiology course material with or without prior foundational knowledge. Specifically, students were asked, to what extent they believe the concepts presented in the anaesthesiology course can be understood without a solid level of knowledge from previous semesters, acquired in anatomy, physiology, and physiopathology? Only 28.1% of respondents (Figure 2) believed that no prior knowledge was necessary. This highlights the positive impact of the teaching approach, which includes a review of concepts from previous years at the start of each course, helping to bridge any gaps in understanding.

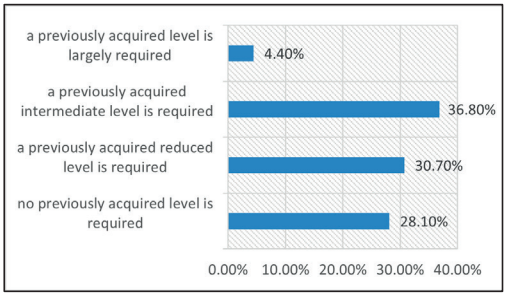

Figure 2. To what extent do you consider that the notions presented in the anaesthesiology course can be understood without having a good level of knowledge acquired in the previous semesters?

Students were also questioned about their practical experience prior to the anaesthesiology course, particularly regarding endotracheal intubation skills. The majority (77.2%) reported having no prior practical experience. Before beginning the course, we assessed students' confidence levels in their theoretical understanding of endotracheal intubation. Results showed that only 22.1% felt confident, while 78.9% expressed low or no confidence in their knowledge.

In the next phase of the questionnaire, we assessed students' practical experience by comparing two methods of teaching endotracheal intubation: using cadavers and a plastic canine model. Students were asked for their opinions on the use of cadavers in anaesthesia training (Figure 3). Before beginning the practical module with cadavers, 16.7% of students expressed feelings of fear, mistrust, or compassion, while the remaining

83.3% reported feeling confident and eager to practice intubation on cadavers.

These findings highlight the varied emotional responses students have towards cadaver-based training, emphasizing the importance of addressing concerns to ensure a supportive learning environment while maintaining the effectiveness of this teaching method.

After completing the practical sessions on intubation using cadavers and a plastic canine model, students were asked to compare the two methods and indicate which they found more effective for developing intubation skills in domestic animals (Figure 4).

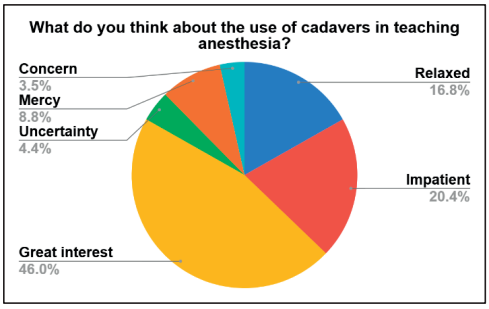

Figure 3. The use of cadavers in anaesthesia training

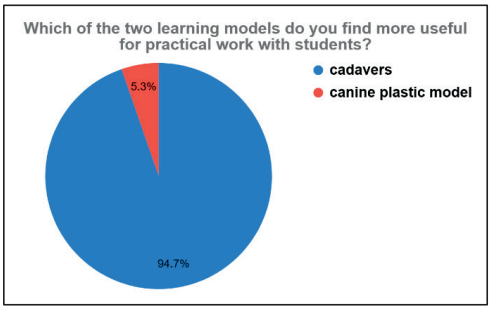

Figure 4. Comparing the two methods for developing intubation skills in domestic animals

The majority of students favoured cadaver-based practice as the more useful method for acquiring intubation competency, with only 5.3% preferring the plastic canine model. This preference may correlate with the small percentage of students who expressed feelings of compassion or discomfort when using cadavers in the educational process.

After completing two hours of practical training on cadavers and plastic models, students were asked to suggest how many hours they felt were necessary to become competent in endotracheal intubation (Figure 5). A majority, 57.5%, believed the allocated time was sufficient to acquire the skill, while 23% indicated they needed more practice time. Following the completion of the entire practical training, students were asked if they felt prepared to perform endotracheal intubation on live animals. Only 6.2% reported feeling unprepared (Figure 6). Notably, this percentage aligns closely with the group that felt more than four hours of training was necessary, highlighting a strong correlation between training duration and confidence in working with live animals.

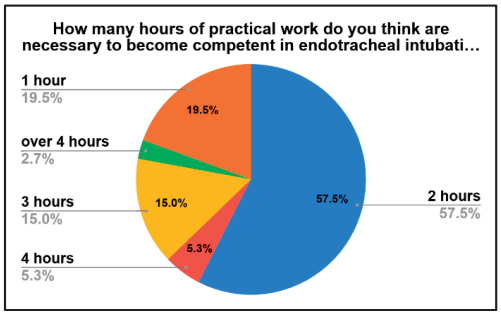

Figure 5. Hours necessary to become competent in endotracheal intubation

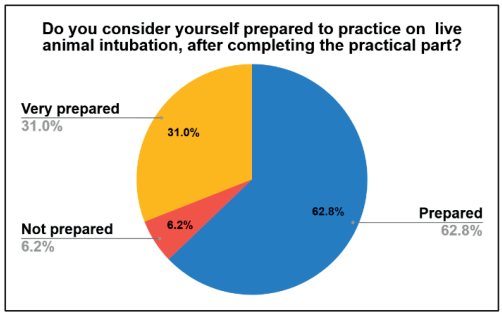

Figure 6. The level of confidence following the completion of the entire practical part

At the beginning of the teaching period, 22.1% of students reported being confident in their theoretical knowledge of endotracheal intubation, while 78.9% felt only slightly confident or not confident at all.

