UNIVERSITATEA DE ŞTIINŢELE VIEŢII
„REGELE MIHAI I" DIN TIMIŞOARA

FACULTATEA DE MEDICINĂ VETERINARĂ

LUCRĂRI ŞTIINŢIFICE

MEDICINĂ VETERINARĂ
TIMIŞOARA
VOLUMUL LVII(4)

SCIENTIFICAL PAPERS
VETERINARY MEDICINE

To be cited: LUCRARI STIINTIFICE: MEDICINA VETERINARA TIMISOARA (SCIENTIFICAL PAPERS: VETERINARY MEDICINE TIMISOARA)**,** vol. LVII(4), 2024

Available online at: **https://www.usab-tm.ro/ro/publicatii-42/revista-47-volume-de-lucrari-stiintifice-10682**
Indexed and/or abstracted in: **CABI Full Text, CAB Abstracts, Ulrich's Periodicals Directory**
Editor: AGROPRINT TIMISOARA ISSN: 2668-2435 and ISSN-L 1221-5295

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2024

ASSESSING THE QUALITY AND SAFETY OF RAW MILK SOLD THROUGH VENDING MACHINES: A CASE STUDY FROM TIMIȘOARA

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Abstract

This study examines the quality and safety of raw milk distributed through vending machines in Timișoara, responding to growing consumer demand for unprocessed dairy products marketed as healthier alternatives. Raw milk vending, which has gained popularity across European nations, allows direct sales from farm to consumer, yet poses potential health risks if strict quality and safety protocols are not maintained. In this research, 13 milk samples from vending machines were collected over two months and subjected to comprehensive physicochemical and microbiological analyses, including fat, protein, lactose, and total dry matter (TDS) content measurements, cryoscopic point, acidity, total mesophilic count (TNGMA), and somatic cell count (SCC). The findings revealed notable variability across samples and locations, even though the milk originated from a single dairy supplier. Inconsistent fat (2.1%-6.4%) and protein (3.2%-3.8%) levels suggest improper homogenization, while high SCC and TNGMA values, with most samples surpassing safety thresholds, indicate issues in milk hygiene practices. Some samples also showed indications of water addition, inferred from cryoscopic point deviations. Furthermore, the potential for unregulated heat treatment was identified, which contravenes raw milk vending standards, and could compromise product integrity and nutritional value. The frequent non-compliance with microbial and somatic cell count standards underscores inadequate adherence to milking hygiene and poor mammary health management within dairy herds. Consequently, while raw milk vending machines offer direct consumer access to fresh milk, this study emphasizes the need for stringent oversight to ensure that marketed products meet safe and consistent quality standards, aligning with regulatory expectations for direct raw milk sales.

Keywords: *milk, freshness, hygiene, microbiology.*

In recent years, consumer interest in minimally processed and locally sourced food has markedly increased, with raw milk becoming a favored option due to its perceived health advantages. In numerous European nations, vending machines have emerged as a prevalent means for farmers to sell raw milk directly to consumers, facilitating access to fresh, unprocessed dairy products (1). Nevertheless, raw milk, although abundant in essential nutrients, poses health hazards if hygiene and safety protocols are not rigorously upheld (2). In Romania, where organized milk sales markets are restricted and producers frequently encounter adverse conditions with large processors, numerous small-scale farmers have resorted to direct sales via vending machines (3).

Despite the advantages this model offers to both farmers and consumers, concerns remain regarding the quality and microbial safety of raw milk available through vending machines (4). Factors such as somatic cell count, bacterial load, and inconsistent nutrient content can significantly impact both the safety and nutritional value of raw milk (5).

This study aims to evaluate the physicochemical properties and microbial quality of raw milk sold through vending machines in Timișoara. By analyzing samples from multiple machines, this research provides insights into the consistency, nutritional profile, and safety of milk available in these outlets. The findings seek to inform regulatory authorities, consumers, and producers, highlighting the necessity for stringent hygiene protocols and regulatory supervision in the distribution of raw milk.

Materials and methods

This study assessed the quality and safety of raw milk sold through 13 vending machines in Timișoara, Romania. Milk samples were collected from each machine over two consecutive months (May and June) to account for any temporal variations. The milk sourced for these vending machines came from a single milk supplier, ensuring a controlled comparison across different machines.

A total of 13 raw milk samples were collected from each vending machine on the same day for each sampling period. To maintain sample integrity, the milk was stored under refrigerated conditions during transport to the laboratory.

Each sample underwent analysis for key components: fat, protein, lactose, and total dry matter (TDS) using the Lactoscope Rapid Analyzer (Delta Instruments, Drachten, the Netherlands). To assess freezing point consistency, the cryoscopic point was measured using a Cryostar device. Acidity was

also measured and expressed in degrees Thörner (°T).

The microbial quality of each sample was determined by analyzing the total mesophilic count (TNGMA) and somatic cell count (SCC). For TNGMA, samples were inoculated on nutrient agar and incubated at 30°C for 72 hours, following serial dilutions to identify bacterial colonies in the 15-300 colony-forming units (CFU) range (6, 7). Somatic cell count was determined using a NucleoCounter SCC analyzer (Chemo Metec, Denmark), which uses fluorescence detection for cellular DNA stained with propidium iodide.

Results were recorded and analyzed for variability across samples and machines, focusing on compliance with European safety standards for raw milk (8). Descriptive statistics, including mean and standard deviation, were used to interpret differences in nutrient composition and microbial load, providing insights into the safety and quality of vending machine milk in the study region.

Results and discussions

The analysis of raw milk samples collected from 13 vending machines across Timișoara in May and June revealed considerable variability in physicochemical properties, indicating inconsistencies in the quality and composition of the milk supplied by these machines (Table 1, Table 2). Although all vending machines reportedly sourced milk from a single supplier, differences in nutrient content, particularly fat, protein, and lactose levels, were observed among samples, which may reflect variations in machine maintenance, milk handling, or initial milk quality.

Fat content varied significantly across samples, ranging from 2.1% to 6.4%, with an overall average of 4.1%. The wide range suggests that the milk may not have been sufficiently homogenized, which could lead to fat separation within the vending machines. This lack of uniformity can cause higher fat concentrations in samples drawn later in the day, as fat rises and accumulates at the top. Fat is a major energy component in milk, and its variability may affect consumer expectations for consistent nutrition, as well as potential health outcomes. Inconsistencies in fat content can diminish the perceived quality of raw milk for consumers who may seek it for its unaltered, natural state and expected nutritional richness.

Protein levels across the samples were relatively stable, with values ranging between 3.4% and 3.7%, and an average protein content of 3.6%. Protein content is a critical indicator of milk's nutritional value and is expected to be fairly consistent if the milk originates from a single source. The minimal variations observed here may reflect consistent protein synthesis across the dairy herd. However, even small fluctuations in protein content could influence the perceived quality and texture of milk products, particularly for consumers who value the high-protein profile of raw milk (9).

Lactose levels in the analyzed milk samples ranged from 4.7% to 5%, with an average of 4.8%. Lactose, the primary sugar in milk, plays an essential role in its energy profile and sweetness (10). The slight variation in lactose content observed among samples could be attributed to differences in milk handling, temperature, or storage conditions within the vending machines.

The Total Dry Matter (TDS) percentage in milk samples varied from 11.6% to 15.6%, with an average of 13.3%. TDS, which includes all non-water components of milk (fat, protein, lactose, minerals, etc.), is an important indicator of milk quality. High TDS can imply a richer nutrient profile, whereas low TDS suggests dilution or potential nutritional inconsistency. Such variations in TDS could be attributed to several factors, including differences in the milking process, cow diet, or milk handling and storage. The inconsistency in TDS could impact consumers who rely on raw milk for its perceived natural and high-nutrient content, as this metric directly reflects the milk's richness and nutritional density. Variations in the TDS percentage can also be used for determining the presence of bovine subclinical mastitis (11).

The cryoscopic point, or freezing point, of milk samples revealed notable variability, with 5% of samples displaying values higher than expected, suggesting possible water adulteration. The freezing point of milk is a standardized measure of quality, as the addition of water would increase it. Normal milk typically has a cryoscopic point between -0.53 and -0.51, while samples with values deviating above these limits may indicate dilution (12). This finding raises concerns about the integrity of milk sold through vending machines, as water addition not only dilutes the nutritional content but may also mislead consumers. Water adulteration could be due to unintentional contamination during handling or deliberate addition to increase product volume.

Acidity levels across the milk samples were within the normal range, with values from 19 to 20 degrees Thörner (°T). Acidity in milk is primarily due to lactic acid resulting from fermentation of the lactose by lactic bacteria, and standard limits are set to ensure freshness and prevent spoilage (13).

The consistent acidity observed in this study suggests that, at the time of sampling, the milk was fresh and had not undergone significant bacterial fermentation.

This result indicates that, despite other variances, the samples were largely within acceptable freshness parameters and had likely been refrigerated adequately to prevent spoilage. The microbial safety of the milk samples was assessed by analyzing the total mesophilic count (TNGMA) and the somatic cell count (SCC), both of which serve as key indicators of milk's microbiological quality and animal health (14, 15). The results raised significant concerns about the safety of raw milk sold through vending machines in Timișoara, with both TNGMA and SCC values frequently exceeding permissible limits.

Table 1

The registered physiochemical and freshness values of the collected raw milk in May according to the sampling location

No. Crt.	Location	Fat (%)	Protein (%)	Lactose (%)	TDS (%)	Cryoscopic point (°C)	Acidity (°T)
1	Timișoara 1	4.4	3.7	4.9	13.8	0.54	19
2	Timișoara 2	4.3	3.6	4.8	13.5	0.51	20
3	Timișoara 3	2.1	3.7	5.0	11.6	0.53	19
4	Timișoara 4	4.2	3.7	4.8	13.5	0.53	20
5	Timișoara 5	4.4	3.7	4.8	13.7	0.50	19
6	Timișoara 6	4.2	3.7	4.9	13.4	0.54	20
7	Timișoara 7	3.1	3.7	4.8	12.4	0.52	19
8	Timișoara 8	2.5	3.7	4.9	11.8	0.52	19
9	Timișoara 9	4.2	3.7	4.8	13.5	0.52	19
10	Timișoara 10	4.4	3.7	4.8	13.7	0.56	20
11	Timișoara 11	4.3	3.4	4.7	13.2	0.52	19
12	Timișoara 12	6.4	3.7	4.7	15.6	0.52	19
13	Timișoara 13	4.4	3.7	4.8	13.7	0.52	20

Table 2

The registered physicochemical and freshness values of the collected raw milk in June according to the sampling location

No. Crt.	Location	Fat (%)	Protein (%)	Lactose (%)	TDS (%)	Cryoscopic point (°C)	Acidity (°T)
1	Timișoara 1	4.4	3.7	4.8	13.7	0.52	19
2	Timișoara 2	4.3	3.6	4.8	13.5	0.52	19
3	Timișoara 3	3.9	3.6	4.8	13.1	0.52	20
4	Timișoara 4	2	3.7	5.0	11.4	0.51	20
5	Timișoara 5	4.4	3.7	4.8	13.7	0.52	19
6	Timișoara 6	3.8	3.2	4.6	12.4	0.53	20
7	Timișoara 7	4.4	3.6	4.8	13.5	0.52	19
8	Timișoara 8	4.2	3.6	4.8	13.4	0.53	20
9	Timișoara 9	4.2	3.6	4.8	13.5	0.52	20
10	Timișoara 10	2	3.8	4.8	11.4	0.52	19
11	Timișoara 11	1.4	3.8	4.8	10.7	0.52	20
12	Timișoara 12	3.8	3.6	4.8	13.5	0.52	19
13	Timișoara 13	3.6	3.6	4.9	11.9	0.53	20

The total mesophilic count (TNGMA) in the milk samples varied widely (Fig. 1). In May, values ranged from 3.2 x10^5 to 5.2 x 10^6 cfu/mL, with an average of 2.1 x10^6 cfu/mL. In June, TNGMA values were even higher, reaching a maximum of 9 x 10^6 cfu/mL. Most samples exceeded the European safety limit for raw milk, which is typically set at 1 x 10^5 cfu/mL, reflecting widespread bacterial contamination likely due to poor hygiene during milking, inadequate cleaning of milking equipment, or improper storage. High TNGMA levels in raw milk can lead to spoilage, off-flavors, and potential health risks, including foodborne illnesses, particularly among vulnerable populations such as children, elderly individuals, and immunocompromised persons.

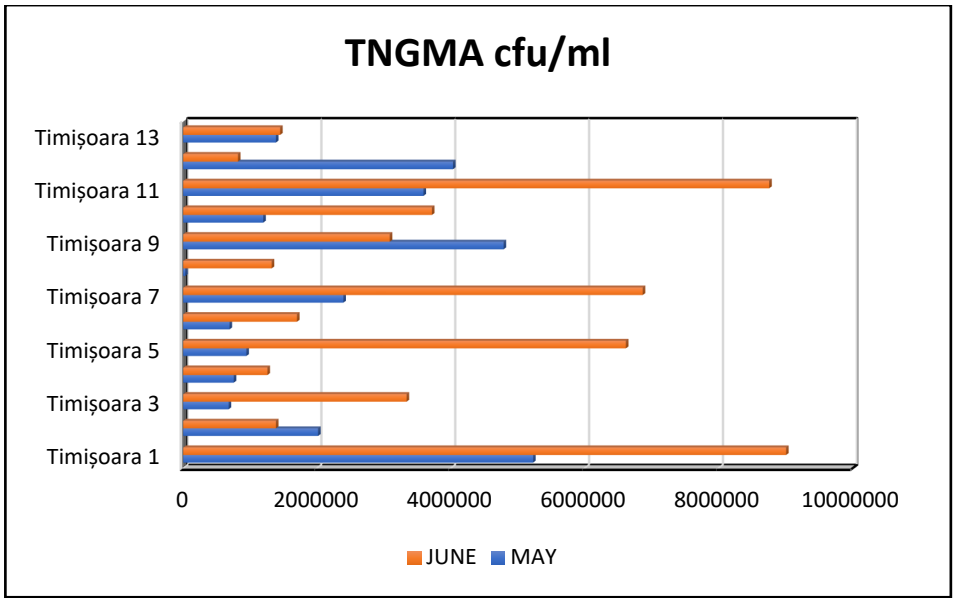

Fig. 1. Variations of the TNGMA in the analysed months

The considerable variability in TNGMA among machines further suggests possible differences in milk handling practices or sanitation across the vending locations. Notably, a sample with an unusually low TNGMA count of 3.2 x 10^4 cfu/mL raised suspicion about possible antibiotic presence, which could inhibit bacterial growth. However, laboratory testing did not confirm antibiotic residues, and the sample was later found to have undergone possible heat treatment, as indicated by a positive peroxidase test. This finding violates raw milk regulations, as heat treatment is not allowed for raw milk sold as such and highlights the need for regulatory oversight to ensure compliance.

Somatic cell count (SCC) in milk is an important indicator of udder health, with elevated SCC levels associated with mastitis, an inflammation of the mammary gland (15). In this study, SCC values in May ranged from 10,000 to 776,000 cells/mL, with an average of 345,000 cells/mL, whereas in June, SCC values ranged up to 929,000 cells/mL (Fig. 2). These values are significantly above the European Union's recommended limit of 400,000 cells/mL for raw milk (8), suggesting that the cows supplying the milk to these vending machines may be experiencing mastitis or other udder health issues.

Elevated SCC can lead to enzymatic changes in milk, resulting in a decreased shelf life and altered flavor. It also suggests compromised animal health and inadequate farm-level disease management practices, particularly regarding milking hygiene and infection control (16). The presence of elevated SCC across multiple samples underscores the need for better herd management practices and routine health monitoring to ensure that milk from mastitic cows is not entering the raw milk supply chain.

One sample raised concerns regarding potential heat treatment, as it displayed both low TNGMA and SCC values and tested positive in a peroxidase assay, a test sensitive to heating above 70°C. Since raw milk vending regulations prohibit heat treatment, this finding suggests a possible non-compliance (17). Heat treatment alters milk's nutritional profile and enzyme activity (18), which conflicts with consumer expectations of raw milk's natural composition. This result emphasizes the need for monitoring and testing to verify compliance with raw milk regulations, ensuring that milk marketed as raw truly meets regulatory definitions.

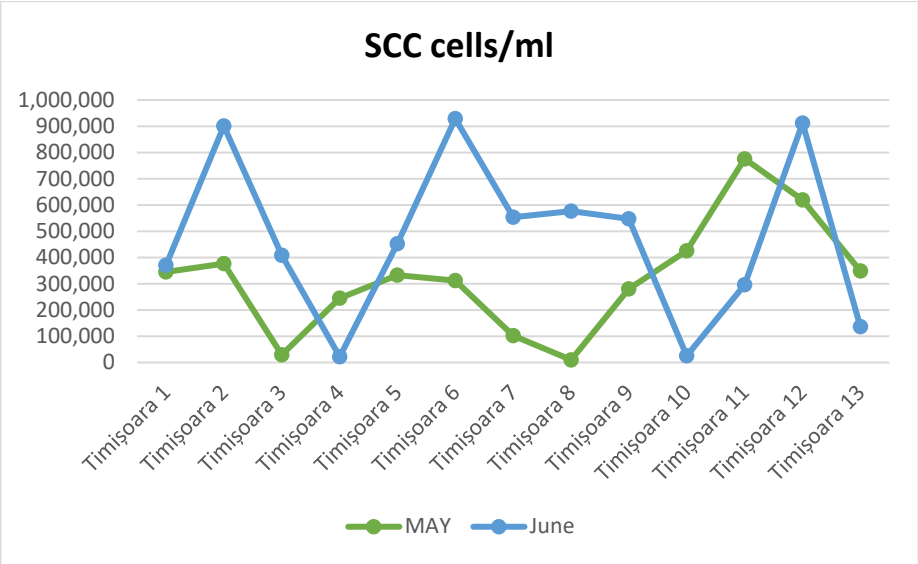

Fig. 2. Variations of the SCC in the analysed months

The high TNGMA and SCC values observed in these samples pose significant concerns for public health, particularly for consumers who choose raw milk for perceived health benefits. Given the elevated microbial loads, consumers could be exposed to pathogens that cause gastrointestinal illness (19), particularly if they consume the milk without further processing. Additionally, the variability in nutrient composition and potential adulteration (e.g., water addition) further reduce the reliability of raw milk from vending machines as a consistent and safe product.

Conclusion

This study highlights significant variability in the quality and safety of raw milk sold through vending machines in Timișoara, underscoring the challenges of maintaining consistent standards in direct farm-to-consumer milk sales. The analysis revealed fluctuations in key nutritional components—such as fat and protein levels—indicating inconsistent homogenization practices. Additionally, high microbial counts and somatic cell numbers in most samples exceeded European safety thresholds, suggesting insufficient hygiene during milking and potential health issues within supplying dairy herds. These factors collectively reduce the reliability of raw milk from vending machines as a consistently safe and nutritious option for consumers.

The study's findings highlight concerns regarding current practices that may compromise product integrity, including potential heat treatment of raw milk and possible mixing from various sources, in violation of direct-sale regulations.

Given the popularity of raw milk vending machines as a fresh, local product source, it is essential to implement and enforce stricter quality controls, including regular testing for microbial and somatic cell counts. Such measures would support small-scale farmers in delivering a safer, higher-quality product and provide consumers with increased confidence in the health benefits of raw milk.

The results of this study emphasize the need for enhanced regulatory oversight and adherence to sanitary practices to safeguard the quality and safety of raw milk in vending machines.