After completing the teaching period, with both theoretical and practical components of the anaesthesiology course, the percentage of confident students rose to 64.6%, with 3.5% feeling slightly confident and 31.9% still not

confident. Although the proportion of confident students tripled following the training, 35.4% indicated that they needed additional preparation to feel fully confident.

The final confidence level for theoretical knowledge (64.6%) closely aligns with the confidence in practical performance (62.8%), emphasizing the complementary role of both theoretical and practical teaching methods. This small difference highlights the need for an integrated approach, where both aspects are equally prioritized to ensure the effective transfer of knowledge and the acquisition of competency (Musk et al., 2017).

It is important to distinguish between acquiring a skill and performing it with professional confidence (Varner et al., 2021).

The analysis highlighted the significant impact of pandemic-related disruptions on students' education, suggesting the need for continued support and adjustments in the educational process to help students fully recover and succeed. The strategy to incorporate a review of foundational concepts from previous years at the beginning of each course plays a crucial role in bridging knowledge gaps and ensuring that students are better equipped to support their learning process.

CONCLUSIONS

The varied academic backgrounds within the cohort indicated the necessity for customized teaching strategies, in order to ensure that all students can build on their prior knowledge and develop the required day one competency in the subject.

While cadaver-based training is generally regarded as more effective (94.7%), addressing emotional concerns for many students (54%) remains essential to optimize their learning experience.

The majority of students (77.2%) lacked prior practical experience with endotracheal intubation, and this was reflected in their initial confidence levels. The close alignment between the final confidence levels for theoretical knowledge and practical performance underscores the interdependence of these two components in skill acquisition, 62.8% declaring that they feel prepared to practice on live animals. This emphasizes the importance of an integrated teaching approach, where theoretical instruction and practical training complement each other to reinforce understanding and competency.

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PATHOLOGICAL AND MOLECULAR INSIGHTS OF ANIMAL MELANOMA CELL CULTURES - A COMPREHENSIVE REVIEW

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Abstract

Melanoma is a particular neoplasm arising from the unregulated proliferation of melanocytes. It affects both humans and different animal species that develop this tumour spontaneously, including canines, equines, and rarely felines. Understanding the pathology and molecular biology of melanoma can be enhanced by studying both two-dimensional and three-dimensional melanoma cell cultures from various species, some animals (e.g. dogs) sharing multiple physiopathological mechanisms with human melanoma. Moreover, established mouse melanoma cell lines from genetically modified models are commonly used for molecular characterization and utility in therapeutic testing, while noting limitations posed by genetic differences. Recent studies regarding isolation protocols for cultivating neoplastic melanocyte cultures show a significant variation including fine-needle aspiration, tissue excision, and enzymatic digestion. Hence, comparative genetic analyses indicate similarities between animal and human melanoma cells, especially regarding mutations in the BRAF and NRAS oncogenes. This review highlights the relevance of melanoma cell cultures across species as significant in vitro models for advanced cross-species melanoma research, enhancing insights into neoplastic initiation and progression and ultimately contributing to improved diagnostic and therapeutic approaches in veterinary and human pathology and oncology.

Key words*: melanoma, melanocytes, cell cultures, oncogenes, animal models.*

INTRODUCTION

Melanoma is a particularly aggressive type of cancer that arises from the uncontrolled growth and neoplastic transformation of melanocytes, which are cells that originate in the basal layer of the epidermis and derive from multipotent neural crest cells (melanoblasts) (Horak et al., 2019; Sforna et al., 2021). Although melanocytes are also naturally present in various parts of the body, such as the iris and inner ear, cutaneous malignant melanoma (CMM) remains the most commonly encountered form in human oncology (Aktary et al., 2023). Among various types of melanomas, mucosal ones are relatively rare in humans, but still considered a highly metastatic and aggressive variant of this neoplasm (Segaoula et al., 2018). Moreover, despite its rarity, uveal melanoma represents one of the most prevalent intraocular tumours in both human and veterinary oncology (Uner et al., 2022). Thus, the development of predictive animal models and cell cultures that accurately mimic the pathogenesis and molecular alterations associated with various types of melanomas presents substantial challenges for further genetic and even clinic investigations.

Melanoma also affects various animal species, including dogs (~7% of malignant pathologies, ~30% of oral tumours), horses, and, to a lesser extent, cats (less than 1%), frequently arising spontaneously (Polton et al., 2024; He at al., 2024; Cristian et al., 2023; Hrițcu et al., 2023; Aktary et al., 2019). This phenomenon of interspecies occurrence presents a distinctive opportunity for the comparative study of melanoma, facilitating the development of improved treatment options for both human and animals.