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MAST CELL TUMORS IN DOGS AND CATS - A REVIEW

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Abstract

A mast cell tumor (MCT) is a type of malignant tumor consisting of mast cells (mast cells are immune cells located in the skin and other tissues, that are normally responsible for allergic reactions). They are usually noticed in middle aged patients but can occur in patients of any age. Mast cell tumors typically form nodules or masses in the skin, but they can also affect other areas of the body, including the spleen, liver, intestine, and bone marrow. MCTs are the most common skin tumors in dogs and the second most frequent ones in cats (following basal cell carcinoma). They can be a raised lump or bump on or just under the skin, and may be red, ulcerated, or swollen. While some may be present for many months without growing much, others can appear suddenly and grow very quickly. They may appear to fluctuate in size, getting larger or smaller, even daily. These size changes can occur spontaneously or when the tumor is handled, which causes degranulation and subsequent swelling of the surrounding tissue due to the histamine release. This cancer is typically diagnosed using fine-needle aspiration (FNA). The whole tumor is graded from I-III, with grade I as much less aggressive than grade III. Higher-grade tumors have a higher tendency to metastasize. Surgical removal of MCT is the preferred treatment once the animal is diagnosed with this disease. MCT invade into surrounding tissues and wide surgical margins are necessary to ensure removal of all cancerous cells. This paper wishes to underline the most important aspects of this tumors in dogs and cats, with a special emphasis on clinical signs, diagnosis, and treatment options.

Keywords*: mast cell tumor, oncology, dog, cat.*

Oncological diseases significantly impact not only patients but also the researchers and clinicians who work to manage and treat these conditions. Tumors, whether benign or malignant, are frequently encountered in general veterinary practice. Among these, skin tumors represent a significant proportion, accounting for approximately 30% of all tumor diagnoses in companion animals. This highlights the importance of skin neoplasms, including mast cell tumors, in clinical and research contexts (6, 22).

Mast cell tumors (MCTs) are one of the most common neoplasms in dogs and a significant yet less frequent malignancy in cats. Originating from mast cells - a component of the immune system involved in inflammatory and allergic responses - these tumors are most commonly found in the skin but can also occur in visceral organs, such as the spleen and liver (1, 3).

These cells originate from bone marrow-derived progenitors and are widely distributed throughout connective tissues, particularly in the skin, gastrointestinal tract, and respiratory system. Mast cell tumors (MCTs) arise from the uncontrolled proliferation of mast cells, often influenced by mutations in the c-KIT proto-oncogene, which regulates cell growth and differentiation. In tumors, biological markers such as mitotic index (MI), Ki-67, and c-KIT localization are used to assess proliferation and predict outcomes (22).

Mast cells contribute to the pathology of MCTs through the release of pro-inflammatory cytokines and mediators, leading to local and systemic effects, including pruritus, ulceration, and gastrointestinal issues (6).

Mast cell tumors (MCTs) are the most frequently diagnosed cutaneous neoplasms in dogs, comprising 16% to 21% of all skin tumors. Although MCTs predominantly affect older dogs, with an average onset age of 8–9 years, they have also been observed in younger animals, and no significant sex predisposition has been noted. While MCTs occur in various breeds, certain breeds show a heightened risk, including those of bulldog lineage (e.g., Boxers, Boston Terriers, Pugs), as well as Labrador Retrievers, Golden Retrievers, Cocker Spaniels, Schnauzers, Staffordshire Terriers, Beagles, Rhodesian Ridgebacks, Weimaraners, and Shar-Peis. This increased prevalence in specific breeds suggests a potential genetic predisposition to the disease (2, 5).

The exact cause of mast cell tumors (MCTs) in dogs remains largely unidentified. Historically, these tumors have been linked to chronic inflammation or exposure to skin irritants. However, current epidemiological data in dogs does not support the hypothesis that topical carcinogens play a significant role in their development. This suggests that the etiology of MCTs is likely multifactorial, potentially involving genetic, environmental, and immunological factors (14, 18).

Unlike mast cell tumors (MCTs) in dogs, which are primarily cutaneous or subcutaneous, feline MCTs typically occur in three distinct forms: cutaneous MCT, splenic/visceral mast cell disease, and

intestinal MCT. These forms may overlap in some cases. The exact cause of MCTs in cats is not yet understood, and there is no evidence suggesting that viral infections are involved in their development (14).

Two distinct types of cutaneous mast cell tumors (MCTs) have been identified in cats: the more common mastocytic MCT, which is histologically similar to canine MCTs, and the less frequent histiocytic MCT. The histiocytic form exhibits features characteristic of histiocytic mast cells and may regress spontaneously within 4 to 24 months. The average age of cats diagnosed with MCTs is between 8 to 9 years, with the mastocytic form typically occurring in older cats (mean age of 10 years), while the histiocytic form is more common in younger cats (mean age of 2.4 years). Siamese cats have been shown to have a predisposition for both types of MCTs, particularly the histiocytic form, which is often observed in young Siamese cats under 4 years of age. However, conflicting reports exist, with some studies indicating no significant difference between the two forms in Siamese cats. While earlier studies suggested a male predilection for developing MCTs, more recent research has not confirmed this trend (6, 20).

Materials and methods

Articles (published mainly in the last 10 years), having as a topic of interest veterinary oncology, most specific mast cell tumors, species - dogs and cats, as well as clinical studies and other publications were selected for this review (using PubMed, Google Academic, Web of Science).

Results and discussions

This review paper focuses on the clinical signs, diagnosis and treatment options of MCT in both dogs and cats.

Mast cell tumors in dogs

Most dogs with mast cell tumors (MCTs) present with a cutaneous or subcutaneous mass. While MCTs are typically solitary, they can develop sequentially or synchronously, meaning multiple tumors may appear over time. These tumors can arise anywhere on the body, with 50-60% occurring on the trunk, 25-40% on the limbs, and about 10% on the head and neck. Less commonly, MCTs affect areas like the scrotum, perineum, back, or tail (6, 22).

Occasionally, MCTs develop in extracutaneous sites such as the conjunctiva, nasopharynx, larynx, oral cavity, gastrointestinal tract, or urinary tract. Although rare, spinal MCTs have been reported. Visceral mastocytosis is also documented, but it typically results from the spread of a primary aggressive cutaneous MCT. Isolated visceral MCTs or primary mast cell leukemia are extremely uncommon in dogs (22).

Mast cell tumors (MCTs) in dogs often present as erythematous (red) or edematous (swollen) masses, which may also be alopecic (hairless). They are notorious for their wide variety of clinical appearances, earning them the reputation of "great imitators." These tumors can resemble soft cutaneous or subcutaneous masses, often mimicking benign conditions like lipomas. If not investigated, this can lead to delayed diagnosis and treatment of more advanced and harder-to-manage disease (4, 8).

The gross appearance of an MCT can provide some insight into its behavior and histological grade. Tumors that appear aggressive are generally aggressive, but even those that appear less active should not be assumed to be benign. Clinical signs of aggressive MCTs include:

- rapid growth
- local irritation and inflammation
- poor demarcation from surrounding tissues, indicating local infiltration
- ulceration of the mass
- presence of satellite nodules (5, 6).

In addition, dogs may exhibit urticarial (hive-like) swelling or diffuse edema, which could mimic conditions like cellulitis or acral lick dermatitis. These varied presentations highlight the importance of thorough investigation to avoid misdiagnosis and ensure appropriate management (6).

Complications resulting from the release of bioactive substances by mast cell tumors (MCTs) occur in up to 50% of dogs with MCTs. The degranulation of mast cells releases various mediators, including histamine, heparin, and proteases, which can cause local effects such as erythema, inflammation, and irritation. Additionally, these substances can lead to local hemorrhage and impair wound healing. The resulting inflammation can complicate the clinical management of MCTs and contribute to the development of secondary conditions such as ulceration or tissue necrosis (7).

The systemic release of histamine from mast cell tumors (MCTs) can cause gastrointestinal ulceration due to histamine's stimulation of H2 receptors. This leads to increased acidity, vascular damage, and heightened gastrointestinal motility, all contributing to the formation of ulcers. Studies have found gastrointestinal ulceration in 35-83% of dogs with MCTs at necropsy, with a

higher incidence seen in dogs with high-grade tumors or more extensive disease. Furthermore, the systemic release of other bioactive compounds can lead to severe reactions, including "flashing syndromes," collapse, or even anaphylactic shock, emphasizing the systemic risks associated with MCTs (19, 22).

Fine-needle aspiration (FNA) is an effective diagnostic tool for mast cell tumors (MCTs), correctly identifying 92-96% of cases. Although mast cell granules may occasionally fail to stain well with rapid Romanowsky-type stains, this issue is uncommon and can typically be addressed by a longer fixation period in alcohol. While FNA is highly accurate for diagnosis, it is not suitable for cytological grading, particularly for Grade I and II tumors. Poorly differentiated tumors can sometimes be identified based on cytological features (9).

Histopathology is essential for grading MCTs, and imaging techniques like ultrasound may help evaluate the extent of the primary tumor, particularly if it has invaded deeper tissues, such as muscle layers. Though pulmonary metastasis is rare in MCTs, thoracic radiographs are often recommended to assess for potential intrathoracic lymphadenopathy or pulmonary interstitial infiltrates. Common sites of metastasis include local lymph nodes, followed by the spleen, liver, or other visceral organs (1, 10).

Mast cell tumors in cats

Mast cell tumors (MCTs) in cats most commonly present as discrete, firm, alopecic (hairless) nodules, which are typically pale or tan in color. These tumors can be solitary or multiple, with approximately 25% of cases involving multiple lesions at the time of presentation. Although distant metastasis is uncommon, certain areas are more frequently affected, including the head, limbs, and tail. Cats with visceral MCTs may develop cutaneous metastases, and animals with multiple nodules or palpable abdominal abnormalities should be thoroughly staged to assess the extent of the disease. This staging process helps guide treatment decisions and determine the prognosis (6, 11).

Grading of mast cell tumors (MCTs) provides valuable prognostic information that helps predict the tumor's biological behavior and aids in selecting the most appropriate treatment plan. Two primary histologic grading systems are used for cutaneous MCTs: the Patnaik grading system and the Kiupel grading system.

- the Patnaik system classifies MCTs into three grades: Grade I (well-differentiated), Grade II (moderately differentiated), and Grade III (poorly differentiated), which correlates with the likelihood of metastasis and the aggressiveness of the tumor.
- the Kiupel system uses a simpler two-tier grading approach, categorizing MCTs as either low grade (well-differentiated) or high grade (poorly differentiated), with the latter being associated with a poorer prognosis (15, 17).

Both systems provide useful insights into the potential course of the disease, and often both are reported to offer a more comprehensive assessment (17).

Cytologic grading systems, which have been studied extensively, show promise in predicting the histologic grade of MCTs and can provide independent prognostic value. When grading MCTs cytologically, key features are evaluated, such as:

- the degree of granulation in mast cells
- presence of mitotic figures (indicative of cell division)
- nuclear size variation and pleomorphism
- presence of binucleated or multinucleated cells
- number of fibroblasts or collagen fibrils present in the tumor's stroma (16).

These cytologic features can help predict both the behavior of the tumor post-surgery and its potential clinical outcome, which is crucial for treatment planning and prognosis.

Treatment

Surgery remains the treatment of choice for primary grade I or II mast cell tumors (MCTs). The traditional approach for MCT removal has been to ensure margins of 3 cm in two planes, as well as the excision of one fascial plane deep to the tumor. However, recent studies suggest that for MCTs up to 5 cm in diameter, wider surgical margins may not be necessary. Instead, a 2 cm lateral margin with one fascial plane deep margin is considered sufficient for effective management. This approach has not been extensively studied for tumors larger than 5 cm, and it is generally considered inadequate for grade III tumors, which require more aggressive surgical margins to ensure complete excision and minimize the risk of recurrence (6, 21).

The primary treatment for feline mast cell tumors (MCTs) is surgical excision. Tumors classified histologically as "diffuse" require wider surgical margins compared to those described as "compact." While there is no established role for chemotherapy, some reports suggest that vinblastine, chlorambucil, and lomustine may be used. Prednisolone is

often part of the treatment regimen as well. Radiation therapy has limited documented use in MCT treatment for cats (23).

Histopathological grading, particularly using the Patnaik criteria, does not reliably predict prognosis. However, histological classification as either compact or diffuse is helpful. Compact mastocytic tumors, which account for 70–85% of cases, are generally minimally invasive and rarely metastasize. In contrast, diffuse tumors tend to be more locally invasive and are more likely to have locoregional metastasis, occurring in 5–10% of cases. Additionally, proliferation markers may provide some insight into prognosis, though more data is needed to fully assess their predictive value (22).

Up to 44% of dogs previously treated for mast cell tumors (MCTs) will develop new MCTs. In some cases, particularly in breeds such as Golden Retrievers, Labrador Retrievers, Weimaraners, and Boxers, dogs may experience multiple MCTs either within a short timeframe or sequentially throughout their lives. These new tumors are considered de novo lesions, not metastases from the original tumor, so dogs with multiple MCTs do not typically have a shorter survival compared to those with a single tumor of the same grade. There is no evidence that systemic therapy, such as chemotherapy, can prevent the development of new MCTs or improve the overall prognosis for these dogs (2, 9).

Cutaneous metastases from MCTs are more likely to occur in association with high-grade, aggressive tumors. These metastatic tumors tend to be multiple, grow rapidly, and are often ulcerated, indicating a more aggressive form of the disease (24).

Some studies have questioned the prognostic value of complete versus incomplete surgical margins in mast cell tumors (MCTs). In approximately 25% or more of tumors with incomplete or narrow margins, en bloc resection of the scar has revealed no mast cells, suggesting that incomplete excision might not always lead to recurrence. One study found a recurrence rate of only 23% for incompletely excised grade II tumors (Séguin et al., 2006). This might indicate that the mast cells at the margins were either non-neoplastic or eradicated by the immune system. The challenge in assessing margins arises because MCTs can release factors that attract normal inflammatory mast cells, making it difficult to differentiate between tumor cells and normal cells, especially in lower-grade tumors (12, 21).

Recurrence can still occur even with complete margins, with local failure rates ranging from 5–11%. However, it is more likely when margins are incomplete or narrow. If there is macroscopic residual disease or signs of recurrence or metastasis, a "wait and see" approach is inappropriate, and definitive surgery should be performed when possible. In cases where surgical excision is difficult, neo-adjuvant chemotherapy (such as vinblastine and prednisolone, or just prednisolone) may help achieve a complete excision. Alternatively, postoperative radiotherapy can address any residual disease. Recurrent MCTs generally have a poorer prognosis (16, 19).

For grade I and many grade II tumors with incomplete margins but no gross disease or complications, options include performing an en bloc excision of the scar or monitoring the site closely. If recurrent disease is detected, it should be resected with adequate margins. If the second surgery achieves clear margins, no further adjunctive therapy is typically required (10, 18).

Radiation therapy is commonly used as an adjunctive treatment for mast cell tumors (MCTs), particularly following surgery. The best results are achieved when radiation is planned upfront as part of the treatment strategy, rather than being considered after an inadequate surgical excision. Radiation therapy alone is generally avoided for bulky MCTs due to the risk of mast cell degranulation, which can lead to severe systemic effects, including fatalities. Mast cell degranulation can release bioactive substances such as histamine, which may result in life-threatening reactions (13).

For cases with extensive disease, neoadjuvant treatments like chemotherapy or prednisolone are typically used before radiation to reduce the risk of severe degranulation during or after radiation. These therapies can help stabilize the tumor and prevent systemic complications associated with radiation-induced mast cell release. Overall, the use of radiation therapy in MCT management requires careful planning to avoid complications, especially in more advanced cases (8, 12).

Chemotherapy is primarily used when systemic treatment is necessary to manage disseminated, non-resectable, or high-grade mast cell tumors (MCTs). It is also employed for treating residual microscopic disease, particularly when radiation therapy is not available. Chemotherapy is indicated for grade II tumors with a high mitotic index, grade III tumors, or tumors that exhibit features suggesting a mix of grade II/III. In these cases, chemotherapy aims to delay or prevent metastatic spread in addition to surgical treatment of the primary tumor (1, 7).

Vinblastine and prednisolone are commonly used as first-line therapies, while

lomustine is often used as a second-line treatment. In some cases, protocols alternating between vinblastine and lomustine are used. A combination of vinblastine, cyclophosphamide, and prednisolone has shown good response rates, but its potential for additional toxicity without a proven survival advantage has limited its widespread use. There is limited anecdotal evidence supporting the use of other chemotherapeutic agents in the treatment of canine MCTs (6, 20).

Good outcomes have been reported in some studies, with all patients surviving at least 3 years following chemotherapy for high-grade MCTs, though the prognosis can still be poor for some high-grade cases (15).

Neoadjuvant chemotherapy, given prior to surgery or radiation therapy, can be beneficial in consolidating the tumor mass, making it more amenable to complete excision. By reducing the tumor's size and improving its consistency, neoadjuvant therapy may increase the likelihood of achieving clear surgical margins during excision. This approach can also facilitate safer and more effective radiation treatment by decreasing the risk of radiation-induced complications, particularly in tumors with significant size or invasive characteristics. This strategy is often employed for high-grade or large MCTs where achieving complete surgical resection can be challenging without prior tumor shrinkage (13, 17).

The activation of the KIT receptor tyrosine kinase (TK) plays a significant role in the development of canine mast cell tumors (MCTs). This activation is primarily caused by genetic mutations in the c-kit gene, which lead to increased cell proliferation independent of ligands, as well as changes in cell migration, maturation, and survival. These mutations are present in approximately 15-40% of canine MCTs, with a higher frequency observed in high-grade tumors. Such mutations are often linked to a worse prognosis (3, 5).

Recent research has explored the use of tyrosine kinase inhibitors (TKIs) in tumors with c-kit mutations. Two TKIs, masitinib mesylate and toceranib phosphate (SU11654), have shown significant effectiveness in treating MCTs harboring c-kit mutations. These agents have been well tolerated in dogs, with controlled studies demonstrating tumor response and delayed progression as well as increased survival, especially in high-grade tumors. Interestingly, some tumors without c-kit mutations have also responded to these drugs, likely due to their ability to inhibit other tyrosine kinases. These findings suggest a promising avenue for MCT treatment, although further research is needed to fully understand the role of TKIs in canine MCT management (3).

Stelfonta (tigilanol tiglate) is an innovative, nonsurgical treatment for canine mast cell tumors (MCTs) that offers an alternative to traditional surgical removal. Approved by the FDA for use in nonmetastatic MCTs up to 10 cm^3 in volume, Stelfonta is injected directly into the tumor, where it works by triggering a local inflammatory response that leads to tumor destruction. This procedure can be performed without general anesthesia, though some sedation may be needed, and it requires a short recovery period with minimal disruption to the dog's lifestyle (25).

Stelfonta has been shown to resolve 75-87% of tumors with a single or sometimes two injections, with most tumors healing completely within 4–6 weeks. It is considered a viable option for tumors in accessible areas such as the skin or subcutaneous tissue, particularly in cases where surgery may be impractical or undesirable (25).

While Stelfonta has proven effective, its use requires careful management, including pre-treatment with corticosteroids and antihistamines to mitigate potential mast cell degranulation effects, which can lead to systemic reactions like vomiting or shock. This treatment has been praised for its ability to avoid the need for surgery, anesthesia, and the long recovery periods associated with traditional approaches (25).

Conclusions

Mast cell tumors (MCTs) are a prevalent concern in the dermatologic pathology of both dogs and cats. Accurate and prompt diagnosis is crucial for managing these tumors effectively, as their biological behavior can be highly variable, ranging from benign to highly aggressive forms. Early and precise identification allows for the selection of the most appropriate therapeutic approach, which can significantly influence outcomes.

Due to the prevalence of MCTs, their potential for local invasion, and the risk of metastasis, timely diagnosis and proper prognostication are essential. The treatment costs, along with the emotional burden on pet owners, make it all the more important to evaluate MCTs accurately, ensuring that the right treatment plan is chosen based on tumor grade and staging. This includes using grading systems like Patnaik or Kiupel, and considering adjunctive therapies such as surgery, radiation, and chemotherapy, depending on the tumor's characteristics. By accurately prognosticating the disease, veterinarians can provide more tailored care and improve the quality of life for

pets while alleviating the financial and emotional stress on owners.