Melanomagenesis, the transformation of melanocytes into melanoma cells, which usually begins with the benign proliferation of melanocytes, leading to the formation of a nevus, where melanocytes cluster and lose contact with surrounding keratinocytes (Aktary et al., 2019; Horak et al, 2019). These nevus cells may subsequently stop proliferation and enter senescence; or bypass senescence and start

actively proliferation and basement membrane invasion (Gao et al., 2024). This process involves alterations in key cellular functions, including proliferation, immortalization, epithelial-mesenchymal transition, and migration, as well as changes in molecular signalling pathways, cell cycle regulation, and adhesion (Aktary et al., 2019). Activation of tyrosine kinase receptors such as KIT, MET, and RET by their respective ligands (SCF - Stem Cell Factor, HGF - Hepatocyte Growth Factor, and GDNF - Glial cell line-derived neutrophic factor) can trigger various signalling pathways (e.g., ERK1/2 MAP-kinase - Extracellular signal-regulated kinase 1/2, mitogen-activated protein kinase; PI3/PTEN-AKT - Phosphoinositide 3-kinase/Phosphatase and Tensin homolog/Protein kinase B; WNT/β-catenin) that play critical roles in melanoma development, both *in vivo* and *in vitro* (Aktary et al., 2019; Sforna et al., 2021).

In recent years, considerable progress has been made in the establishment and characterization of melanoma cell lines, which have become essential for functional assays, molecular characterization, and therapeutic testing. Among the various models used in melanoma research, mouse melanoma cell lines have gained particular prominence due to their genetic similarity to human melanomas and the ease with which they can be genetically modified. However, it is crucial to acknowledge the limitations that arise from the genetic differences between species (Aktary et al., 2023; Uner at al., 2022; Kuzu et al., 2015).

Thus, the role of animal models in melanoma research is no longer limited to murine species; it consists of a bigger spectrum of animals which naturally develop melanomas. Notably, canine melanoma, especially the mucosal oral and acral variants, exhibits multiple shared molecular and pathological mechanisms with human melanoma, including genetic alterations in key oncogenes such as BRAF, NRAS, PTEN, and KIT and involving the same signalling pathways as MAPK, PI3K and WNT (Gao et al., 2024; Uner et al., 2022; Nishiya et al., 2016).

Melanoma cell cultures allow researchers to thoroughly analyse the pathways involved in tumoural progression by providing controlled environments for studying both the molecular and cellular levels of melanoma (He at al., 2024; Marconi et al., 2018). The development of both two-dimensional (2D) and three-dimensional (3D) cell cultures has been noted, the 3D models allowing more relevant representations by mimicking the tumour *in vivo* architecture. Furthermore, different methods have been utilized in recent studies for isolating neoplastic melanocytes, including tissue excision, fine-needle aspiration, and enzymatic digestion (Lo-Giudice et al., 2024; Correa et al., 2009). Therefore, the establishment of permanent animal melanoma cell lines has represented an important advancement in melanoma research, enabling in-depth functional further assays.

This review offers a thorough examination of the pathological and molecular insights derived from mammalian melanoma cell cultures, underscoring their translational significance for both veterinary and human melanoma research.

MATERIALS AND METHODS

An extensive bibliographic study was conducted to select scientific articles from the Web of Science, Scopus, Science Direct and PubMed databases, focusing on key words such as “animal melanoma cell cultures”, “canine melanoma cell cultures”, “murine melanoma cell cultures”, “equine melanoma cell cultures”, “feline melanoma cell cultures”, “swine melanoma cell cultures”, “melanoma molecular biology”, “melanoma pathology” and “comparative oncology”. A noticeable increase in publications related to these topics, particularly in studies conducted on murine, canine and equine models, has been observed since the early 2000s to the present year (2025), highlighting a growing interest among researchers from various fields. This increasing trend emphasizes the importance of mammalian permanent melanoma cell cultures in advancing the understanding of melanoma biology, tumour microenvironment, and further developing of new therapeutic strategies.

RESULTS AND DISCUSSIONS

In the last decades, significant efforts have been invested in the development of suitable animal models for the study of various types of melanomas. Thus, murine melanoma cell lines, such as B16-F10 and B16-LS9, derived from

genetically engineered models, are widely utilized nowadays in melanoma research (for both cutaneous and uveal melanomas), due to their genetic similarity to human melanoma, enabling investigations into tumour progression, metastasis, and the tumour microenvironment (Olbryt et al., 2006; Aktary et al., 2019). Their usage has previously advanced the investigation of the dysregulated PI3K/AKT and MAPK signalling pathways, which play particular roles in melanoma development, being also encountered in the pathogenesis of human melanoma (Richards et al., 2020).

In contrast with human sporadic melanoma, in murine melanoma lines, genetic studies have revealed that no BRAF (v-Raf murine sarcoma viral oncogene homolog B) mutation was involved (Melnikova et al., 2004). However, mutations of the MAPK pathway (BRAF V600E, HRAS - Harvey Rat Sarcoma Viral Oncogene Homolog, NRAS - Neuroblastoma RAS Viral Oncogene Homolog) can be generated in murine melanocytes through genetic engineering (Aktary et al., 2023).

Regarding canine melanoma, dogs serve as an important model for studying melanoma due to the natural spontaneous incidence of this neoplasm in dogs. Mucosal melanomas are recognized for being particularly aggressive and for their tendency for early metastasis (Gao et al., 2020; Pérez-Santana et al., 2024). Additionally, ocular melanoma is the most common primary intraocular tumour encountered in this species, sharing multiple molecular characteristics with human uveal melanoma, although in dogs these melanomas usually arise in the anterior uvea site, while in humans they commonly develop in the choroid (Polton et al., 2024; Singh et al., 2014; Nishiya et al., 2016).

Canine and human melanoma cell lines share mutations in key carcinogenic pathways, including the MAPK (mitogen-activated protein kinase; 43% of canine mucosal melanoma) and PI3K/AKT/mTOR (phosphoinositide 3-kinase/protein kinase B) signalling cascades, which play crucial roles in tumour proliferation and progression (He et al., 2024; Aktary et al., 2019). In addition, both species have comparable sensitivity in cell cultures to therapeutic inhibitors (e.g. rapamycin and AZD6244) targeting these previously mentioned carcinogenic pathways (van der Weyden et al., 2016). Mutational activation of the proto-oncogene BRAF, NRAS, KRAS (Kirsten Rat Sarcoma Viral Oncogene Homolog), PTEN, and KIT has been identified in both species, although with varying frequencies, being more common in sun-damaged skin melanomas in humans (Dobrin & Militaru, 2023; van der Weyden et al., 2020). In contrast, oncogene BRAF V600E mutation is frequently encountered in human cutaneous melanoma due to the mutagenesis produced by ultraviolet irradiation, but it is less common in canine melanoma (~6%). However, canine melanoma often exhibits NRAS and KIT mutations, which are also found in certain subtypes of non-UV induced human melanoma, particularly mucosal and acral melanomas (Gao et al., 2024).

The establishment of two canine oral melanoma cell cultures, Ocr_OCMM1X and Ocr_OCMM2X, has been obtained from surgically excised melanocytic lesions localized within the oral cavity (Segaoula et al., 2018). Furthermore, a mucosal melanoma cell line, COMM6605, has been developed from a fresh tumoral tissue sample obtained from a male Bichon Frise dog. This process has employed enzymatic digestion techniques, specifically utilizing type IV collagenase within the cellular medium to facilitate tissue breakdown (Li et al., 2024).

In an additional study involving five canine melanoma cell cultures, two of which were derived from cutaneous melanomas and other two were mucosal melanomas, has showed that enzymatic digestion is preferred for obtaining a larger number of cells (Sforna et al., 2021).

Regarding collection techniques, fine-needle aspiration (FNA) was also utilized in some oral melanoma cases, where samples has been obtained from primary tumours and/or lymph nodes metastasis (Lo Giudice et al., 2024). While FNA technique provides a minimally invasive method for cell collection, it still presents limitations regarding collecting an adequate cell number. However, direct fine-needle aspiration from the inner part of the tumour mitigated has helped in obtaining cleaner samples, lowering the bacterial contamination. Additionally, three-dimensional (3D) cell cultures obtained with FNA have been established to better replicate the tumour

microenvironment and enhance the study of cellular interactions *in vitro* in the future. Spheroids, which are fundamental 3D clusters created by aggregating cells of a single type, have been successfully developed from canine oral melanoma metastases, forming aggregates of round to polygonal neoplastic cells, while organoids, which consists of multiple types of cells and present self-differentiation capabilities that enable them to develop structures resembling tissues and organs, have been established from both primary melanomas and metastases (Lo Giudice et al., 2024; Lee et al., 2023).

In feline population, melanoma is an uncommonly encountered tumour, representing less than 1%, the most frequently reported variant being the ocular melanoma which commonly affects the uveal tract and the iris (Polton et al., 2024). Unfortunately, the only notable characterisation studies of feline melanoma cell cultures have been conducted in 1983 by Rasheed S. and later, in 2002, by Mayr B. and his collaborators.

As for translational relevance, feline *in vivo* models for uveal melanoma have been developed by inoculating a strain of FeSV (Feline Sarcoma Virus) in the iris, obtaining tumours in 90% of the inoculated cats, illustrating atypia of melanocytes in the uveal site (Uner et al., 2022).

Previous molecular studies of feline uveal melanoma showed that the most commonly oncogenes mutations known in human ocular melanomas (BRAF V600E, GNAQ - Guanine Nucleotide-Binding Protein Alpha-Q, NRAS, KIT, MEK1 - Mitogen-activated protein kinase kinase 1) are rarely encountered in feline melanoma samples, only one case of feline cutaneous melanoma with mutation of BRAF oncogene being described (Kuroki et al., 2024; Kayes & Blacklock, 2022).