As research continues to advance, a better understanding of the molecular mechanisms driving MCTs and the development of more effective targeted therapies will hopefully improve outcomes and provide additional treatment options for affected animals. For now, treatment protocols should be individualized based on tumor grade, location, and response to therapy, with close monitoring to manage potential recurrence or metastasis. The growing body of literature on MCTs emphasizes the importance of a multimodal approach in their management, incorporating both traditional and emerging therapies to optimize patient survival and quality of life.

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STATISTICAL ANALYSIS OF MILK COMPOSITION AND DAIRY COW MANAGEMENT PARAMETERS: INSIGHTS FROM LINEAR REGRESSION MODELS

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Abstract

This study aims to determine specific relationships between milk composition variables and dairy cow health indicators, focusing on identifying significant predictors through correlation analysis and linear regression models. Milk composition plays a key role in determining dairy quality and nutritional value, directly affecting taste, shelf life, and the suitability of milk for processing into various dairy products. Key constituents such as fat, protein, lactose, and somatic cell count (SCC) serve as indicators of both milk quality and cow health, reflecting aspects such as metabolic efficiency and immune response. Fat content, protein content, lactose, and SCC were analyzed to identify significant predictors of milk quality. Robust predictive models were developed for fat content (adjusted $R^2 = 0.9649$) and protein content (adjusted $R^2 = 0.8928$), with total solids and protein content emerging as strong predictors. In contrast, models for SCC and lactose showed weaker fits (adjusted $R^2 = 0.4004$ and 0.5231, respectively), highlighting the need for additional predictors or alternative modeling approaches. The study indicates that leveraging easily measurable parameters can streamline the assessment of key milk quality indicators while maintaining precision.

Keywords: *milk composition, linear regression models, fat, protein, lactose, somatic cell count.*

The quality and biochemical composition of cow's milk are fundamental for assessing its nutritional value and suitability for dairy product processing. Key milk components, such as fat, protein, and lactose, significantly influence both the stability and organoleptic characteristics of milk, with variations arising from factors like the cow's lactation stage and individual physiological differences (1, 2). Proteins, particularly casein, play an essential role in coagulation, a process vital for producing high-quality cheeses and other dairy products (3, 4). Casein, the predominant protein in milk, not only contributes to texture and stability but also impacts the nutritional value of dairy products. This importance of casein has been highlighted in recent studies focusing on its structural role in micelle formation, essential for maintaining milk's physical properties and stability during processing (16).

In this study, Spearman correlation analysis and multiple regression models were employed to explore relationships among milk composition variables and to identify significant predictors of milk quality. Multicollinearity was evaluated using the Variance Inflation Factor (VIF), with a threshold of 5 to exclude highly correlated predictors, ensuring stability in the regression models (7, 8). To further refine model selection, the Akaike Information Criterion (AIC) was applied, prioritizing models that balanced complexity with performance, where lower AIC values indicated an optimal model fit (9, 10). Additionally, Cook's Distance was used to identify and remove influential data points, preventing any disproportionate effect on the regression coefficients (11).

Additional milk quality indicators, such as pH and differential somatic cell count (DSCC), provide insights into various biochemical factors in milk that can affect its composition and stability (12, 13). Analyzing these components alongside biochemical markers like urea and beta-hydroxybutyrate (BHB) helps deepen understanding of key biochemical influences on milk composition (14, 15).

The aim of this study is to develop predictive models that facilitate efficient milk quality assessment by identifying key biochemical predictors within milk composition. By examining the relationships among components such as total solids, protein, fat, somatic cell count(SCC), and Lactose, the study seeks to provide a basis for streamlined quality analysis methods. These methods leverage easily measurable indicators to predict more complex quality metrics, potentially offering practical tools for routine monitoring.

Materials and methods

Study Design and Sample Collection

This study investigates the biochemical composition and quality indicators of milk from dairy cows on a farm in the Banat region, Romania. All Milk samples were collected from 98 cows of the same breed (Romanian Spotted Cattle). All milk samples were collected from each cow on the same day to minimize environmental and temporal variability, controlling for feed, milking practices, and potential diurnal shifts in milk composition. After data cleaning (eliminating outliers, missing data, and influential points identified using Cook's Distance), 54 samples

remained for analysis. The biochemical analyses were performed by the Official Dairy Control service.

Data Preprocessing

Several preprocessing steps were applied to prepare the dataset for analysis. Key variables were converted to numeric format, and rows with missing values were excluded to ensure complete data. Outliers were removed to reduce skew and enhance reliability, with values beyond *1.5xIQR* excluded. A Spearman correlation analysis was conducted to assess associations between milk composition and quality indicators, informing predictor selection for regression models, a significance level of $p < 0.05$ was used to determine statistically significant relationships.

Statistical Modeling

Multiple linear regression models were developed to identify predictors for key milk composition variables: fat content, protein concentration, SCC, and lactose content. Each target variable was initially modeled with predictors chosen based on correlation analysis. Stepwise regression was applied to refine predictors using forward selection and backward elimination, retaining variables that significantly improved model fit. Predictors were assessed for multicollinearity using the Variance Inflation Factor (VIF), with a threshold of 5. Predictors exceeding this value were reviewed and, if necessary, removed to maintain model stability.

Influential data points were identified using Cook's Distance, and observations exceeding the threshold (4/n, where n is the number of observations) were flagged and removed to refine models. Models were then refitted to assess the impact on adjusted R^2 and AIC values.

These diagnostics confirmed that the models met the assumptions of linear regression after removing influential data points, improving the stability of each final model.

Statistical Analysis Software

The statistical analysis was conducted using R software (version 4.3.1), an open-source programming environment widely utilized for statistical computing and data visualization. R software is available through the Comprehensive R Archive Network (CRAN) at https://cran.r-project.org. Key packages facilitated each step: "readxl" enabled data import from Excel, while "dplyr" managed data cleaning and manipulation. The "Hmisc" package calculated Spearman correlations, and "GGally" and "ggplot2" provided visualization tools for displaying correlations and model diagnostics. To check for multicollinearity, "car" was used to calculate Variance Inflation Factors (VIFs). Model selection and optimization, including stepwise regression, were handled by "MASS", while "broom" helped tidy model outputs. The "patchwork" package allowed for combining plots, enhancing the clarity of presented results. This integrated approach supported both the exploration and rigorous analysis of milk composition and quality indicators.

Integration of Artificial Intelligence

To streamline workflow and enhance precision, ChatGPT, an AI-powered tool developed by OpenAI, was employed as an assistant throughout the study. ChatGPT, accessible at https://openai.com/chatgpt, contributed to statistical analysis with R by providing guidance and clarification on performing statistical analyses and data visualization within the R environment. It also supported the identification and retrieval of current scientific literature relevant to the study's objectives, and helped refine, structure, and enhance the clarity of the manuscript.

Results and discussions

Correlation Analysis

The Spearman correlation analysis (as shown in Fig. 1) revealed several significant relationships between milk composition and quality indicators, guiding the selection of predictors for each regression model.

Positive correlations were observed between fat and total solids, with a high positive correlation *(*$\rho = 0.941$, $p < 0.001$*)*, indicating that as total solids increase, fat content also increases significantly. Similarly, casein and protein were nearly perfectly correlated *(*$\rho = 0.994$, $p < 0.001$*)*, suggesting a close relationship and similar contributions to milk composition.

Negative correlations included a moderate negative relationship between lactose and fat *(*$\rho = -0.490$, $p < 0.001$*)*, implying that higher fat content is associated with lower lactose levels. Additionally, lactose and somatic cell count (SCC) showed a moderate negative correlation *(*$\rho = -0.556$, $p < 0.001$*)*, suggesting that increased SCC is linked to reduced lactose content.

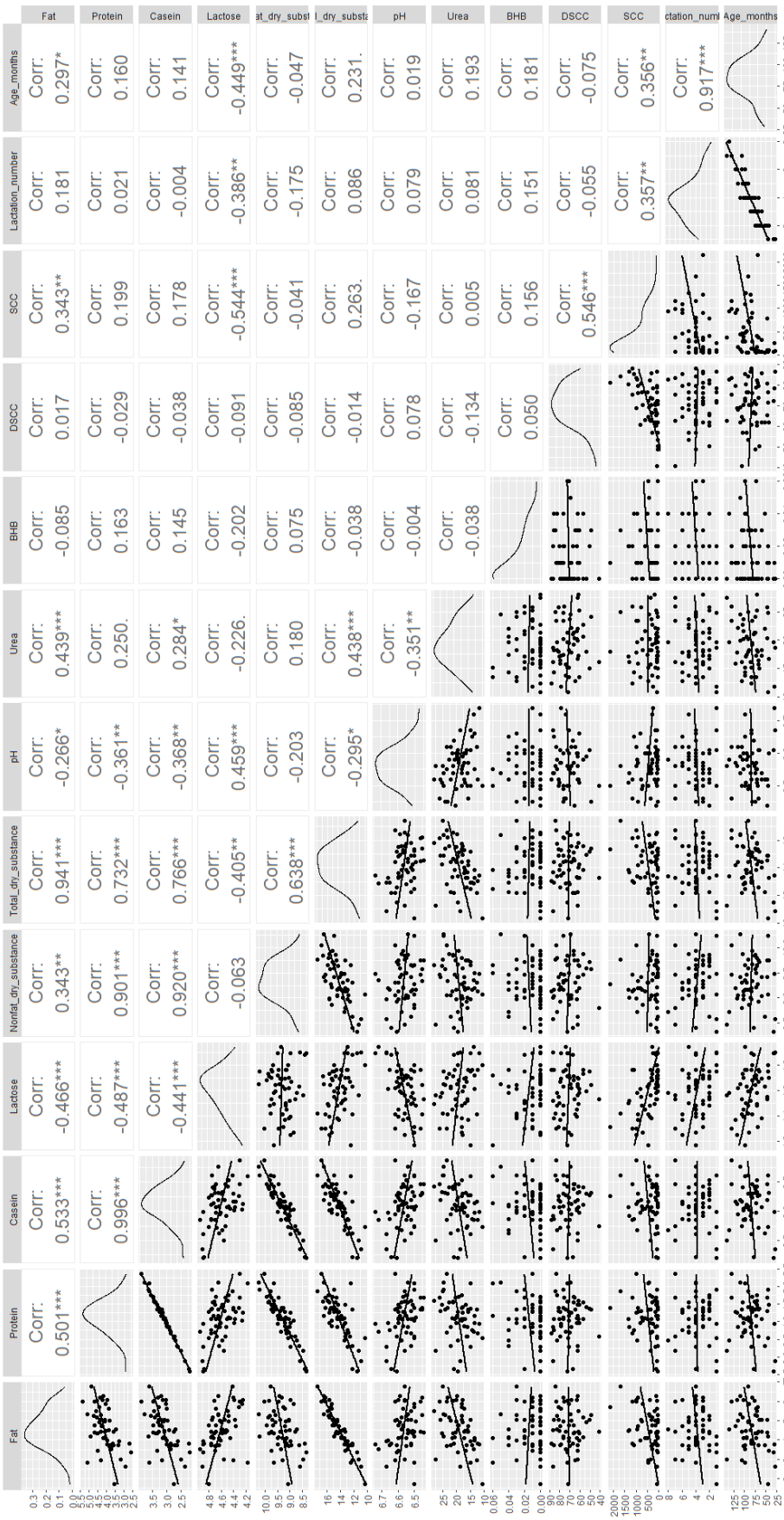

Fig. 1. Scatter plot matrix showing pairwise correlations among milk composition variables. Density plots on the diagonal represent the distribution of each variable, while scatter plots and correlation coefficients summarize relationships between pairs of variables.

Regression Models

In this study, regression analysis was employed to explore the relationships between milk composition variables and associated predictors. The general form of the multiple linear regression model used is:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \ldots + \beta_n X_n + \varepsilon$$

Where Y represents the dependent variable (e.g., Fat, Protein, SCC, or Lactose), β_0 is the intercept, β_1, β_2, …, β_n are the coefficients associated with the predictors X_1, X_2, …, X_n, and ε denotes the error term. This approach quantifies the effects of changes in one or more predictors on the response variable, holding other predictors constant.

Fat Content

The regression model for fat content initially used *Total Solids* and *Protein* as predictors. After identifying and removing influential points, the final refitted model for *Fat* is:

$$\text{Fat} = -4.256 + 0.852 \cdot \text{Total Solids} - 0.794 \cdot \text{Protein} + \varepsilon$$

Interpretation of Coefficients.

The intercept (β_0 = *-4.256*) represents the expected fat content when all predictors are zero, though this value primarily serves as a baseline in the model. Each unit increase in Total Solids is associated with a *0.852*-unit increase in Fat, holding Protein constant. Each unit increase in Protein is associated with a *0.794*-unit decrease in Fat, holding Total Solids constant.

Model Fit

The refitted model has an Adjusted R^2 of 0.9649, indicating that the model explains 96.5% of the variability in Fat. This high Adjusted R^2, along with an AIC of -13.46, suggests a strong and reliable model. The observed vs. predicted values for Fat are shown in Figure 2, where the close alignment of points along the diagonal line indicates a strong predictive capability.

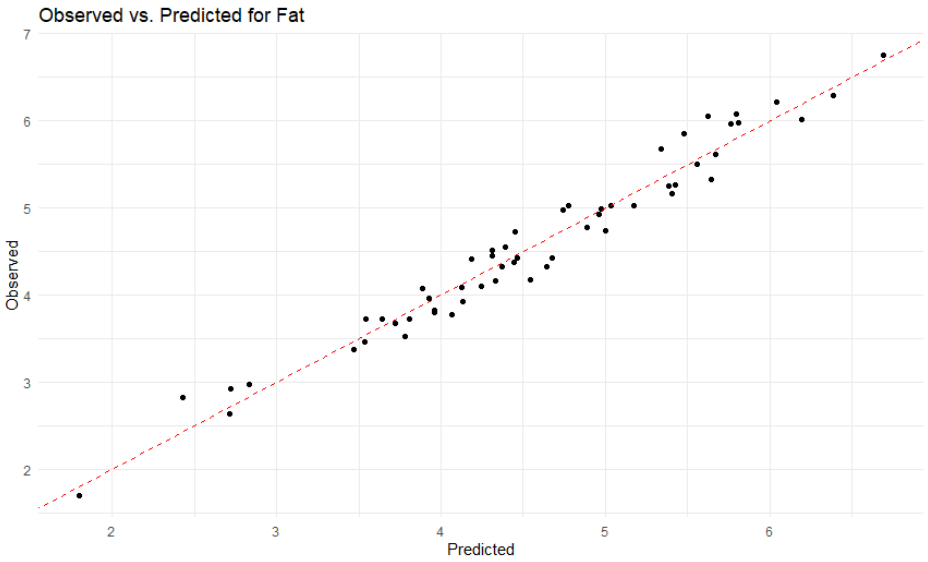

Fig. 2. Observed vs. Predicted plot for Fat

Protein Concentration

The regression model for protein concentration used Fat and Total Solids as predictors. The final refitted model is:

$$\text{Protein} = -2.733 - 0.704 \cdot \text{Fat} + 0.709 \cdot \text{Total Solids} + \varepsilon$$

Interpretation of Coefficients

The intercept (β_0 = *-2.733*) represents the baseline level of Protein. Each unit increase in Fat is associated with a *0.704*-unit decrease in Protein, holding Total Solids constant. Each unit increase in Total Solids is associated with a *0.709*-unit increase in Protein, holding Fat constant.

Model Fit

The model explains *89.3%* of the variance in Protein (Adjusted R^2 = *0.8928*), with an AIC of *-0.95*. This high Adjusted R^2 suggests that the model has strong explanatory power. The observed vs. predicted values for Protein are shown in Figure 3, indicating a reliable model, with most points clustering around the line of equality.

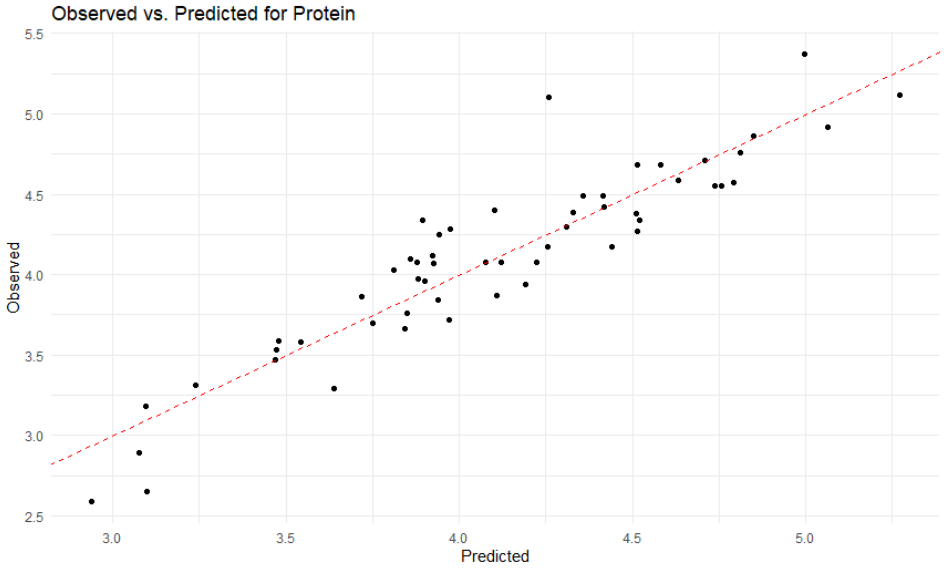

Fig. 3. Observed vs. Predicted plot for Protein

Somatic Cell Count

The somatic cell count model included Lactose as a predictor. After removing influential points, the final refitted model is:

$$SCC = 6597.3 - 1348.1 \cdot Lactose + \varepsilon$$

Interpretation of Coefficients

The intercept (β_0 = *6597.3*) represents the baseline SCC. Each unit increase in Lactose is associated with a decrease of *1348.1* units in SCC.

Model Fit

The refitted model has an Adjusted R^2 of *0.4004*, indicating that Lactose explains approximately *40%* of the variability in SCC. Despite an AIC of *834.5*, this model has limited predictive power. The observed vs. predicted values for SCC are shown in Figure 4, revealing some dispersion from the line of equality, suggesting the need for additional predictors to improve model fit.

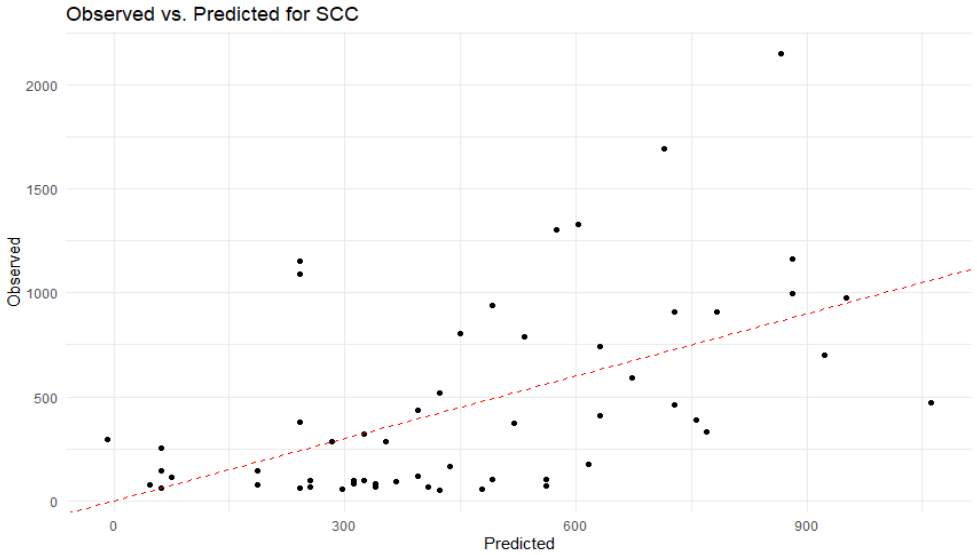

Fig. 4. Observed vs. Predicted plot for SCC

Lactose

The lactose content model initially used Protein, pH, and SCC as predictors. After removing influential points, the final refitted model is:

$$\text{Lactose} = -0.446 - 0.067 \cdot \text{Protein} + 0.825 \cdot \text{pH} - 0.000194 \cdot \text{SCC} + \varepsilon$$

Interpretation of Coefficients

The intercept (β_0 = *-0.446*) serves as the baseline lactose content. Each unit increase in Protein is associated with a decrease of *0.067* units in Lactose, holding pH and SCC constant. Each unit increase in pH is associated with an increase of *0.825* units in Lactose, holding Protein and SCC constant. Each unit increase in SCC is associated with a decrease of *0.000194* units in Lactose, holding Protein and pH constant.