Equine melanomas can spontaneously occur, being distinguished 5 types of melanocytic tumours in this species: melanocytoma, dermal melanoma, dermal melanomatosis, anaplastic malignant melanoma and benign cutaneous/mucosal melanoma (Dobrin et al., 2024; Aktary et al., 2019; Chapman et al., 2009). While human melanoma is commonly associated with UV irradiation and unfrequently genetic mutations, equine melanomas, most encountered in aging grey-coated horses, are commonly strongly linked to a genetic alteration in the STX17 gene (which encodes for *syntaxin 17*) (Seltenhammer et al., 2014). It has been previously noted that this mutation implies further activation of the ERK1/2 MAPK pathway, pointing out the importance of this signalling pathway in melanoma development regardless the species (Pimenta et al., 2023; van der Weyden et al., 2020).

One study revealed that in three equine melanoma cell lines (MelDuWi, eRGO1, eRGO6) mutant BRAF oncogene was detected, along with one encountered mutation of KRAS proto-oncogene in MelDuWi cell line (Gao et al., 2024). Additionally, such as in human and canine melanoma, in 2019, Wong. K. et al. demonstrated through next generation sequencing on equine melanomas the presence of mutations in the same oncogenes (NRAS, TP53 - tumour protein p53, KIT, BRAF) that could activate the ERK pathways implicated in melanomagenesis. Hence, the same signalling cascade and mutations of proto-oncogenes are involved in both human, canine and equine melanomas.

Regarding the protocols for obtaining equine melanoma cell lines, more studies have made notable advancements. One study revealed that for establishing a routine method for isolation of primary horse melanoma cells, surgically excised tissues from 13 grey horses with melanomas were used (Chapman et al., 2009). Their approach involved mechanically mincing the tissues with a scalpel blade and subsequently subjecting the obtained pieces through enzymatic digestion with collagenase. Afterwards, the resulting melanoma cell suspension has been filtered through nylon cell strainers to further separate the cells. In order to confirm the melanoma origin of the obtained equine cell cultures, these researchers have proven the presence of melanoma-associated antigens (Melan-A, MAGE-1 - melanoma-associated antigen 1, MAGE-3 - melanoma-associated antigen 3) (Chapman et al., 2009). Other conducted studies on this topic revealed that the process of trimming the epidermis and the surrounding connective and adipose tissue is favourable in order to obtain pure melanoma cells in the culture (Metzger et al., 2007).

Briefly, most protocols for obtaining equine melanoma cell cultures use enzymatic digestion, being also mentioned mixed solutions with collagenase, hyaluronidase and pronase.
Nevertheless, in 2014, Seltenhammer and her collaborators established for the first time both a primary (HoMel-L1) and a metastatic equine melanoma (HoMeL-A1) cell line only by mechanical mincing the excised tissues and washing the obtained pieces several times in phosphate-buffered saline solution to remove the excessive melanin pigment.
In swine pathology, spontaneous melanoma is rarely encountered, yet it has been occasionally observed in pigmented pig breeds (e.g. Duroc) (Horak et al., 2019).
Due to skin depigmentation in their lifetime and the presence of more actively cytotoxic CD8+ T lymphocytes in tumour microenvironment, more than 85% of swine melanomas can spontaneously regress (Horak et al., 2019).
Additional studies demonstrated that cutaneous melanoma in dark skin miniature breed pigs (e.g. Sinclair, Munich Miniature Swine Troll) have similar histopathological characteristics with human tumours (Aktary et al., 2019; Green et al., 1992).
A pig model for studying hereditary melanoma has been developed in Czech Republic, known as Melanoma-Bearing Libechov minipig (Horak et al., 2019). Further genetical studies on this pig model has demonstrated overexpression of MC1R (melanocortin 1 receptor), RACK1 (receptor for activated C kinase 1), MITF (microphthalmia-associated transcription factor) and KIT genes which has been also found to be implied in human melanoma development.
Although swine melanoma cells might represent a valuable model for conducting studies about tumour immune response, due their numerous physiological similarities within species and their tendency of undergoing spontaneous regression, pig melanoma cell lines have been lastly mentioned in articles from 1979 and 1992 (Green et al., 1992; Berkelhammer et al., 1979).
The most encountered variants of melanomas in animals, along with their molecular characteristics and translational potential, are summarized in Table 1. These similarities between animal variants and human melanoma promote future research, highlighting the potential use of animal models in translational studies and the further development and establishment of more animal melanoma cell cultures.