Model Fit

The refitted model has an Adjusted R^2 of *0.5231*, explaining approximately *52.3%* of the variance in Lactose. The AIC of *-60.73* suggests a moderate fit, and additional predictors could be explored for further refinement. The observed vs. predicted plot for Lactose is shown in Figure 5, which demonstrates an acceptable fit, though variability around the line of equality suggests moderate predictive strength.

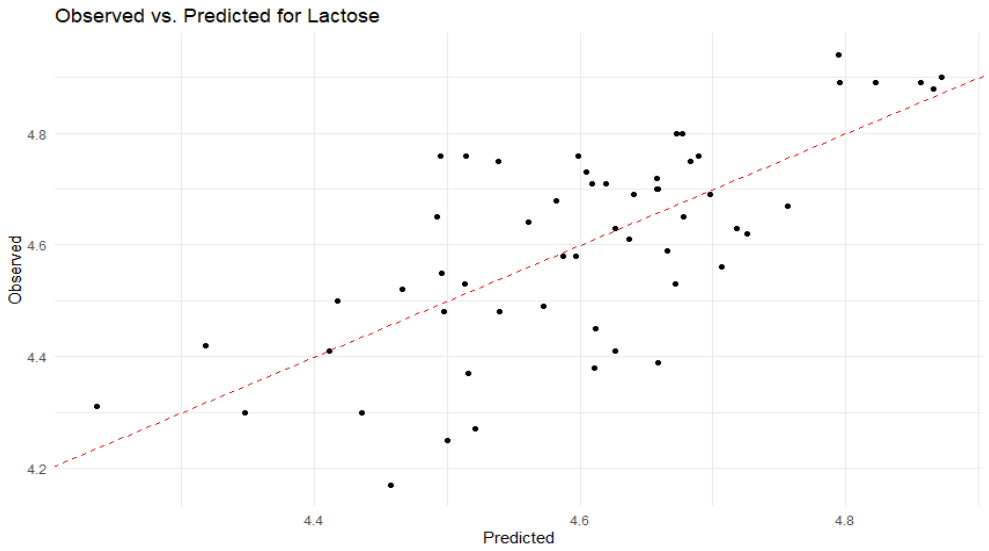

Fig. 5. Observed vs. Predicted plot for Lactose

The Spearman correlation analysis revealed significant relationships that helped in selecting predictors for each regression model. The positive correlation between Fat and Total Solids (ρ = 0.941, p < 0.001) aligns with findings that higher total solids often increase fat content, enhancing milk's caloric and nutritional profile (2, 5). Similarly, the nearly perfect correlation between Protein and Casein (ρ = 0.994, p < 0.001) is consistent with research showing that casein is a dominant protein influencing overall protein levels and nutritional quality in dairy products (16).

In the regression model for Fat, the strong relationship between Fat and Total Solids, with an Adjusted R^2 = 0.9649, supports the importance of solids in determining fat content and, consequently, the energy density of milk (9). For Protein, the model highlights that while protein concentration rises with total solids, an increase in Fat leads to a slight decrease, likely due to the dilution of protein in high-fat samples (3, 10).

The negative association between Lactose and SCC (ρ = −0.556, p < 0.001) reflects studies indicating that higher SCC, often linked to udder health issues, reduces lactose content due to compromised milk production processes (11). In the SCC regression model, Lactose was a moderate predictor, explaining about 40% of its variability, suggesting additional health-related factors might be needed for stronger predictive power (6).

These findings underscore the interconnectedness of milk composition factors in determining quality and nutritional value, emphasizing the importance of integrated quality control measures in dairy science (15).

Limitations. While the study's regression models provide robust predictors of milk composition, the analysis could be further refined by incorporating additional factors, such as genetic factors, could improve model robustness, especially for SCC and lactose, where predictive power was weaker.

Conclusions

This study identified key relationships between milk composition and cow health indicators, showing that Total Solids and Protein are strong predictors of fat and protein levels,

while SCC and lactose are influenced by additional factors.

The interconnectedness of milk components suggests that changes in one component can significantly affect others, future research could refine the analysis by incorporating additional factors, such as genetic influences. Given the strong predictive relationships found in the regression models, simpler methods for determining certain milk components could be proposed. In our study, Total Solids and Protein can reliably predict fat content, reducing the need for more complex or time-consuming fat analysis.

However, since the regression model for SCC showed weaker predictability, direct measurement of lactose may still be necessary for accuracy. These regression models suggest that a more streamlined approach to milk quality analysis is feasible, leveraging easily measurable parameters to predict other key quality indicators effectively.

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THE ACTION OF OINTMENT WITH EXTRACTS OF *CENTELLA ASIATICA* AND *CROTON LECHLERI* ON THE INTEGUMENT IN SEVERE THERMAL BURNS

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Abstract

The health of the skin, both in humans and in animals, has been and continues to be an ongoing concern for the medical and pharmaceutical fields. Accidental or inflicted, wounds are represented by cuts, lacerations, burns, tears or other injuries, and for them to heal without complications, certain trauma management protocols must be followed. In the present study, the action of the ointment in which were incorporated two extracts obtained from *Centella asiatica* and from *Croton lechleri* was followed on the integument of laboratory mice. The thermal burn was treated for 7 days by twice daily application of the ointment and to assess its action samples were collected from the integument on day 0, day three and day seven for histological investigations. Because the use of the respective ointment in burns requires additional care, future investigations will be conducted to elucidate the action on the skin in the case of this type of trauma.
Keywords: integument, thermal burns, *Centella asiatica*, *Croton lechleri*.

By knowing and understanding the mechanisms regarding the homeostasis of the skin, over time, solutions and remedies have been sought in terms of preventing the installation of conditions at the level of the best represented organ of the body, with multiple functions (among which the sensory and barrier are the best known), conditions that are as numerous as the causes that can produce them.

The reasons why skin care is vital are plentiful, starting with the fact that, first and foremost, it is a barrier against external threats such as pathogenic bacteria, allergens and trauma. At the same time, the skin is an indicator of internal unbalance regarding the proper functioning of the body, such as allergies or nutritional and/or drug intolerances, or various diseases.

Thus, multiple causes can determine the weakening of this barrier, starting from mild, reversible ones, such as nutrition, to serious, irreversible ones, such as trauma or hereditary disorders.

Open wounds often need to be treated for days, weeks, or even months before they can be closed. Many wound care products are available that can debride the wound without damaging the adjacent unaffected tissue, that can decrease infection, and that can increase the rate of healing.

It is known that *Centella asiatica* extracts have been traditionally used for wound healing by increasing cell proliferation and collagen synthesis, much more stable in the injured region, with much faster epithelization and wound contraction compared to the untreated model, and with a positive effect on angiogenesis (2, 9, 13, 14, 15). Also, one of the components of *Centella asiatica* extract, asiaticoside, has an antioxidant effect (16).

Although *Croton lechleri* has medicinal properties, being a good analgesic, antioxidant and immunomodulator, with antitumor, antimutagenic and cytotoxic effects, it is mainly used in the treatment of various types of wounds, where its antibacterial and antiviral capacity is also added (1, 7, 11). Thus, the specialized literature presents studies in which after only one day of treatment with Dragon's blood, the wound contracts and a dark crust forms on the surface of the wound, thus preventing the establishment of a secondary infection. Studies also show that Dragon's blood stimulates the proliferation and migration of fibroblasts, stimulates collagen synthesis, accompanied by rapid epithelial regeneration (7).

Materials and methods

The present study was performed on a total of 12 Balb/c mice, which were separated into two groups: the experimental and the control.

The duration of the experiment was 7 days, in which period the skin samples were collected day 0, 3, and 7 respectively. The lab animals were housed individually, in standard plastic cages in the Toxicology/Pharmacology/Biobase laboratory from Faculty of Veterinary Medicine Timișoara, where the temperature conditions (20°C) were constant. Each animal included in the study was clipped before the mechanical burn was applied by application of a hot round iron, with known diameter. Under the effect of narcosis (isoflurane), the hot iron was applied for 5 seconds to the skin of the animals, in the lumbar zone. Immediately after the induction of

the mechanical burn, the phytotherapeutic ointment made at the Department of Pharmacology of Faculty of Veterinary Medicine Timișoara was applied, for each individual of the experimental group. The ointment was applied 2 × 1/day, in an equal amount (1g/application), for each mouse. For the euthanasia of the animals, the combination of Ketamine (50-100 mg/kg) and Xylazine (2-8mg/kg IM) was used. The skin samples were fixed in ethyl alcohol 80^0 for 7 days, after which they were washed, dehydrated and embedded in paraffin. The sections were processed for the histological study by the standard Hematoxylin – Eosin method.

Results and discussions

From a structural point of view, mammalian skin has a similar architecture, even if there are differences (especially in thickness) between species, within the same species or between different regions of the body (4). The lab mice are the animal model frequently used in the study of physiological and biochemical mechanisms of skin diseases and healing stages, since they are easy to handle, maintain and reproduce, and are economically accessible (3, 5, 10, 18). In rodents, the epidermis and dermis are much reduced in thickness, compared to other mammals and humans (12). Thus, the epidermis, thin, has a basal layer, resting on the basement membrane, consisting of a single layer of cubic cells. Above the basal layer is the spinous layer, which has 1-2 rows of larger keratinocytes, followed by the granular layer, with 1-2 rows of squamous keratinocytes, the last layer being the corneous layer, with flattened keratinized cells. The absence of the *stratum lucidum* (usually this layer is present only in areas where the epidermis is thick and devoid of glandular and corneous productions) and melanocytes was also noted. The superficial dermis does not form dermal papillae, its junction with the epidermis being almost rectilinear. In the structure, the typical, non-oriented arrangement of collagen fibers can be observed, between which fibroblasts are localized. Microscopically, the absence of sweat glands was identified, and the sebaceous glands are simple alveolar, holocrine type, and are attached to the hairs (Fig. 1).

In rodents, sweat glands are located only on the palmar and plantar pads level (17).

Under the hypodermis, represented by the fibro-adipose connective tissue, the presence of the muscle layer (*panniculus carnosus*) was noted, containing striated myocytes, whose contraction, independent of the deep muscles, is important in the synthesis of collagen and in the wound healing process (Fig. 2).

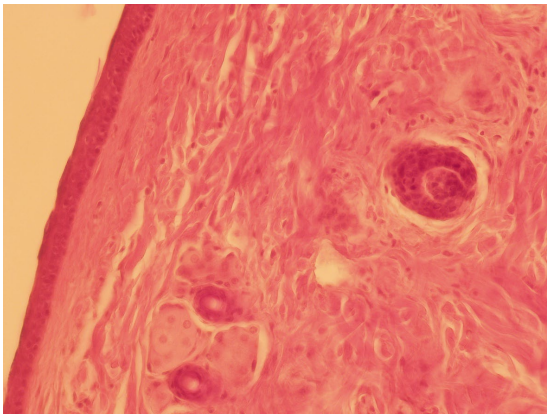

Fig. 1. Histological section through the skin from the control group: normal structural appearance, H.E., 200X

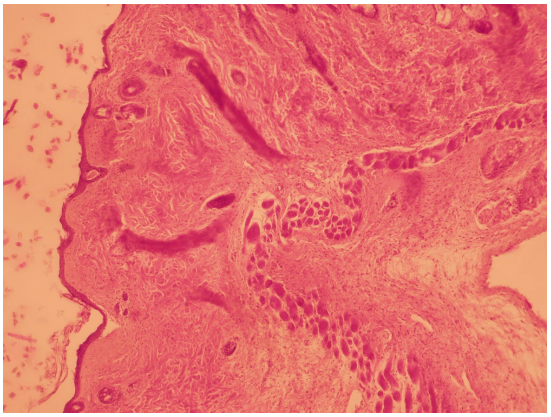

Fig. 2. Histological section through the skin from the control group: muscular layer, H.E., 40X

From a physiopathological point of view, the thermal aggressor induces the alteration of the protective and sensory functions of the skin, at the point of impact, distinguishing three concentric zones, which extend both horizontally and in depth, vertically. Thus, the central area of the lesion, of maximum impact, also called the coagulation zone, is characterized by the total and irreversible loss of the component tissue structures, together with the coagulation and denaturation of the matrix proteins, including those that constitute the cell membranes, where the installation of necrosis can also be found. The stasis zone surrounds the coagulation zone, and is characterized by the occurrence of vascular phenomena, such as vasoconstriction and ischemia. A correct management of the burn will make the alterative phenomena in this area to be able to improve; otherwise they will progress to necrosis, edema and the

appearance of infections. The third, peripheral zone, also called the hyperemia zone, presents viable cells, and vasodilatation mediated by cells and local inflammatory mediators (8).

Microscopically, after the application of the thermal aggressor, at the level of the impact zone, the installation of alterative changes was observed, affecting the epidermis and the dermis (Fig. 3 and 4). Thus, the epidermis is absent almost entirely, the area being covered by a thin, detached crust due to reduced adhesion. Following coagulation, the collagen fibers in the dermis formed a homogeneous mass. Sebaceous glands and hairs have undergone necrosis phenomena.

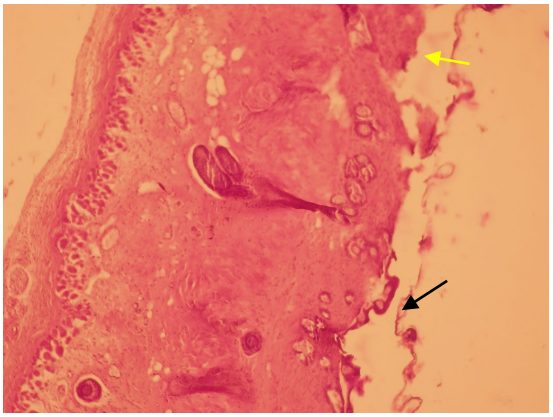

Fig. 3 Histological section performed through the skin from the positive control group: absence of epidermis (→), thin, detached crust (→), H.E., 100X

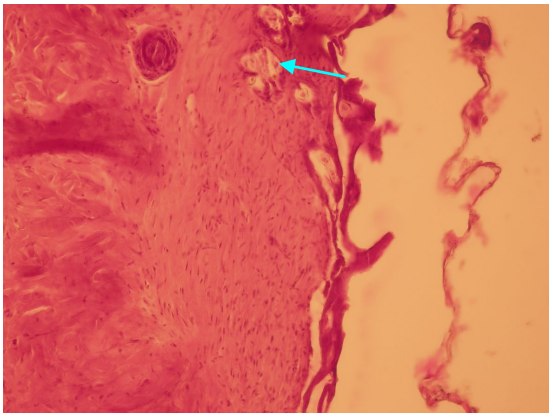

Fig. 4. Histological section performed through the skin harvested from the positive control group: necrosis of the sebaceous glands (→), H.E., 200X

In the deep area, vascular phenomena can also be noted, expressed by congestion (Fig. 5). In the adjacent area, where the thermal aggressor propagates, the spaces that separate the epidermis from the dermis appeared, being a sign that indicates the destruction of the junctional complex between the two structures (Fig. 6).

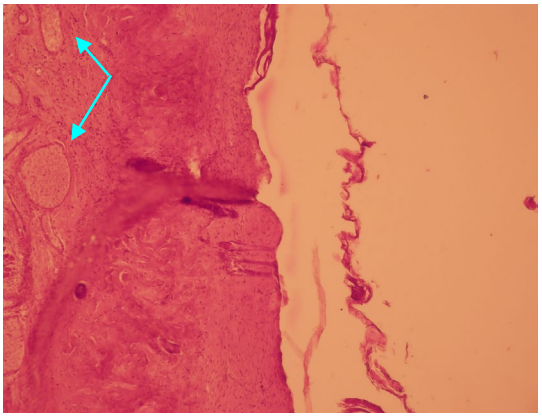

Fig. 5. Histological section performed through the skin from positive control group: vascular congestion (→), H.E., 100X

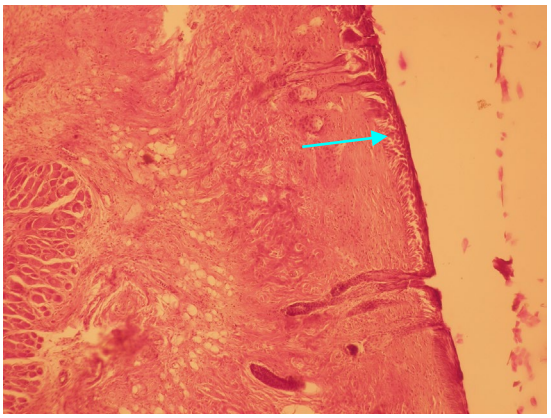

Fig. 6. Histological section performed through the skin harvested from the MA group: destruction of the dermo-epidermal junctional complex (→), H.E., 200X

The burn, as an acute pathological entity, entails a dynamic healing process, which, like any other reparative process, involves the passage of four phases, which can evolve distinctly, gradually or overlapping, respectively: the hemostasis phase, the inflammatory phase, the of proliferation and tissue remodeling phase. In the impact area, three days after the application of the thermal aggressor, without being followed by the institution of any treatment, microscopically, the absence of the epidermis was found, which signifies the absence of the initiation of epithelization.

The accentuation of the process of coagulation and protein denaturation was revealed by the appearance of a compact mass of collagen fibers, which are not individualized and not anastomosed. In the dermis, localized glandular productions and hairs were completely necrotic. In the deep dermis, adipocytes were the predominant cells, among which inflammatory cell infiltration was

observed (Fig. 7). At the periphery of the affected area, the inflammatory infiltrate was very obvious (Fig. 8). All these identified elements represent moment 0 of the initiation of the healing process (6).

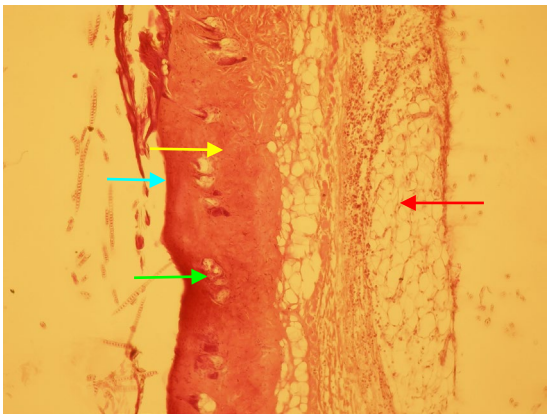

Fig. 7. Histological section performed on the skin from the positive control group, three days post-aggression: absence of epidermis (→), accentuated coagulation of collagen fibers (→), necrosis of sebaceous glands (→), presence of adipocytes (→), H.E., 40X

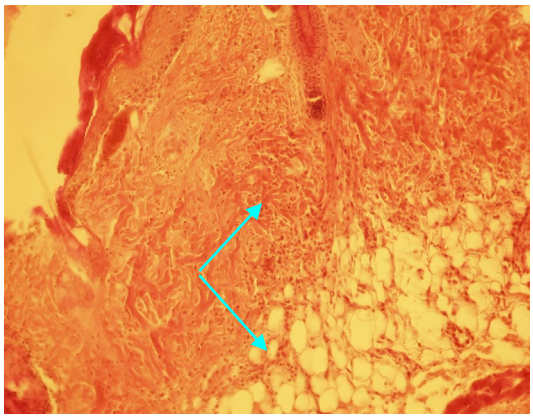

Fig. 8. Histological section performed on the skin from the positive control group: inflammatory infiltrate in the peripheral area of the lesion (→), H.E., 200X

After three days of treating the wound caused by a thermal aggressor with the ointment obtained at the Discipline of Pharmacology, which contained extracts of *Centella asiatica* and *Croton lechleri*, by applying it twice a day, the microscopic examination revealed the reduction of necrotic tissue in the area of impact, under which an extensive layer of adipocytes is found. At the base of this layer, the appearance of immature hair follicles was noted (Fig. 9). The massive presence of adipocytes is a specific element of the third day post-burn, their involvement in the healing process being still unknown (6). After three days of treatment, the epidermis is absent in the affected area, and a thin, adherent crust is found in its place. The compact mass of collagen fibers is reduced, with inflammatory cellular infiltrates (Fig. 10).

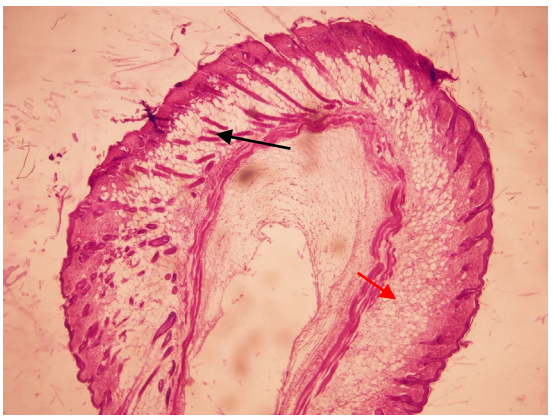

Fig. 9. Histological section performed through the skin from the group treated with ointment for three days post-aggression: immature hair follicles (→), massive presence of adipocytes (→), H.E., 40X

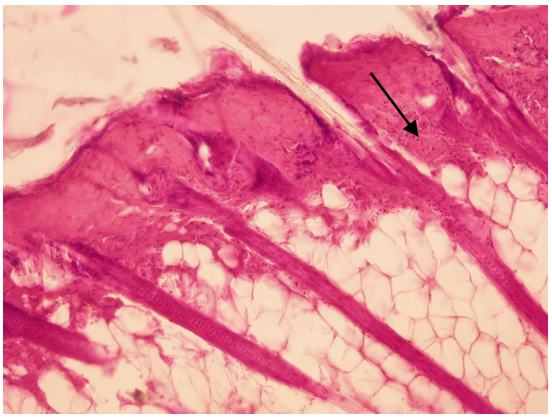

Fig. 10. Histological section performed through the skin from the group treated with ointment for three days post-aggression: much reduced compact mass of collagen (→), H.E., 200X

Structurally, the peripheral area to the aggression showed much more advanced changes in the healing process, with a thickened epidermis, as a result of the proliferation of keratinocytes, which form dermal papillae. Inflammatory cells invaded the entire structure of the skin, the cells being present under the epidermis, among the collagen fibers, among adipocytes, above and below the muscle layer (Fig. 9, 11 and 12).

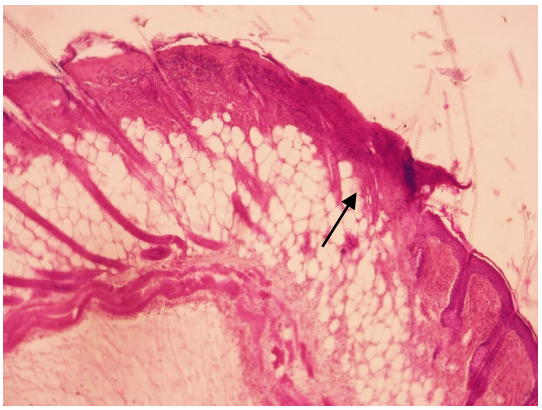

Fig. 11. Histological section performed through the skin harvested from the group treated with ointment for three days post-aggression: reduced mass of collagen (→), H.E., 200X

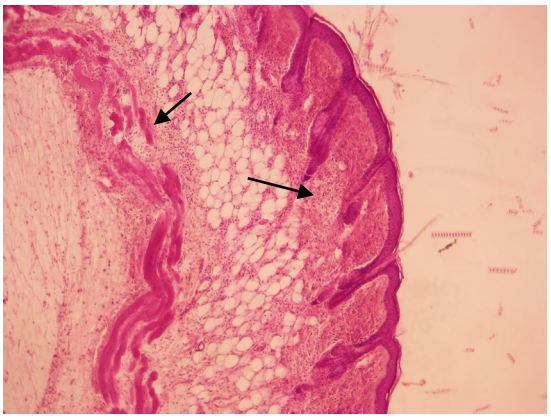

Fig. 12. Histological section performed through the skin harvested from the group treated with ointment for three days post-aggression: inflammatory infiltrate (→), H.E., 100X

Seven days after the action of the aggressor agent on the skin, at the structural level the numerical reduction of adipocytes and the significant increase of the inflammatory infiltrate were evident. In the impact area, a thick crust was present, with relative adhesion, arranged over the inflammatory infiltrate under which rare collagen fibers and sebaceous glands are found, the two structures being newly formed (Fig. 13). In the region adjacent to the impact zone, an exaggerated proliferative phenomenon of keratinocytes was found, with thickened epidermis and the appearance of dermal papillae. The dermis shows newly synthesized collagen fibers and keratinocyte proliferations that will form hair follicles and sebaceous glands. The ratio of adipocytes/inflammatory cells is in favor of the second category, and vascular phenomena were observed under the muscle layer, expressed by hyperemia/vasodilatation (Fig. 14).

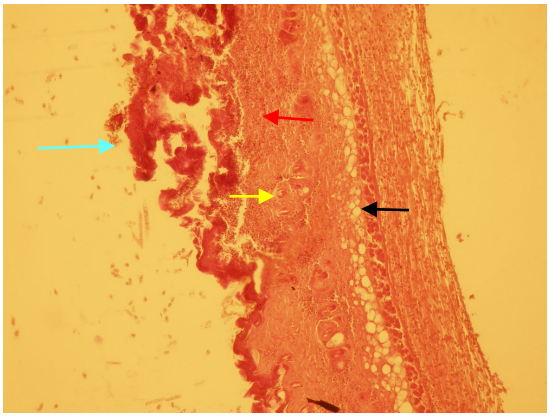

Fig. 13. Histological section performed through the skin from the positive control group, seven days post-aggression: inflammatory infiltrate (→), numerical reduction of adipocytes (→), thick crust (→), newly formed glandular structures (→), col. H.E., 40X

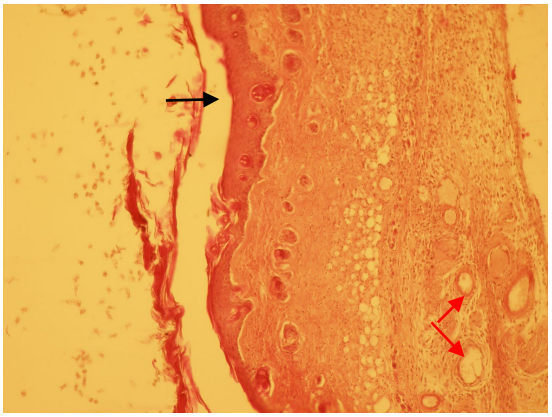

Fig. 14. Histological section performed through the skin harvested from the MA group, seven days post-aggression: thickened epidermis (→), vascular congestion (→) H.E., 40X

After 7 days of application of the ointment with extract of *Centella asiatica* and *Croton lechleri*, the microscopic examination revealed denudation of the impact area, of direct action of the thermal aggressor (Fig. 15). Thus, after this time interval, the lysis of the healing components was found, although the peripheral zone of the thermal trauma is in the phases of tissue proliferation and remodeling, revealed by the thickened epidermis, with the formation of dermal papillae, a sign of complete epithelization and by the presence of inflammatory infiltrate. The number of adipocytes is relatively increased compared to that of the untreated group, in this case, the layer formed by them being sensitive to lytic processes (Fig. 16). Denudation of the impact area will generate the establishment of additional protection methods.

In both cases, the maintenance of

environmental hygiene conditions and appropriate food prevented the installation of secondary infections, which would have hindered the healing processes.

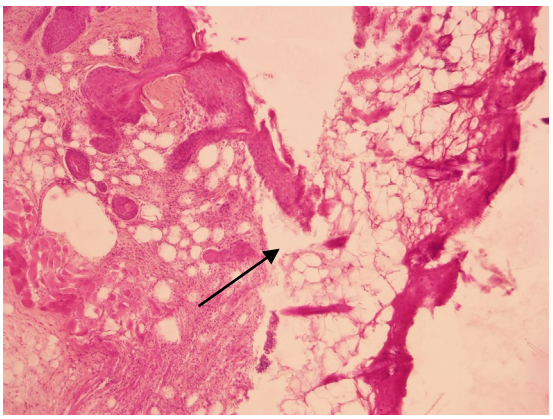

Fig. 15. Histological section performed through the skin harvested from the batch treated with ointment for seven days post-aggression: denudation of the impact area (→), H.E., 100X

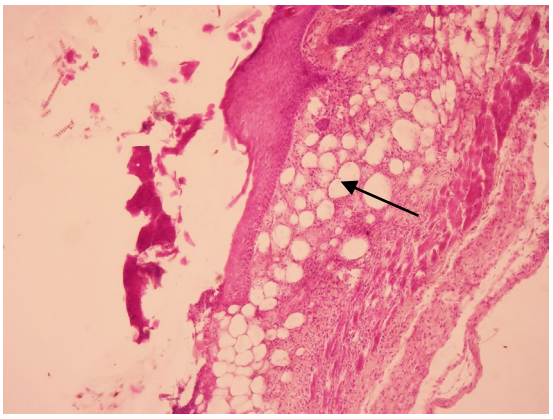

Fig. 16. Histological section performed through the skin harvested from the batch treated with ointment for seven days post-aggression: numerous adipocytes (→), H.E., 100X

Conclusions

The absence of the establishment of an infectious process that could have caused complications and that could have interrupted or hindered the reparative phases of the skin in the area affected by the thermal agent highlighted the importance of respecting certain hygiene conditions of the microclimate. Although, separately, the two plants studied are used in the treatment of various skin conditions, together, after an interval of 7 days, the tissue components in the impact area suffered a lysis phenomenon, the area remaining completely denuded. To overcome this obstacle, it is necessary to establish additional protection and/or treatment measures, even if in the peripheral area, the contraction reparative events follow their normal course. We recommend a reorientation of the use of this type of ointment towards other skin conditions, which manifest with hyperkeratosis, such as callus, for example.

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ANTIMICROBIAL RESISTANCE IN SOME *E. COLI* STRAINS ISOLATED FROM DOGS WITH DIFFERENT CLINICAL SIGNS

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Abstract

Lately, the antimicrobial resistance in a large number of bacterial germs has become a global threat to public health. Among the bacteria that represent the greatest threat to human health, due to the increase of antimicrobial resistance, are also those included in *Enterobacteriaceae* family, especially *Escherichia coli*, *Salmonella* spp. and *Klebsiella* spp. The aim was to identify the *Escherichia coli* strains isolated from dogs, as well as to establish the frequency of resistance phenotypes of these strains. A total of 50 samples with pathological material were taken from dogs with different clinical signs and a number of 43 strains were isolated on Levine medium. With Vitek 2 Compact the isolated strains were included in the Gram-negative *Escherichia coli* species. The results of the disk-diffusimetric Kirby-Bauer method showed that the frequency of lincomycin resistance phenotypes was the highest (65.11%), followed by the one of ampicillin and clindamycin resistance phenotypes (62.79%). Also, *E. coli* strains, isolated from dogs, developed a resistance to enrofloxacin, regardless of the origin of the samples with pathological material. Thus, the results confirm an marked increase of resistant *E. coli* strains to a large variety of antibiotics, frequently used in the therapy of infectious diseases in dogs

***Keywords*:** *antimicrobial resistance, dogs, E. coli strains, resistance phenotypes.*

Antibiotic resistance to bacteria represents a very topical problem both in veterinary, as well as in human medicine, because it is considered a phenomenon with pronounced zoonotic risk. Resistance phenotypes in animal pathogenic bacteria, both Gram-positive and Gram-negative bacteria, are increasing in frequency due to the use of antibiotic-based veterinary medicinal products, especially in farm animals (1, 9, 19).

The spread of multiple antibiotic resistance, in bacterial species pathogenic for animals and humans, determined the performance of extensive phenotypic and genotypic studies to elucidate, as deeply as possible, this phenomenon. Thus, it was demonstrated that antibiotic resistance is genetically coded, supported by many resistance genes present in the bacterial chromosome and in mobile genetic elements (plasmid R, integrons, transposons). Through them, genes can be transferred between strains of the same bacterial species, respectively intraspecific transmission, as well as between strains included in other bacterial species (interspecific transmission) (1, 2, 9, 19).

In the last few years, the antibiotic resistance in a large number of bacterial germs has become a global threat to public health. Among the bacteria that represent the greatest threat to human health, due to the increase in antibiotic resistance, are those included in *Enterobacteriaceae* family, especially *Escherichia coli*, *Salmonella* spp. and *Klebsiella* spp. (5, 6, 9, 10, 12, 19).

Currently, on a global level, approximately 50% of the total number of antibiotics are used in veterinary medicine. Bacteria, which inevitably develop resistance to the antibiotics used in animals, include food-borne pathogens in humans, opportunistic pathogens and commensal bacteria (1, 4, 8, 14, 19).

Last but not least, is worth mentioned the upward trend of using antibiotics in animals which is a direct consequence of the unprecedented increase in global demand for animal protein. A clear certainty is the fact that resistant bacteria, which appear in animals, can represent a risk, not only for animals, but also for veterinarians, farmers and consumers (1, 8, 13, 19, 21).

This research aimed to establish the phenotypes of antibiotic resistance, specifically to determine the frequency of resistance phenotypes in *Escherichia coli* strains isolated from dogs with different clinical signs.

Materials and methods

The samples, represented by pharyngeal exudate, fecal matter, pus, conjunctival secretion, milky secretion, perianal gland secretion and skin samples (interdigital wound and skin pustules) were taken from 50 dogs with various clinical signs.

In order to isolate the primary cultures of *E. coli* strains, the samples with pathological material were inoculated in nutrient broth and incubated at temperatures of 37°C, under aerobic conditions, for a period of 18-20 hours. Next, in order to obtain pure strains of *E. coli*, inoculations on Levine medium were made. The characteristic colonies were subsequently

inoculated on agar with defibrinated sheep blood 7% for the definitive identification of *E. coli* strains, based on Vitek® 2 Compact equipment.

All 43 identified strains of *E. coli* were tested for antimicrobial resistance, using 13 antibiotics from the following classes:

- aminoglycosides: gentamicin (GM);
- *β*-lactams: ampicillin *(*AM), amoxicillin with clavulanic acid (AMC) penicillin *G* (P);
- cephalosporins: cephalexin (CN);
- phenicols: chloramphenicol (C);
- lincosamides: lincomycin (L);
- macrolides: clindamycin (CD), erythromycin (E);
- quinolones: enrofloxacin (ENR);
- sulfonamides: trimethoprim/sulfamethoxazole (SXT);
- tetracyclines: doxycycline (DO);
- other substances: metronidazole (MTR) (22, 23).

The resistance profile was made by the disk-diffusimetric method, respectively the KIRBY-BAUER method, using the Mueller-Hinton medium and biodiscs with the antibiotics mentioned above (24).

According to the EUCAST standard, respectively based on the diameter of the inhibition zone, the results were classified as sensitive/susceptible (S) or resistant (R) (22).

Results and discussions

According to bacteriological and microscopic examinations, respectively based on the morphological and cultural characters developed by the inoculated bacteria, a total of 43 bacterial strains, included in genus *Escherichia*, were isolated, while a number of seven samples were sterile. Furthermore, with the Vitek 2 Compact equipment, the *E. coli* species was identified in all of the 43 strains isolated from dogs (Table 1).

Table 1

***E. coli* strains isolated from dogs and identified with the Vitek 2 Compact system**

Crt. no.	Sample	Identified bacterial species	No. of positive samples	
			No.	%
1.	Pharyngeal exudate	*E. coli*	31	72.11
2.	Feces		5	11.63
3.	Interdigital wound		1	2.32
4.	Purulent material		1	2.32
5.	Skin pustules		1	2.32
6.	Conjunctival secretions		1	2.32
7.	Perineal gland secretion		2	4.66
8.	Milk secretion		1	2.32
TOTAL			**43**	**100**

Thus, the E. coli species was identified in 31 strains isolated from pharyngeal exudate, five strains from faecal matter, two strains from the secretion of the perianal gland and one strain each from the interdigital wound, milk secretion, conjunctival secretion, skin pustules, respectively purulent material.

These results regarding the identification of the *E. coli* species in the strains isolated from dogs are similar to the existing data in the literature, where several researchers focused on the field of canine infectious pathology.

Thus, the results obtained from the samples collected from the nasopharyngeal region of dogs, were also confirmed by the results obtained from other authors. For example, in a study, Moonsun et al. (14) reported that bacterial pathogens were isolated from 36 dogs with respiratory symptoms. Of the 36 positive samples, 16 (44.4%) were Gram-positive and 20 (55.6%) were Gram-negative. The identification of the species was carried out with API 20E or API NE identification systems, which are based on biochemical characters. From the total number of Gram-negative bacteria, a percentage of 16.7%, was identified as *Escherichia coli* species (14).

Researches carried out on the identification of the *E. coli* species, in dogs with various clinical signs of the digestive tract or in healthy dogs, were also confirmed by other research teams. Thus, Marchetti et al. (13) in 2016, in Buenos Aires, Argentina, conducted a study on fecal samples collected from both pet dogs (n = 50) and stray dogs (n = 50). A number of 197 bacterial strains were isolated, of which 95 strains were biochemically identified as *E. coli*, respectively 46 strains of *E. coli* in the case of stray dogs and 49 strains in the case of pet dogs. Another study, conducted by Banik et al. (5) was conducted to detect *E. coli* strains from dogs with diarrhea symptoms, from South Bengal, India.

Therefore, 112 fecal samples collected from these dogs were tested between May and September 2012, identifying a percentage of 63.4% (71) positive samples for Escherichia coli. (5, 13).

The results regarding the identification of resistance phenotypes, depending on the classes of antibiotics, as well as the frequency of this resistance were processed and shown in Table 2.

Table 2

Antibiogram results for *E. coli* strains isolated from dogs

Antibiotic	C (µg)	Diameter of inhibition zone (mm)		S		R		Tested strains
		S≥	R <	No.	%	No.	%	
Gentamicin	10	17	17	25	58.13	18	41.86	**43**
Ampicillin	10	14	14	16	37.20	27	62.79	**43**
Amoxicillin+ clavulanic acid	20-10	19	19	23	53.48	20	46.51	**43**
Penicillin G	6	-	-	19	44.18	24	55.81	**43**
Cephalexin	30	14	14	23	53.48	20	46.51	**43**
Chloramphenicol	30	17	17	23	53.48	20	46.51	**43**
Lincomycin	15	-	-	15	34.88	28	65.11	**43**
Clindamycin	2	-	-	16	37.20	27	62.79	**43**
Erythromycin	15	-	-	18	41.86	25	58.13	**43**
Enrofloxacin	-	-	-	31	72.09	12	27.90	**43**
Doxycycline	30	-	-	23	53.48	20	46.51	**43**
Trimethoprim/ Sulfamethoxazole	1.25/23. 75	14	11	20	46.51	23	53.48	**43**
Metronidazole	4	-	-	17	39.53	26	60.46	**43**

Legend: C = concentrație; S = susceptible strains; R = resistant strains (22)

A number of 14 antibiotics from several classes were used to identify the resistance phenotypes, as multiple antibiotic resistance can be transmitted (through R factor) both interspecifically and intraspecifically.

Based on the results, was noticed that susceptible strains had a frequency between 34.88% (L) and 72.09% (ENR), while resistant strains had a frequency between 27.90% (GM) and 65.11% (L), the interpretations being made according to EUCAST recommendations (22).