Table 1. Comparative molecular features of principal melanoma variants across species

Species	Variant	Spontaneous occurrence	Genetic mutations	Translational potential for further research
Murine (B16)	Cutaneous Uveal	Yes (genetically engineered)	Induced BRAF, NRAS, HRAS	Drug testing Progression and tumour microenvironment
Canine	Oral/mucosal Acral Uveal Cutaneous	Yes	BRAF, NRAS, KRAS, PTEN, KIT	Further therapy improvement (particularly for mucosal, acral and uveal variants)
Feline	Uveal Cutaneous	Yes	BRAF (rare)	Progression and therapy of uveal melanoma
Equine	Dermal	Yes	NRAS, TP53, KIT, BRAF, KRAS	Therapy improvement
Swine	Cutaneous	Yes, and spontaneous regression	MC1R, RACK1, MITF, KIT	Tumour microenvironment and immune response

CONCLUSIONS

Variants of animal melanoma cell cultures have proven significant contribution for understanding the complexity of pathological and molecular insights of melanoma biology. Thus, these in vitro models facilitated the understanding of signalling pathways (e.g. MAPK/Akt), principal proto-oncogenes mutations and their inhibitors involved in melanomagenesis. Additionally, cross-species studies of melanoma tumours have been improving diagnostic tests and therapeutic strategies in both veterinary and human oncology.

While murine cells remain pivotal for preclinical testing, they do have limitation regarding genetic particularities. Therefore, canine, equine and swine *in vitro* models can enhance the further development of targeted therapies involving the tumour microenvironment. Moreover, swine melanoma spontaneous regression can provide supplementary insights regarding the tumour microenvironment and immune response that can be later translated into specific immunotherapy for all species.

The present review also highlights that feline melanoma is not yet extensively studied, further research is needed in order to better understand the molecular characteristics of melanoma in this species and to develop permanent feline melanoma cell lines.

Lastly, standardization of culturing protocols and further development of three-dimensional cultures and organoids can contribute effectively in advancing studies of tumour microenvironment composition, molecular changes and pathological insights involved in melanoma evolution.

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RESEARCH ON CERTAIN INSURANCES WITH APPLICABILITY IN THE FIELDS OF VETERINARY MEDICINE AND AGRICULTURE

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Abstract

The paper presents a review of several types of insurance applicable to general fields but also specifically for veterinary medicine and agriculture. At the same time, some mathematical calculation formulas for life insurance, single premium and pension insurance are presented. Finally, two reports issued by Financial Supervisory Authority for the years 2022, 2023 are presented regarding the subscriptions related to optional home insurance, respectively the number of contracts in force

Key words*: insurances, veterinary medicine, agriculture, life, diseases.*

INTRODUCTION

The insurance represents a classic form of risk transfer from the insured to specialized companies, called insurers.
Insurance has been around since ancient times.
Insurance began to appear when people became aware of the risks to which their activity, goods, health or even their lives could be subjected. The main threats in those days were represented by extreme weather phenomena, natural catastrophes or diseases.
The Code of Hammurabi (1750 BC) included the first regulations regarding insurance activity, especially as a result of the development of maritime transport. In that code it was regulated how merchants could be exempted from repaying credits if their goods were stolen during transport. Merchants could pay creditors a certain amount of money. The amount of money they paid then corresponds to the insurance premium today.
The loss of harvests as a result of pests, the death of animals, fire were the main risks to which the Romanian household was subjected till the year of 1744.
The first insurance company in Romania appeared in 1744 in Brașov and was called 'Casa de Incendiu'. In 1906, the Agricola Company was established, which was oriented towards agricultural insurance. The Autonomous Directorate of State Insurance appeared in 1942. This company had a diversified portfolio of all types of insurance. The State Insurance Administration (ADAS) was the only insurance company in the period 1952-1990.
After the Revolution of 1989, the insurance market in Romania diversified, both in terms of number of companies and types of insurance products existing in the companies' portfolios. (Ciurel et al., 2022)

MATERIALS AND METHODS

We have to look at the insurance activity from several perspectives: the legal perspective, the financial perspective and the economic perspective.
Classification criteria and categories of insurance are given by the object and the legal form of realization.
According to the object of the insurance:
- property insurance (buildings and other constructions, household goods, fixed or circulating assets, vehicles, agricultural crops, animals, etc.);
- personal insurance (death, temporary or permanent loss of work capacity, reaching a certain age). Personal insurance can be: life insurance or accident/health insurance;
- third-party liability insurance - the insurer undertakes to compensate material damages or bodily injuries that the insured has caused to third parties. The object of these insurances is