From the group of aminoglycosides, resistance phenotypes were observed only against gentamicin. Thus, the resistance of *E. coli* strains was reported at a frequency of 41.86%, while the susceptibility was identified at a frequency of 58.13%.

In the case of the β-lactam group, the most antibiotics were selected to determine the resistance phenotype, namely ampicillin, amoxicillin with clavulanic acid and penicillin G, considering that in the therapy of some infections, produced by Gram negative bacteria in dogs, β -lactams are recommended.

Therefore, in the case of ampicillin, the susceptibility had a frequency of 37.20%, that of amoxicillin with clavulanic acid a frequency of 53.48%, respectively for penicillin G a frequency of 44.18%. Regarding the antibiotic resistance, the highest frequency was in *E. coli* strains resistant to ampicillin (62.79%), followed by penicillin G (55.81%) and amoxicillin with clavulanic acid (46.51 %).

From the group of cephalosporins, the antibiotic cephalexin was selected to identify resistance phenotypes. Based on obtained results, was observed that the frequency of resistant strains (46.51%) was lower than that of susceptible strains (53.48%).

Also, in the case of the group of phenicols, where resistance phenotypes were followed only to chloramphenicol, the same values presented in the case of the antibiotic cephalexin were found.

The results obtained for the lincosamide group, regarding the resistance phenotypes to lincomycin, reported a frequency of 65.11% for resistant strains and a frequency of 34.88% in the case of susceptible strains.

Two antibiotics were selected from the macrolide group, namely erythromycin (which indicates the inducible resistance to 14-atom macrolides) and clindamycin (indicating the inducible resistance to 16-atom macrolides). Of the total number of strains, the resistant ones had a frequency of 62.79% to clindamycin, respectively 58.13% to erythromycin. The frequency of *E. coli* strains susceptible to erythromycin was 41.86%, and the one of *E. coli* strains susceptible to clindamycin was 37.20%.

Phenotypes of resistance to the quinolone group were only observed for enrofloxacin. Thus, analyzing the results, was found that the frequency of susceptible strains (72.09%) was higher than that of resistant strains (27.90%).

Resistance phenotypes to tetracycline antibiotics was established for doxycycline. The results obtained revealed a frequency of 53.48% of susceptible strains, higher than that of resistant strains (46.51%).

From the sulfonamide group, the antibiotic trimethoprim with sulfamethoxazole was selected to identify resistance phenotypes and the following values were identified: a frequency of 53.48% for resistant *E. coli* strains, respectively a frequency of 46.51% for susceptible *E. coli* strains.

Another antimicrobial substance, such as metronidazole, was used to establish the resistance phenotypes. Thus, the frequency of isolated *E. coli* strains resistant to metronidazole was 60.46%, while the frequency of susceptible strains was 39.53%.

The results obtained revealed the presence of certain resistance phenotypes in *E. coli* strains isolated from dogs. Multidrug-resistant *E. coli* strains are increasing in frequency, thus the identification of these resistance phenotypes to antibiotics used in therapy indicates a continued expansion of this phenomenon through a complex, two-way animal-human epidemiological circuit. Therefore, there are numerous research groups that focus on identifying resistant *E. coli* strains, as well as their transmission from animals to humans and vice versa (9, 12, 15).

For example, a study by Roca L. et al. (18) aimed to determine the antibiotic resistance of 81 pathogenic bacterial strains isolated from dogs. The most common species isolated were *Staphylococcus intermedius, Pseudomonas aeruginosa* and *Escherichia coli*. The results indicated the resistance of these species to some antibiotics, namely: *S. intermedius* was resistant to trimethoprim/sulfamethoxazole (31%) and enrofloxacin (23%), *P. aeruginosa* was resistant to cephalexin (86%) and clindamycin (76%), and *E. coli* was resistant to clindamycin (78%) and trimethoprim/sulfamethoxazole (75%). Mainly, the species *P. aeruginosa* and *E. coli* showed the highest frequencies of resistance (18).

Also, Qekwana et al. (17) reported data on antibiotic resistance in *E. coli* strains from urine samples of dogs (168/755). Therefore, the authors revealed high levels of antibiotic resistance to penicillin G (99%), clindamycin (100%), tylosin (95%), cephalothin (84%), but also relatively low levels of resistance to enrofloxacin (16%) (17).

Another study, conducted by Thungrat et al. (20) described the antimicrobial susceptibility patterns for *E. coli* isolates from dogs (n = 2392) and cats (n = 780), collected from 6 different regions of the United States of America. Thus, the frequency of antibiotic resistance was as follows: doxycycline (100%), ampicillin/clavulanic acid (48%), enrofloxacin (10%), chloramphenicol (9.6%), trimethoprim-sulfamethoxazole (7.9%), gentamicin (7.9%) and amikacin (0.7%) (20).

According to studies conducted by various research groups, multiple resistance to antimicrobial substances, within *E. coli* serotypes isolated from dogs, was different, depending on the site of sample collection, but also on the used antibiotic (3).

Research conducted by Daodu et al. (7) on the antibiotic resistance profile confirmed the presence of 41 *E. coli* strains from 173 samples collected from the respiratory tract of clinically healthy dogs. Thus, the antibiotic resistance had values such as: amoxicillin with a number of 22 resistant *E. coli* strains and a frequency of 53.7%, chloramphenicol with 9 resistant strains and a frequency of 22%, respectively gentamicin with a number of 12 resistant strains and a frequency of 29.3% (7).

Hata et al. (10) conducted a study on the antimicrobial resistance of *E. coli* strains from 61 fecal samples from dogs in a shelter located in Kanto, Japan. Thus, antimicrobial resistance was detected in 18 dogs, including the following antibiotics: ampicillin with a frequency of 55.3%, gentamicin with a frequency of 14.1%, and chloramphenicol with a frequency of 12.6% (10).

Nocera et al. (16) conducted a 4-year retrospective study at the University of Veterinary Medicine of Naples, in which a total of 189 bacteriological samples were collected from 171 dogs and 18 cats with different skin infections. Thus, regarding the antibiotic resistance of *E. coli* strains, downward trends were observed during these years for amoxicillin/clavulanic acid, ampicillin, enrofloxacin, and trimethoprim/sulfamethoxazole, ranging from 100% to 71%, from 100% to 57%, from 50% to 29%, and from 100% to 43%, respectively. Meanwhile, Hewitt et al. (11) conducted a study, aiming to identify the bacterial strains and antibiotic susceptibility in 476 pathological samples from dogs suspected of bacterial keratitis, in Iowa, USA. As a result, the antibiotic resistance of the identified *E. coli* strains (10 isolates) was to the following antibiotics: chloramphenicol with a frequency of 100% (10/10), doxycycline 90% (9/10), gentamicin 100% (10/10),

trimethoprim/sulfamethoxazole 90% (9/10) (11, 16).

Therefore, the results obtained regarding the resistance phenotypes of *E. coli* strains isolated from dogs highlight the importance of identifying these strains, which may have a zoonotic character. Thus, pets can act as a true microbial reservoir for humans, especially for their owners, but also vice versa, demonstrating the complex epidemiological circuit existing in Gram-negative bacteria.

Conclusions

The highest frequency of resistant *E. coli* strains (65.11%) was towards lincomycin.

The *E. coli* strains isolated from dogs showed resistance phenotypes to enrofloxacin, regardless of the collection site of the pathological samples.

The results regarding the research conducted confirm an obvious increase in resistance phenotypes to a wide range of antibiotics, frequently used in combating infectious diseases in dogs.

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COMPLETE BLOOD COUNT CHANGES IN THE BITCH WITH PYOMETRA

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Abstract

Pyometra is a common pathology of the reproductive system and represents the primary emergency amongst intact bitches. This study aimed to determine the main changes in the complete blood count in the bitch with pyometra and observe the differences in the complete blood count between patients evolving with open-cervix pyometra and patients with closed-cervix pyometra. For carrying out this study, 16 bitches of different breeds and ages affected by pyometra were selected and underwent a complete blood count analysis. Five evolved with closed-cervix pyometra, and 11 evolved with open-cervix pyometra. Neutrophilic leukocytosis was found in both groups of bitches, but there were no significant differences in the values of leukocytes, neutrophils, lymphocytes and monocytes. However, the mean values of eosinophils and basophils were significantly higher in the bitches with pyometra with a closed cervix. The mean values of erythrocyte count, haematocrit and haemoglobin were within physiological limits in both groups of bitches.

Keywords: *pyometra, complete blood count, reproductive system, bitch.*

Pyometra represents an acute or chronic infection of the uterus with an accumulation of puss in the lumen. Pyometra is the pathology with the most considerable prevalence amongst reproductive system pathologies in the bitch affecting around 25% of intact bitches. Moreover, it is the primary reproductive emergency of the intact bitch (3, 5, 11).

The disease primarily occurs in diestrus bitches, its onset being closely correlated with the female's estrous cycle. The high level of progesterone, specific to diestrus, favours the secretory activity of the endometrial glands, thus promoting endometrial proliferation, decreasing myometrium contractility and inducing cervical closure. The pathology is usually diagnosed between 4 weeks and 4 months after estrus, and in the early stages, it causes subtle changes. Therefore, the diagnosis is often made late in the course of the disease, making the patients present to the clinic with an altered general condition. Pyometra-diagnosed bitches can be categorized as closed-cervix or open-cervix, with the first one being considered an emergency having more severe symptoms, a more altered general state and a greater risk of sepsis and death (10, 11).

Pyometra generally affects adult, senior or geriatric bitches with a mean age of diagnosis of 7 years, but the age of the patient can range from 4 months to 18 years (15). Affected bitches may exhibit symptoms such as lack of appetite, altered general condition, polydipsia, lethargy and abdominal distention, with or without vaginal discharge.

The bitches are usually afebrile but may have an increased white blood cell count and prerenal azotaemia accompanying dehydration (with hyperproteinemia and hyperglobulinemia) (11). In addition to the clinical signs common in pyometra, one study reports the presence of lameness for the first time in 16% of the animals included in the study (8). The main changes in total blood count parameters described in the literature are low red blood cells, lymphocytosis, neutropenia, leucocytosis and eosinophilia (9). As the clinical signs of the closed-cervix pyometra are nonspecific, ultrasound correlated with a complete blood count analysis plays an important role in confirming the diagnosis.

Materials and methods

The study occurred between June 2021 and October 2023 at Prime Veterinary Practice in Chesterfield, United Kingdom. In this study, 16 bitches affected by pyometra were selected and underwent a complete blood count analysis. The presence of intrauterine content was determined by abdominal ultrasonography. Every patient underwent a complete clinical exam at admission, and anamnesis was taken from each owner. The presumptive diagnosis was based on clinical signs, history and ultrasonography. The confirmation of the diagnosis was determined by post-ovariohysterectomy gross examination of the intrauterine puss collection.

Fig. 1. Sonoscape S9Pro ultrasound and micro convex C612 probe

In order to perform the abdominal ultrasonography to determine the dimensions of the uterine horns and the presence of secondary pathologies and simultaneous neoplasms, Sonoscape S9Pro ultrasound and a micro convex C612 probe were used (Fig. 1). 2 ml blood samples were collected with aseptic precautions from each patient using cephalic vein catheterization. The samples were collected in EDTA k3 anticoagulant vials. The complete blood count parameters like WBC (leucocytes), LYM (lymphocytes), MON (monocytes), NEU (neutrophils), EOS (eosinophils), BAS (basophils), RBC (erythrocytes), HGB (haemoglobin), HCT (haematocrit), MCV (mean corpuscular volume), MCH (mean corpuscular haemoglobin), MCHC (mean corpuscular haemoglobin concentration), PLT (platelets), MPV (mean platelet volume) were estimated using Abaxis VetScan HM5c haematology machine.

The elected treatment for all the patients included in the study was surgical treatment based on OVH (ovariohysterectomy). The anaesthetic protocol consisted of premedication with Methadone 1% (Insistor®, Axience) at a dose of 0.3 mg/kg i.v., and Medetomidine 0.1% (Domitor®, Orion Pharma) at a dose of 10 µg/kg (0.01ml/kg) i.v., induction with Propofol MCT/LCT 1% (Fresenius Kabi) 2 mg/kg i.v. and maintenance with Izoflurane 1000mg/g (Isothesia® NXT, Covetrus). In the cases were there were observed structural changes in the mammary gland, a regional mastectomy or a complete mammary chain excision was performed. In the cases where a perforation in the uterine horn or the existence of purulent collection in the abdominal cavity were noticed during the OVH procedure, the purulent collection was removed via aspiration and an abdominal cavity lavage was performed using warmed saline (NaCl 0.9%).

For all statistical measurements and data analysis IBM SPSS Statistics program, version 23.0 was used. All statistical analyses were considered significant at $p<0.05$.

Results and discussions

Out of the 16 bitches selected, five manifested closed-cervix pyometra and were assessed to group 1 and 11 open-cervix pyometra and were assessed to group 2. The incidence of closed-cervix pyometra was 31.25 % (5/16) while open-cervix incidence was 68.75% (11/16). The youngest patient diagnosed was 3 years of age, the oldest being 8 years old and the mean age of diagnosis was 5.6 years ± 1,76 SD.

The results of the present study are consistent with other studies from the literature. Verma et al. (13) reports a three times higher incidence for open-cervix (73.77%) than closed-cervix (26.23%) as well as Vidya et al. (14) who also found a higher incidence of open cervix pyometra than closed cervix pyometra (13, 14). The literature reports a mean age of diagnosis of 7 years, ranging from 3 months to 20 years of age but in Sweden, only 20% of pyometra infected bitches, are diagnosed under the age of 10 years old (4, 7).

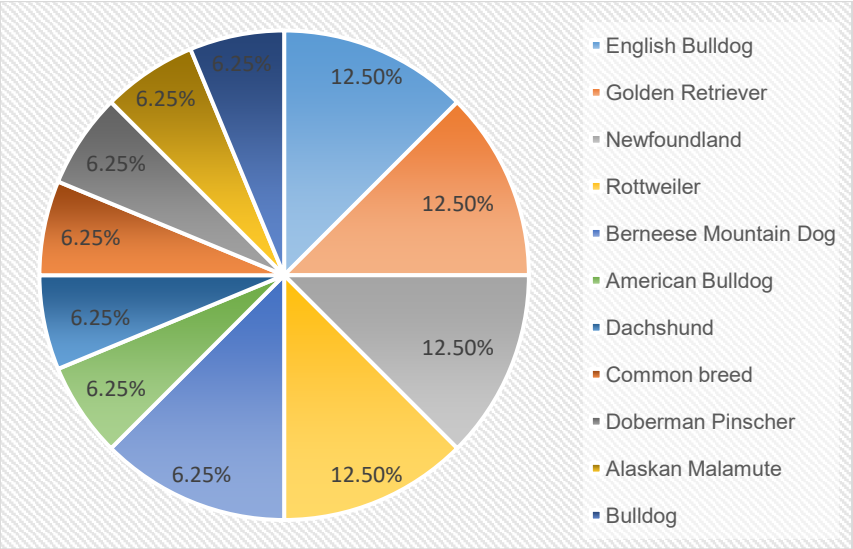

Fig. 2. Percentage of breeds included in the study

The breed prevalence of the patients included in this study reveals a 12.5% for English Bulldog, Golden Retriever, Newfoundland, and Rottweiler with two patients for each breed and a 6.25% for Bernese Mountain Dog, Bulldog, American Bulldog, Dachshund, Doberman Pinscher, Alaskan Malamute, and mixed breed with one patient of each breed (Fig. 2). Even though breed predisposition is speculative varying from one study to another, the literature reports over-representation within certain breeds such as Rottweiler, Golden Retriever and Cavalier King Charles spaniel. (2, 15). The only potential explanation for the predisposition of a particular breed was revealed in a study of 194 Golden Retriever females in which a potential correlation between pyometra and specific changes in the ABCC4 gene located on chromosome 22 was identified (1).

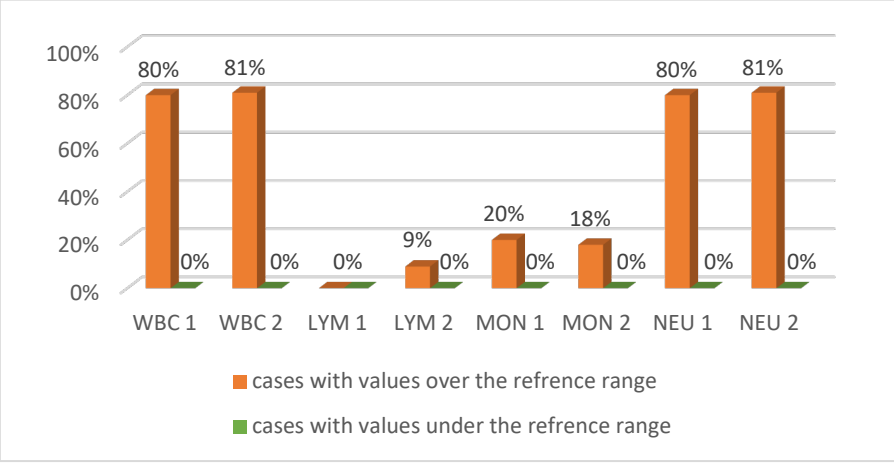

Fig. 3. Percentage of cases showing changes in leukogram parameters in bitches of groups 1 (closed cervix) and 2 (open cervix)

The most remarkable hematologic changes were leukocytosis and neutrophilia, which occurred in 80% (4/5) of the cases in group 1 and 81% in group 2, while monocytosis was found in only 20% of cases in group 1 and 18% in group 2, respectively. In group 1, no lymphocytosis was found in any case, while in group 2, only 9% had lymphocytosis (Fig. 3). Comparing the mean values of the leukogram parameters between the two groups, only the mean values of eosinophils and basophils, which, although were within physiological limits, were significantly higher ($p<0.05$) in the bitches with closed cervix pyometra than in those with open cervix. There were no significant differences in mean leukocyte, monocyte and lymphocyte counts ($p<0.05$) (Tabel 1). Unlike our reports, Jitpean et al. (7) 2017 report total white blood cell count (WBC), segmented neutrophils, and monocyte numbers were

significantly higher in dogs with closed cervix pyometra (6). Also, Paudel et al., 2023 report leucocytosis in 64% of closed-type pyometra patients compared to 20% of open-type pyometra patients. Lymphocytosis is reported at 72% for closed type compared to 15% of open-type pyometra patients, and neutropenia at 64% versus 25% (9).

Table 1

Descriptive statistics of the complete blood count parameters values in the bitch with pyometra

Haematological parameters	Groups	Mean	Std. Deviation	Std. Error	Minimum	Maximum	Reference Range
WBC (10^9/l)	1	25.55	10.25	4.58	10.51	39.20	6.0-17.0
	2	25.11	11.17	3.37	8.01	44.79	
LYM (10^9/l)	1	1.90	0.50	0.22	1.36	2.43	1.0-4.8
	2	1.82	1.03	0.31	0.70	3.93	
MON (10^9/l)	1	0.95	0.69	0.31	0.27	1.89	0.2-1.5
	2	1.29	0.86	0.26	0.41	3.09	
NEU (10^9/l)	1	22.17	9.74	4.35	8.37	35.47	3.0-12.0
	2	21.91	9.51	2.87	6.86	37.85	
EOS (10^9/l)	1	0.39	0.21	0.09	0.18	0.71	0.0-0.8
	2	0.07	0.09	0.03	0.03	0.35	
BAS (10^9/l)	1	0.14	0.12	0.05	0.04	0.33	0.0-0.4
	2	0.02	0.04	0.01	0.00	0.13	
RBC (10^12/l)	1	6.60	1.40	0.62	4.32	7.69	5.5-8.5
	2	6.88	0.98	0.30	5.26	8.60	
HGB (g/dl)	1	14.20	3.12	1.39	9.30	17.00	12.0-18.0
	2	15.06	1.87	0.56	11.90	18.60	
HCT (%)	1	42.94	8.79	3.93	29.22	49.32	37.0-55.0
	2	46.74	5.03	1.52	37.77	55.14	
MCV (fl)	1	65.40	2.30	1.03	63.00	68.00	60.0-77.0
	2	68.36	5.57	1.68	61.00	75.00	
MCH (pg)	1	21.48	0.82	0.37	20.60	22.80	19.5-24.5
	2	21.98	2.25	0.68	18.50	25.20	
MCHC (g/dl)	1	33.00	1.59	0.71	30.80	34.60	31.0-39.0
	2	32.21	1.65	0.50	29.70	35.30	
PLT (10^9/l)	1	265.8	84.03	37.58	148.0	352.00	165-500
	2	213.6	178.87	53.93	4.00	640.00	
MPV (fl)	1	10.44	0.78	0.35	9.30	11.30	3.9-11.1
	2	10.87	1.25	0.38	9.20	12.90	

The results of our study differ from those reported by other authors. From a hematological perspective, Hagman (3) found that 61% of bitches diagnosed with pyometra exhibited leukocytosis, while 4% showed leukopenia and neutropenia. Additionally, 55% had neutrophilia and anemia, 3% experienced monocytopenia, 50% had monocytosis, 37% were thrombocytopenic, and 9% displayed toxic changes (3).

It is to be noted that anaemia was found in only 20% of bitches in group 1 and only 9% of bitches in group 2 (Fig. 4). There were no significant differences in the mean values of erythrocyte count, hematocrit and haemoglobin between the two groups of bitches (Table 1). The results in our study are in contradiction with those reported in a study of 12 bitches with pyometra, in which hypochromic and microcytic anaemia was found (12). Furthermore, Hagman (3) reports the presence of anaemia in 55% of bitches with pyometra, while Paudel et al. (9) report a low RBC value in 93.3% of patients and low haemoglobin in 57.8% (3, 9, 12).

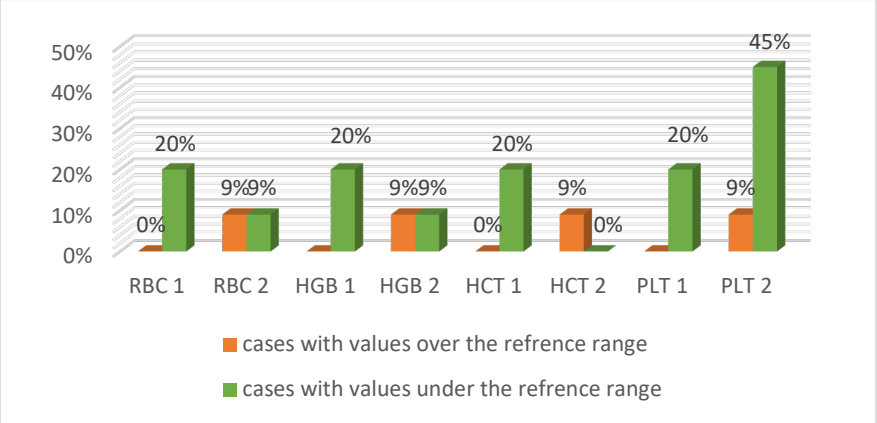

Fig. 4. Percentage of cases with changes in erythrocyte count, haemoglobin, haematocrit and platelet count in bitches of groups 1 (closed-cervix) and 2 (open-cervix)

Conclusions

The main haematological change was neutrophilic leukocytosis in both closed and open cervix pyometra bitches. Anaemia was not consistently observed in bitches with pyometra, regardless of the type of evolution.

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PATTERNS OF ANTIMICROBIAL RESISTANCE IN *E. COLI* ISOLATES FROM EUROPE'S WILD BIRDS

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Abstract

The presence of antimicrobial-resistant (AMR) *E. coli* in wild birds represents a growing risk to ecosystem health and public safety, as migratory species may transmit resistant bacteria across borders and habitats, impacting both wildlife and human populations. To address this issue, a systematic literature review was conducted using the PubMed database, which revealed significant findings that include high rates of multidrug-resistant (MDR) *E. coli*, particularly in species such as yellow-legged gulls (*Larus michahellis*), mallards (*Anas platyrhynchos*), northern goshawks (*Accipiter gentilis*), common buzzards (*Buteo buteo*), and Eurasian Griffon Vultures (*Gyps fulvus*). This review provides an extensive analysis of antibiotic resistance patterns in wild bird populations across Europe from 2013 to 2024, a period marked by rising awareness of AMR in natural ecosystems. Over this period, numerous studies have documented not only the prevalence of antibiotic resistance but also variations in resistance levels across diverse avian species inhabiting different ecosystems. Key topics addressed include the emergence of MDR strains in response to environmental pressures, the impact of anthropogenic factors on resistance spread, and the role of wild birds as reservoirs and vectors of antibiotic-resistant bacteria, posing risks for cross-species transmission. These findings underscore the critical role of wild birds as reservoirs of antibiotic-resistant bacteria, with significant implications for public health and wildlife conservation.

***Keywords**: wild birds, E. coli, antimicrobial resistance, multidrug-resistant, Europe.*

Escherichia coli is responsible for both posing a public health risk and for many economic losses in the industry of various livestock species. The declining efficiency of antimicrobials, correlated with the increasing antibiotic resistance of microorganisms, concerns that the available treatment options for infections caused by bacteria, including *E. coli*, will be significantly limited.

Moreover, birds may play an important role in the dissemination of microorganisms during migration. The fact that antibiotics enter the environment, e.g. into terrestrial waters, also poses a threat of acquiring drug resistance by bacteria. Despite growing interest in studying the occurrence and pathogenicity of bacterial strains isolated from wild birds, there is still little data on their prevalence, transmission routes, and drug resistance (17).

Antibiotic resistance (AMR) presents a multifaceted challenge involving humans, animals, and the environment. However, the contribution of wildlife to the dissemination of antibacterial resistance may be underestimated. Reports of antibiotic-resistant (AR) bacteria in wild fauna have been increasingly documented across various animal species and habitats since the identification of chloramphenicol-resistant *Escherichia coli* isolates in Japanese wild birds in 1977 (21, 25).

Wildlife populations may not directly encounter antibiotics; however, they are susceptible to AR bacteria due to indirect exposure through the environment. Antibiotic residues and AR bacteria from human and animal waste contaminate the environment, posing a risk of transmission to wildlife. The emergence of third-generation cephalosporin-resistant (3GC-R) *Enterobacteriaceae*, initially observed in hospitals and more recently in communities, highlights the global spread of AR bacteria.

Given the potential role of wildlife in AR transmission, it is imperative to consider their protection from exposure. Addressing AMR requires a comprehensive approach that encompasses the interconnectedness of human, animal and environmental health to effectively mitigate the spread of antibiotic resistance (12, 24).

The primary objective of this study was to determine the spread of AR *E. coli* in wild birds across Europe countries in the last decade.

Materials and methods

The systematic literature review was conducted to identify publications reporting on the presence of *Escherichia coli* in wild birds across Europe. Using the PubMed database, we searched from 2013 to March 2024, with the keywords '*E. coli*', '*wild birds*', and '*Europe'*, with no restrictions on language or article type. This review targeted studies containing *Escherichia coli* isolates from European wild birds within the last decade.

The selection process included two stages: (i) an initial screening of titles and abstracts to exclude publications not meeting the inclusion criteria (e.g., those not focused on *E. coli*, wild birds, or Europe), and (ii) a full-text review of relevant studies to extract pertinent data. All

query results were manually verified to exclude duplicates and ensure accuracy in data collection.

Following this process, the initial set of publications was narrowed down to 34. A subsequent detailed review of these full texts resulted in 22 studies meeting the inclusion criteria, with further refinement isolating a subset focused explicitly on antimicrobial resistance (Fig. 1).

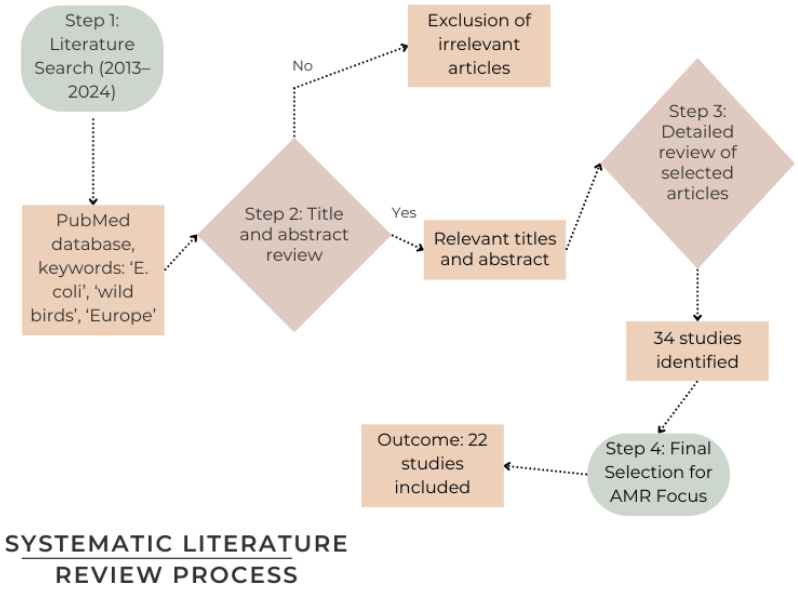

Fig. 1. Methodology of the review process

Wild birds antimicrobial resistance in Europe

Antibiotic resistant bacteria present a growing global healthcare challenge. In Europe, antimicrobial resistance in Gram-negative bacteria is on the rise, particularly in *E. coli*, which constitutes a majority of invasive Gram-negative isolates in European countries represented in Figure 2 (3, 7).

Environment plays a key role in the spread of antimicrobial resistance serving as an unlimited reservoir of antimicrobial resistance genes (10).

Escherichia coli is typically chosen as the representative indicator of antimicrobial resistance in Gram-negative bacteria, and may be relevant to human as well as veterinary medicine (22).

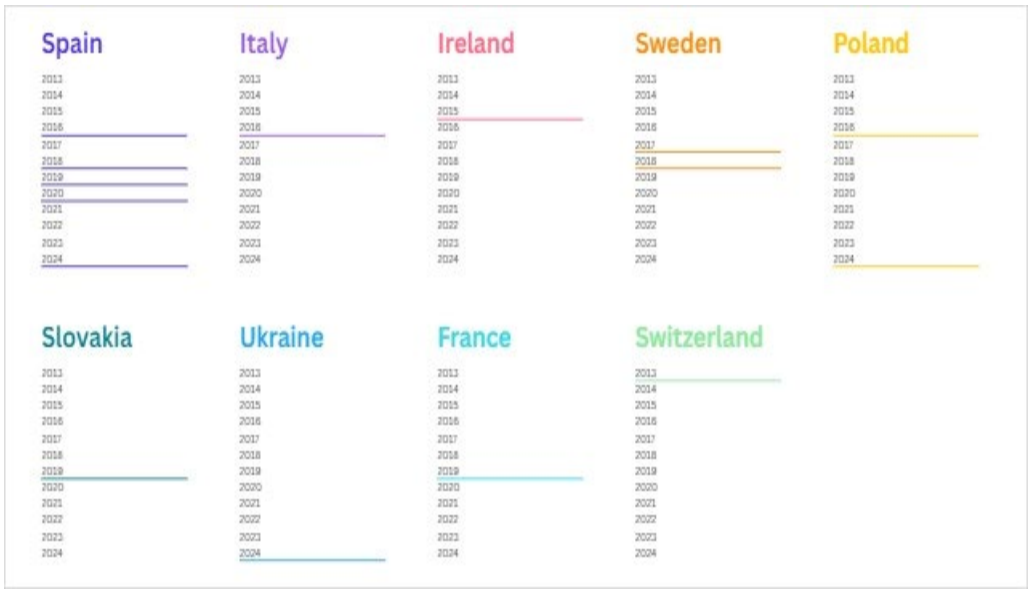

Fig. 2. Timeline of *E. coli* isolation from wild birds in Europe (2013–2024)

Poland

During the years 2011 to 2013 in Poland, isolates of *E. coli*, obtained from Mallards, showed the highest resistance to tetracycline. This resistance was found in 15.8% of all *E. coli* collected from this birds species (16).

Another study titled "Occurrence and Molecular Characterization of *Escherichia coli* Strains Isolated from Black Grouse (*Lyrurus tetrix*) in the Karkonosze National Park, Poland" investigated the presence and characteristics of *E. coli* strains in fecal samples collected during the winter months of 2017 and 2018. A total of 27 *E. coli* isolates were obtained from these samples. Antibiotic resistance testing using the disk-diffusion method revealed that 2 strains were resistant to amoxicillin with clavulanic acid (7.4%), while one exhibited intermediate resistance (3.7%). Additionally, 14 strains (51.9%) were resistant to sulfamethoxazole, with 3 strains (11.1%) showing intermediate resistance. Three strains (11.1%) displayed intermediate resistance to ampicillin, and 6 strains (22.2%) were resistant to gentamicin. Interestingly, all tested strains (n = 27) were susceptible to ciprofloxacin, tetracycline, and nalidixic acid, with 8 strains (29.6%) showing sensitivity to all tested antibiotics. Furthermore, in a broth microdilution test, all isolates (100%) were found to be susceptible to colistin (17).

Switzerland

Following into 2013, it was found that all isolates were classified as multidrug resistant due to their resistance patterns. Specifically, all strains were resistant to ampicillin and the first-generation cephalosporin cephalothin.

Furthermore, three isolates (one from a pigeon and two from cormorants) were resistant to the third-generation cephalosporin cefotaxime in the disk diffusion test but were susceptible to ampicillin-clavulanic acid. Additionally, the strains from pigeons, showed resistance to ampicillin-clavulanic acid in the disk diffusion test. However, all isolates remained susceptible to the fourth-generation cephalosporin cefepime and the carbapenem antibiotic imipenem.

Resistance to nalidixic acid was detected in 75% of the pigeon strains and in one cormorant strain, with the latter also showing resistance to ciprofloxacin. All isolates were resistant to tetracycline, while one pigeon isolate and both cormorant strains were resistant to sulfamethoxazole and trimethoprim. However, all strains tested fully susceptible to chloramphenicol (26).

Ireland

In Ireland, between May 18, 2013, and June 28, 2013, sampling was conducted at seven different sites. The susceptibility testing involved a panel of seven antimicrobial agents, including amoxicillin-clavulanic acid (AMC), ampicillin (AMP), ciprofloxacin (CIP), penicillin G (P), streptomycin (S), and tetracycline (TET). Penicillin G, chosen for comparison and to demonstrate *E. coli*'s intrinsic resistance, was administered at 5 µg. The other antibiotics were given at the following concentrations: amoxicillin-clavulanic acid (20/10 µg), ampicillin (10 µg), ciprofloxacin (5 µg), streptomycin (10 µg), and tetracycline (30 µg).

Eight *E. coli* isolates were found to be resistant to two or more antimicrobial compounds, excluding Penicillin G (all isolates were resistant to penicillin G.). In total, resistance to more than one antimicrobial agent was detected in 10 isolates (5.4%) representing eight out 146 samples. The dominant resistance type (excluding penicillin G) was tetracycline, which was detected in all 10 isolates (5.4%, n = 146), followed by streptomycin, which was detected in seven isolates (4.7%).

In this study, AMR was found in all four-bird species (herring gulls (*Larus argentatus*), black-headed gulls (*Larus ridibundus*), lesser black-back gulls (*Larus fuscus*) and twenty-six from starlings (*Sturnus vulgaris*)) with the highest prevalence in starlings (11.5%, n = 26) (5).

Italy

At a rescue center for injured wild birds in the Province of Messina, located in Sicily, Southern Italy, cloacal swabs were collected from various common European wild bird species undergoing entrance examinations during the period from March to June 2013.

Among the antibiotic resistances observed, the most common was against trimethoprim/sulfamethoxazole, followed by notable resistance to streptomycin, amoxicillin/clavulanic acid, and ampicillin. Resistance to tetracycline was lower in comparison. However, all isolates remained susceptible to meropenem. The highest levels of susceptibility were seen against ceftazidime, norfloxacin, cefotaxime, ciprofloxacin, ceftriaxone, and imipenem.

Multiresistance, defined as resistance to three or more classes of antibiotics, was prevalent in strains from raptors (69.4%), waterbirds (83.3%), and passerines (62.5%). Interestingly, among the strains resistant to cephalosporins, eight were identified as *E. coli* out of the ten strains isolated from raptors and waterbirds (9).

Sweden

In a study involving Swedish gulls, the susceptibility of *E. coli* isolates was evaluated to 10 antibiotic classes. It was found that 83% of these isolates were classified as multiresistant, meaning they were resistant to three or more antibiotic classes. Common resistance traits included resistance to fluoroquinolones, aminoglycosides, tetracycline, trimethoprim, and sulfamethoxazole. However, all isolates remained susceptible to amikacin, colistin, fosfomycin, nitrofurantoin, ertapenem, meropenem, and imipenem (2).

The susceptibility of *E. coli* to carbapenems was assessed using meropenem disc diffusion. Among 391 isolates from Urban Swedish Mallards tested, 18 (4.6%) showed reduced susceptibility to meropenem. Notably, all 18 isolates with decreased susceptibility to meropenem tested negative for carbapenemase genes. However, none of these isolates met the criteria for meropenem resistance according to the EUCAST (the carbapenemase screening cutoff recommended by the European Committee on Antimicrobial Susceptibility Testing) (14).

Spain

Between April 2013 and June 2014, one hundred cloacal swabs were collected from wild birds at the Aragón Reference Centre of Wildlife Recovering (CRFS) in La Alfranca, Zaragoza, Spain, upon their arrival at the center. These swabs were obtained from 100 wild birds representing 15 different families. Upon examination for cefotaxime resistant (CTXR) *E. coli*, sixteen birds (16%) were found to harbor CTXR *E. coli* in their feces, spanning 9 species including griffon vulture (*Gyps fulvus*), yellow-legged gull (*Larus michahellis*), black kite (*Milvus migrans*), red kite (*Milvus milvus*), white stork (*Ciconia ciconia*), spotless starling (*Sturnus unicolor*), golden eagle (*Aquila chrysaetos*), common cuckoo (*Cuculus canorus*), and barn owl (*Tyto alba*). Subsequently, one CTXR *E. coli* isolate was selected and studied from each positive sample.

Among these isolates, 14 were found to contain Extended-spectrum beta-lactamase (ESBL)-positive *E. coli*. Notably, those producing sulfhydryl reagent variable (SHV-12) were resistant to both cefotaxime and ceftazidime. Conversely, most bacteria with CTX-M ESBLs were resistant to cefotaxime but exhibited lower resistance to ceftazidime. However, one ESBL-positive strain showed resistance to both cefotaxime and ceftazidime. Additionally, two other types of resistant *E. coli* were identified conferring resistance to cefoxitin, cefotaxime, and ceftazidime, and another with genetic changes resulting in resistance to cefoxitin and partial resistance to cefotaxime. The majority of antibiotic-resistant *E. coli* strains also exhibited resistance to other antibiotic families including β-lactams, quinolones, tetracyclines, and sulfamethoxazole/trimethoprim (1).

During 2015–2016, wild avian species in Spain were examined. Following overnight incubation at 37°C, bacterial growth was detected on screening plates from 95 birds, representing 14.2% of the sample. Among these, seven birds showed nonfermentative gram-negative rods, while the remaining 88 birds (13.2%) exhibited cefotaxime-resistant enterobacterial isolates.

These 88 specimens were obtained from 28 different species of wild birds, distributed as follows: diurnal birds of prey (28 specimens, 11 species), storks and jackdaws (17 specimens, two species), seagulls (14 specimens, two species), scavenger birds (12 specimens, two species), nocturnal birds of prey (6 specimens, three species), and others (11 specimens, eight species) (20).