legal and/or professional civil liability.
According to the legal form of realization:
- compulsory insurances (by the effect of the law) - the relations between the insured and the insurer, as well as the rights and obligations of each party, are established by law, for example: car liability insurance, home insurance against natural disasters;
- optional insurances - are the insurances in which the terms of the insurance contract are established based on the agreement of will between the insurer and the insured/contractor.
Insurances are divided into - general insurance and life insurance.
We consider that the following insurance classes are of particular importance in the veterinary medical field:
Class 1. Accidents, including accidents at work and occupational diseases;
Class 2. Health;
Class 3. Land vehicles, excluding railway rolling stock, covering damage or loss related to: motor vehicles, other vehicles;
Class 4. Railway rolling stock covers damages or losses related to it;
Class 5. Aircraft cover damages or losses related to them;
Class 6. Maritime, lake, and river vessels cover damages or losses related to them;
Class 7. Goods in transit, regardless of the mode of transport, which cover damage or losses related to merchandise, luggage, and other goods;
Class 8. Fire and natural calamities, which covers damage or loss to property, other than those mentioned in classes 3-7, caused by: fire, explosion, storm or other natural calamities, nuclear power, subsidence and other landslides;
Class 9: Other damages or losses related to goods other than those mentioned in classes 3-7, caused by: hail, frost, theft, other events not covered by class 8;
Class 10. Motor civil liability, for the use of land motor vehicles, including the liability of the carrier;
Class 11. Civil liability for aircraft insurance, including the liability of the carrier;
Class 12. Civil liability for the insurance of maritime, lake, and river vessels, including the liability of the carrier;
Class 13. General civil liability, exclusively those mentioned in classes 10-12;
Class 14. Credit assurances;
Class 15. Guarantees assurances;
Class 16. Various financial losses related to unemployment, unstable weather conditions, failure to achieve benefits, current expenses, unforeseen commercial expenses, depreciation of market value, rents and other income, other indirect commercial losses, other financial losses;
Class 17. Legal protection: expenses related to judicial procedures and other judicial expenses;
Class 18. Assistance available for people in difficulty during travel or absence from home or usual residence.
Regarding personal insurance, the contract composition theorem can be applied:
If A is a life insurance composed of the partial insurances A_1, A_2....A_n and P_1, P_2....P_n are the premiums corresponding to these partial insurances for the same insured, then the value of the total premium for A is:

$$P = P_1 + P_2 + ... P_n$$

Sum assured in case of survival at the end of the insurance term.
Calculation of the single premium:

$$P = M(X)$$
$$X: \begin{pmatrix} v^n & 0 \\ np^x & nq^x \end{pmatrix}$$

Retirement insurance (Popescu et al., 1999):

$$P_x = Sa_x = S\frac{N_x}{D_X}$$

Insurance class 9: animal insurance, represents a form of insurance through which animals can be insured, belonging to natural and legal persons; animals received for use by legal entities and belonging to other legal entities, as well as animals received for breeding or fattening by natural or legal entities, based on contracts concluded with legal entities.
The following categories of animals can be included in the insurance, grouped according to the criteria of species, quality classes, age, exploitation and destinations: cattle and buffaloes; pigs for breeding; fattening pigs; young pigs; sows with piglets until weaning; draft horses; thoroughbred horses; purebred dogs; bee families/hives; fish from farms and fish farms.
In general, insurance companies grant compensation for damages directly resulting

from the occurrence of the following risks:
Accidents occurring inside the shelter:
- acute flatulence of animals due to feed; dystocia; internal injuries caused by swallowing objects (except reticulitis or traumatic pericarditis injuries); sudden hitting or injury; attack by wild animals, including poisoning from snake bites and venomous insect stings; injury or death caused both directly and as a result of snake bites and venomous insect stings. Injury or death caused both directly and as a result of the destruction (collapse) of the shelter, as a result of natural events or phenomena: fire (including the release of smoke, gas or vapours, as a result of the fire); explosion; lightning; crashes, falling bodies (aircraft parts, meteorites, building construction elements); accidental damage to the installations and/or technical equipment related to shelters; earthquake, flood, storm, hail; extreme natural phenomena (tornado, hurricane); weight of ice and/or snow layer, snow avalanche; landslides and/or landslides.
Accidents occurring outside the shelter:
- sudden hitting or injury; attack by wild animals, including poisoning from snake bites and venomous insect stings; road accidents; the drowning; the effects of temperature factors (sunstroke, frostbite).
Most insurance companies do not cover damage caused, produced or aggravated by, or as a consequence of:
- war, invasion, action of an external enemy; nuclear reaction or radiation or radioactive contamination; pollution, leakage or contamination of any nature; pyrotechnic devices and materials, ammunition, explosive devices and firearms; parasitic diseases for which treatments have not been carried out; diseases already existing at the conclusion of the policy; theft under any conditions and in any form; empirical treatments, unauthorized or carried out by unqualified persons; events occurring during transport; lack of fodder, contraindicated or defective fodder or insufficient in quantity or quality; not notifying the veterinarian/competent authorities in time/ if the symptoms were obvious and/or not carrying out the treatments recommended by the doctor.
Agricultural crop insurance provides coverage for a wide range of risks that can cause material damage related to agricultural crops, vegetable crops, fruit of vines and trees. Agricultural crop insurance can be concluded for any of the following plant categories: cereal crops, vegetable crops in the field, seed crops and hybridization lots, legumes for grains, aromatic and medicinal plants, annual and perennial fodder plants, industrial, oleaginous or textile plants, the fruit of vines, fruit trees and hops, fruit shrubs, rootstocks for orchards and vineyards, established vineyards with grafted vines, protected crops in greenhouses, solariums and nurseries.
In general, crop insurance provides protection against the following insured risks:
- torrential rains, which can lead to: washing the soil around the plants, uprooting the plants, breaking the flower shoots, breaking or trampling the plants (at an advanced stage of vegetation), without further recovery, covering the plants with mud from the slopes, shaking the fruits, seeds;
- storm, gale, hurricane or tornado having the following effects: breaking stems, shoots, flowers, bunches of vines; fruit fall, shaking of seeds, covering of young plants with floods of earth or sand, plant debris, etc.;
- hail that can cause: destroying the vegetative apparatus, breaking the plants, shaking the seeds.
The main exclusions, present in the case of most agricultural insurances, are: pastures and natural hay; grasses sown for fertilization or grazing; decorative plants (flowers, bushes, trees, meadows, etc.); agricultural crops under protective plants; orchards of fruit trees and vines; the species or varieties of cultivated plants considered unsuitable to the area where they are cultivated; agricultural crops not sprouting or with inadequate sprouting due to the quality of seeds or planting material, poor germination, the quality of fertilizers or pesticides used; agricultural crops already affected by climate factors; agricultural crops located at distances less than 500 m from river beds.
General/professional civil liability insurance - for doctors, pharmacists and other persons in the field of medical assistance - Law 95/2006.
Professional liability insurance is a type of mandatory insurance that provides protection for material and financial damages caused by professionals to third parties, beneficiaries of

their services. According to the legislation, a number of professions require the conclusion of civil liability insurance against clients in case of: error, mistake, omission or negligence, situations resulting from service contracts. Among the professions for which there is a legal obligation to rent such insurance are: doctors and medical personnel; architects, builders; lawyers, notaries, accounting experts, auditors, secondary intermediary persons; software developers, managers or business administrators.

In the case of doctors, errors in the prescription of a treatment, negligence in consultations, unsuccessful surgical interventions are covered.

Liability insurance for dangerous dogs and other obligations imposed on owners

Dangerous dog liability insurance is an essential tool for pet owners that can cover damage caused by them.

By Emergency Ordinance no. 55 of April 30 2002, regarding the possession of dangerous or aggressive dogs, republished, the obligation of the owners of animals belonging to category I of dangerous dogs to conclude a civil liability insurance for animals was established.

In the Ordinance, the following are mentioned in category I of dangerous dogs: fighting and attack dogs, assimilated by their morphological characters to dogs of the Pit Bull, Boerbull, Bandog type and their hybrids (those dogs resulting from the crossbreeding of breeds).

In addition, a series of obligations for the masters are also mentioned, such as: minimum age of 18 years; full exercise capacity; lack of criminal convictions.

The certificate also includes data on the mandatory sterilization of category I dogs, "the performance of anti-rabies vaccination and boosters, proof of civil liability insurance and the identification number of the dog applied by tattoo or microchip".

RESULTS AND DISCUSSIONS

Since the mandatory insurances should be renewed annually and as a result their volume should be approximately constant, nevertheless we note an increase in the number of insurance policies issued and also in their volume.

However, according to the statistics for the year 2023 carried out by ASF Romania (Financial Supervisory Authority), the subscriptions related to optional home insurance, in the year 2023, recorded a higher value by approximately 24% compared to the year 2022, standing at approximately 572 billion RON, while the gross allowances paid increased by about 44% to the value of 145 billion RON. The number of new contracts concluded during the reporting period was around 1.7 billion, up 18.5% compared to 2022 (Table 1).

Table 1. Subscriptions related to optional home insurance*

Period	Number of contracts in force at the end of the reporting period	Number of new contracts, completed in the reporting period	Gross written premiums (lei)	Gross compensations paid (lei)
2023	1.685.094	1.714.246	572.308.044	145.037.335
2022	1.616.633	1.447.078	459.891.122	100.476.331

*Incl. Euroins Romania data in the first quarter of 2023, before the withdrawal of the operating authorization, with the exception of the number of contracts in force (asfromania.ro)

In 2023, the number of health insurances was on 231.108 contracts, in 2022 it was 220.332 contracts and in 2021 were 382.261 contracts (Figure 1).

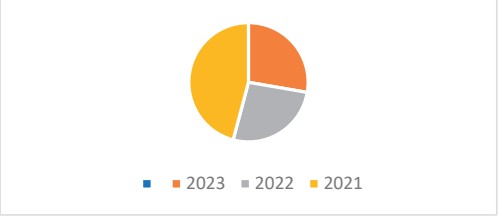

Figure 1. Represents Number of contracts in force at the end of the reporting period (asfromania.ro)

CONCLUSIONS

Insurance represents a classic form of risk transfer from the insured to specialized companies, called insurers.

The first insurance company in Romania appeared in 1744 in Brașov and was called 'Casa de Incendiu'. In 1906, the Agricola Company was established, which was oriented towards agricultural insurance. The Autonomous Directorate of State Insurance appeared in 1942. This company had a diversified portfolio of all types of insurance.

The most important types of impact insurance for agriculture and the veterinary medical field have been listed.

In the two reports issued by Financial Supervisory Authority - ASF Romania, we note an increase in the number of policies issued and the volume of sales in 2023 compared with 2022.

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***Emergency Ordinance no. 55 of April 30 2002, regarding the keeping regime of dangerous or aggressive dogs, republished.

***Law 381/2002 regarding the granting of compensation in the case of natural calamities in agriculture.

***Law 95/2006 on health reform.

***Law 235/2015 on the authorization and supervision of insurance and reinsurance activity.

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