Darwich et al. conducted research on wild animals brought to the Wildlife Rehabilitation Centre (WRC) of Torreferrusa in Catalonia, located in the northeastern Iberian Peninsula. Their analysis, conducted between November 2016 and May 2017, revealed a prevalence of cephalosporin-resistant (CR) phenotype of 11.5% among wild birds, with 22 out of 191 individuals exhibiting this resistance represented in Figure 3 (6).

This chart shows the prevalence of antimicrobial resistance (AMR) genes among various species of birds, along with their common names and scientific names. The percentages represent the proportion of individuals within each species that carry AMR genes.

- *Accipiter gentilis* (northern goshawk): 23% of individuals carry AMR genes.
- *Accipiter nisus* (Eurasian sparrowhawk): 38% of individuals carry AMR genes.
- *Bubo bubo* (Eurasian eagle-owl): None of the individuals carry AMR genes (0%).
- *Buteo buteo* (Common buzzard): 12% of individuals carry AMR genes.
- *Strix aluco* (Tawny owl): 17% of individuals carry AMR genes.
- *Tyto alba* (Barn owl): 67% of individuals carry AMR genes.
- *Larus michahellis* (Yellow-legged gull): 14% of individuals carry AMR genes.
- *Sylvia melanocephala* (Sardinian warbler): 33% of individuals carry AMR genes.

- *Turdus merula* (Common blackbird): 13% of individuals carry AMR genes.

These percentages indicate the prevalence of antimicrobial resistance within each bird species, which can be important for understanding the spread of AMR in avian populations and its potential implications for human and animal health (Fig. 4) (6).

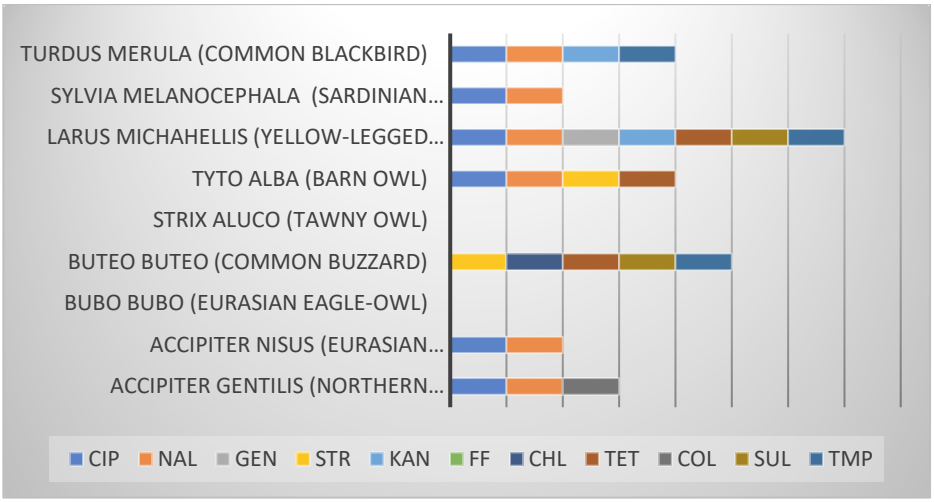

Fig. 3. Resistance phenotype to non-Beta-lactams of wildbirds from Catalonia by Darwich et al. (6)

Abbreviations: CIP, Ciprofloxacin; NAL, Nalidixic acid; GEN, Gentamicin; STR, Streptomycin; KAN, Kanamycin; FF, Florfenicol; CHL, Chloramphenicol; TET, Tetracycline; COL, Colistin; SUL, Sulphametoxazole; TMP, Trimethoprim; ND, not detected.

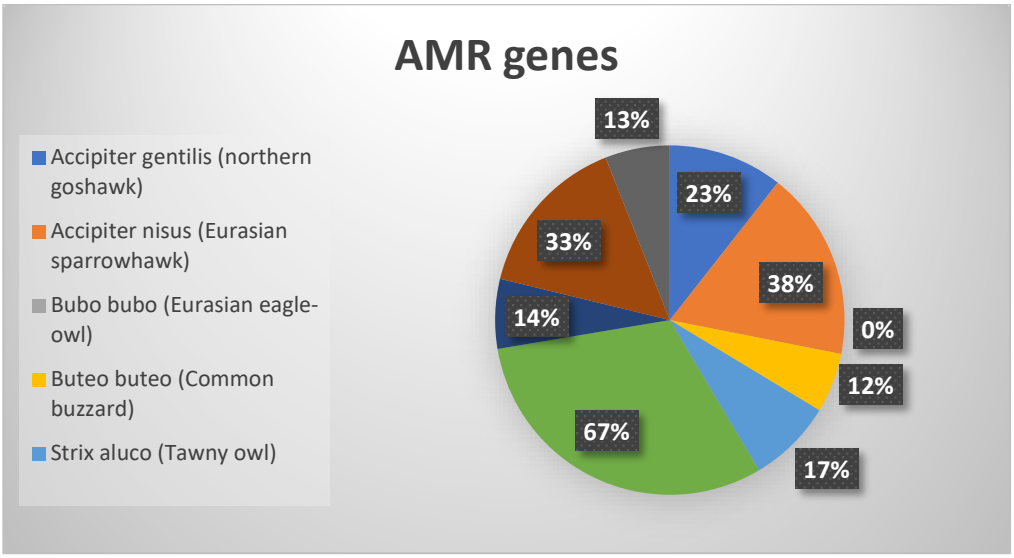

Fig. 4. Antimicrobial resistance genes procents of wildbirds from Catalonia (6)

High rates of resistant *E. coli* isolates to amoxicillin, cotrimoxazole, and tetracycline were observed in Egyptian vultures, with rates generally around 50%, varying depending on the species and age. Conversely, no isolates resistant to imipenem, meropenem, colistin, and amikacin were detected in either species. *E. coli* isolates from adult Egyptian vultures showed a higher relative risk of resistance to tobramycin and gentamicin compared to adult griffon vultures. When comparing resistance between different age groups of Egyptian vultures, nestlings exhibited higher resistance to amoxicillin/clavulanic acid compared to adults, whereas isolates from adults displayed a higher relative risk of resistance to cotrimoxazole compared to those from nestlings.

Overall, 64 *E. coli* isolates from 33 samples (1–3 isolates per sample) from griffon vultures and 65 *E. coli* isolates from 31 samples (1–3 isolates per sample) from Egyptian vultures

were analyzed from the Castilian Highlands in central Spain (4).

The study titled "High-risk Lineages of Extended Spectrum Cephalosporinase Producing *Escherichia coli* from Eurasian Griffon Vultures (*Gyps fulvus*) Foraging in Landfills in Northeastern Spain" investigated the prevalence and resistance profiles of extended-spectrum cephalosporinase-producing *Escherichia coli* in Eurasian Griffon Vultures. Out of 218 cloacal swabs, a total of 87 isolates (39.9%) representing one individual per isolate were recovered on MacConkey agar supplemented with ceftriaxone and confirmed to be *E. coli* by PCR. These isolates exhibited high levels of resistance, with all being resistant to ampicillin, cefotaxime, and ceftazidime, except for 8 isolates that were susceptible to ceftazidime. Additionally, a significant proportion of isolates showed phenotypic resistance to ciprofloxacin (95.4%), tetracycline (87.3%), sulfamethoxazole (85.0%), trimethoprim (83.9%), nalidixic acid (71.2%), chloramphenicol (68.9%), gentamicin (41.3%), and azithromycin (35.6%) (11).

France

According to Ngaiganam et al. (19) bacterial strains isolated on MacConkey agar were the only ones showing reduced susceptibility to third-generation cephalosporin (ceftriaxone), with *E. coli* strains being resistant to fourth-generation cephalosporin (cefepime).

Their findings revealed that ESBL-producing *Enterobacteriaceae* were more commonly isolated from beach gulls compared to chickens and pigeons (Table 1), as previously reported by Stedt et al. (19, 23), the interaction between gulls, their feeding habits, environmental exposure, and migratory patterns likely contribute.

Table 1

Antibiotic resistance phenotype of yellow legged gull and pigeon

Sources from bacterial strains	Antibiotic resistance phenotype
Pigeon	AX, KF, CRO, FEP
Yellow-legged gull	AX, AMC, CRO, FEP, STX, CIP
Yellow-legged gull	AX, AMC, CRO, FEP, STX
Yellow-legged gull	AX, AMC, CRO, FEP

Abbreviations: AX amoxicillin, AMC amoxicillin–clavulanic acid, KF cefalotin, CRO ceftriaxone, FEP cefepime, SXT trimethoprim–sulfamethoxazole, CIP ciprofloxacin.

Slovak Republic

E. coli strains isolated from goshawk feces exhibited the highest resistance levels to ampicillin (AMP, ≥16 mg/L; 6 out of 9 strains) and tetracycline (TET, ≥16 mg/L; 6 out of 9 strains), followed by nalidixic acid (5 out of 9 strains) and streptomycin (5 out of 9 strains). Additionally, four isolates displayed resistance to enrofloxacin, ciprofloxacin, and cotrimoxazole. Among the ten isolates from eagles, four strains were resistant to both ampicillin and tetracycline, while three strains showed resistance to nalidixic acid. No resistant strains were detected in one isolate from a goshawk and four isolates from eagles (13).

E. coli strains isolated from rook feces (15) in the Slovak Republic showed high resistance to ampicillin (60%) and streptomycin (40%), followed by resistance to fluoroquinolones (ciprofloxacin-22%, enrofloxacin-24%), tetracycline (18%), cotrimoxazol (17%) and florfenicol (14%).

Handrova and Kmet discovered that resistance to third and fourth generations of cephalosporins was not observed. However, they confirmed widespread resistance to ciprofloxacin and enrofloxacin in six strains, with four originating from goshawks and two from eagles (13).

Ukraine

Recently, the prevalence of multidrug-resistant (MDR) *Escherichia coli* in wild and domestic waterfowl from Ukraine was investigated. The prevalence of MDR *E. coli* varied from 10.0% to 31.8% across distinct regions: 20% of wild mallard specimens in the Kherson region, 31.8% of domestic ducks, and 10% of geese in the Kharkiv and Cherkasy regions were identified as MDR.

Isolates from wild birds showed high resistance to ampicillin (AMP), amoxiclav (AMC), amoxicillin (AMX), doxycycline (DO), and chloramphenicol (C), while isolates from poultry were resistant to ampicillin, amoxiclav, doxycycline, amoxicillin, chloramphenicol, and enrofloxacin (EX). Most of the *E. coli* isolates from wild waterfowl were classified as non-multidrug-resistant (non-MDR) forms.

Analysis of antibiotic sensitivity phenotypes revealed that among non-MDR bacteria, only four antibiotic-resistant phenotypes were detected, whereas among MDR bacteria, two

antibiotic-resistant phenotypes were detected in mallards and six in domestic waterfowl (8).

Results and discussions

Wild birds interact with a variety of other wildlife species, including predators, prey, and competitors. The presence of AMR bacteria in bird populations can influence these interactions in several ways. Predators consuming birds carrying AMR bacteria may be exposed to these resistant pathogens, potentially leading to the spread of AMR within predator populations.

Prey species may experience changes in predation pressure if bird populations decline due to AMR-related health issues. Competition among bird species for resources such as food and nesting sites may be altered if certain species are disproportionately affected by AMR.

Tetracycline appears frequently in the data (Table 2) with high rates of resistance observed across different bird species and countries (Poland, Switzerland, Ireland, Sweden, Spain, France). This resistance is concerning because it's a broad-spectrum antibiotic commonly used in both human and veterinary medicine (18). Its widespread use likely contributes to the high prevalence of resistance.

Table 2

The most commonly repeated antibiotics that have been at the top of antimicrobial resistance over the last decade related to the studies involved in this review

No.	Author	Country	Bird Species	Antibiotics	Prevalence Rates
1	**Kuczkowski et al. 2016 (16)**	Poland	Mallards	Tetracycline	15.8% of isolates from mallards
2	**Zurfluh et al., 2013 (26)**	Switzerland	Pigeons, Cormorants	Tetracycline	All isolates resistant
3	**Carroll et al., 2015 (5)**	Ireland	Black-headed gulls Starlings Herring gulls Lesser black-back gulls	Tetracycline Streptomycin	10 isolates, respectively 7
4	**Giacopello et al., 2016 (9)**	Italy	Raptors and Waterbirds	Trimethoprim/Sulfamethoxazole	Most common resistance
				Streptomycin, Amoxicillin/Clavulanic acid, Ampicillin	Notable resistance
5	**Atterby et al., 2017 (2)**	Sweden	Gulls	Fluoroquinolones, Aminoglycosides, Tetracycline, Trimethoprim, Sulfamethoxazole	Common resistance traits
6	**Hessman et al., 2018 (14)**	Sweden	Urban Mallards	Meropenem	No resistance
7	**Alcala et al., 2016 (1)**	Spain	Various Wild Birds	Cefotaxime Ceftazidime	High resistance
8	**Oteo et al., 2018 (20)**	Spain	Various Wild Birds	Cefotaxime-resistant enterobacteria	13.2% resistance in 88 isolates
9	**Darwich et al., 2019 (6)**	Spain	Wild Birds	Cephalosporin-resistant E. coli	11.5% prevalence in wild birds
10	**Handrova & Kmet, 2019 (13)**	Slovakia	Goshawks, Eagles	Ciprofloxacin, Enrofloxacin	Common resistance in 6 strains (4 from goshawks, 2 from eagles)

11	**Ngaiganam et al., 2019 (19)**	France	Beach Gulls, Chickens, Pigeons	Ceftriaxone, Cefepime	Resistance
12	**Blanco et al., 2020 (4)**	Spain	Egyptian Vultures, Griffon Vultures	Amoxicillin, Cotrimoxazole, Tetracycline	High resistance rates in Egyptian vultures
14	**Guitart-Matas et al., 2024 (11)**	Spain	Eurasian Griffon Vultures	Ampicillin, Cefotaxime, Ceftazidime	High resistance levels
				Ciprofloxacin	95.4% resistant
				Tetracycline	87.3% resistant
				Sulfamethoxazole	85.0% resistant
15	**Kwaśna et al., 2024 (17)**	Poland	Black Grouse (*Lyrurus tetrix*)	Sulfamethoxazole	51.9% resistant, 11.1% intermediate resistant
16	**Eckenko et al., 2024 (8)**	Ukraine	Wild Mallard, Domestic Ducks, Geese	Ampicillin, Amoxiclav, Amoxicillin, Doxycycline, Chloramphenicol	High resistance in wild birds
				Enrofloxacin	Resistance observed in poultry
				MDR* prevalence	Wild mallards 20%, Domestic ducks 31.8%, Geese 10%

MDR- multidrug resistance*

Trimethoprim/Sulfamethoxazole resistance was notable among raptors and waterbirds in Italy (9). This combination is often used to treat a range of bacterial infections, and its high resistance suggests the need for alternative treatment options.

Cephalosporins (Cefotaxime, Ceftazidime, Ceftriaxone, Cefepime), particularly third-generation cephalosporins, is another concerning trend observed because are important for treating serious infections in humans, and their high resistance rates in wild birds from Spain and France (1, 9, 11, 19) suggest potential transmission of resistant bacteria from humans or livestock to wildlife.

Fluoroquinolones (Ciprofloxacin, Enrofloxacin), another class of broad-spectrum antibiotics, have also shown significant resistance patterns and are commonly used in both human and veterinary medicine. The presence of resistance in wild birds from Sweden and Slovakia (11, 13) indicates a potential spill-over of resistant bacteria from domestic animals to wildlife or vice versa.

Ampicillin, Amoxicillin, Amoxicillin/Clavulanic Acid, these beta-lactam antibiotics are also widely used in both human and veterinary medicine. High resistance levels observed in Eurasian Griffon Vultures in Spain and in wild birds from various countries suggest the need for surveillance and prudent use of these antibiotics.

The absence of resistance to meropenem in urban mallards in Sweden is an interesting finding, as it's a critically important antibiotic in human medicine. This suggests limited exposure of these birds to this antibiotic or a lack of selective pressure favoring resistance.

The prevalence of multidrug resistance in wild mallards, domestic ducks, and geese from Ukraine indicates widespread resistance to multiple classes of antibiotics (8). This highlights the complexity of AMR in wild bird populations and the urgent need for comprehensive surveillance and control measures.

Birds play important roles in ecosystem functioning, including seed dispersal, pollination, and pest control. Changes in bird populations due to AMR-related health issues can disrupt these ecological processes, leading to cascading effects on plant communities, insect populations, and other components of the ecosystem.

Overall, the data indicate widespread resistance to a range of antibiotics in wild bird

populations (Fig. 5), with some antibiotics showing particularly high rates of resistance. This underscores the importance of understanding the transmission dynamics of AMR between humans, domestic animals, and wildlife and implementing strategies to mitigate the spread of resistant bacteria in natural ecosystems.

Fig. 5. Geographic distribution of *E. coli* isolation studies in wild birds across Europe (2013–2024)

These reports suggest that anthropogenic factors, such as agricultural practices and waste management, play a crucial role in the dissemination of antibiotic-resistant bacteria in the environment. The ecological impacts of AMR in wild birds are profound, potentially affecting bird populations and their interactions with other wildlife.

Highlighting the significant presence and implications of antimicrobial-resistant *E. coli* in wild bird populations across Europe demonstrates the role of wild birds as reservoirs and vectors of antibiotic-resistant bacteria, which has important public health and ecological implications. There is a clear need for enhanced surveillance and research efforts to better understand the dynamics of AMR in wildlife. Additionally, integrating AMR monitoring into wildlife conservation strategies is crucial to safeguard both ecosystem health and human health.

Conclusions

This study reveals extensive antibiotic resistance (AMR) across diverse classes of antibiotics in wild bird populations throughout Europe. High resistance rates to commonly used antibiotics such as tetracycline, trimethoprim/sulfamethoxazole, and beta-lactams (ampicillin, amoxicillin) were observed in several bird species, particularly raptors, waterbirds, and scavenging species like Eurasian Griffon Vultures. Additionally, significant resistance to third-generation cephalosporins and fluoroquinolones—critical antibiotics for human medicine—was detected, suggesting possible AMR transmission from human and livestock sources to wildlife.

Tetracycline resistance was notably widespread among different bird species and countries, likely due to its common use in both human and veterinary medicine. This trend is concerning as it points to the potential overuse of this broad-spectrum antibiotic.

The high prevalence of multidrug resistance (MDR) in species such as wild mallards and domestic waterfowl emphasizes the complexity of AMR dynamics in wild avian populations and the potential for these species to act as reservoirs or vectors of resistance genes. While the absence of resistance to meropenem in urban mallards in Sweden offers a positive finding, the widespread resistance observed in other antibiotic classes highlights an urgent need for continued AMR surveillance in wildlife.

The presence of AMR in wild birds poses a potential ecological risk, as these species are integral to ecosystem functions like seed

dispersal and pest control. AMR-related health issues in bird populations could lead to cascading ecological impacts, underscoring the need for a broader ecological approach to AMR monitoring and management.

Overall, this study underscores the importance of understanding the transmission dynamics of AMR among humans, domestic animals, and wildlife, and highlights the need for proactive strategies to mitigate the spread of resistant bacteria in natural ecosystems. Targeted efforts in surveillance and responsible antibiotic use across sectors are crucial to managing AMR and preserving ecosystem health.

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CASE REPORT OF UROABDOMEN IN INTACT MALE YORKSHIRE TERRIER WITH URETHRAL RUPTURE AFTER VEHICULAR ACCIDENT

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Abstract

A 5- month- old entire male Yorkshire terrier was presented for anuria and lethargy of four days duration which had progressed to anorexia, halitosis, abdominal distension and abdominal pain after a vehicular accident. A diagnosis of uroabdomen was made following triage ultrasound, classic radiograph, urethral catheterization and abdominocentesis. The diagnosis of uroabdomen was established after inability to perform urethral catheterization and after abdominocentesis, the resulting liquid had an ammonia smell. The definite diagnosis was established during the abdominal exploratory surgery.

Keywords*: uroabdomen, urethral rupture, urethral reconstruction.*

Uroabdomen is an accumulation of urine in the peritoneal cavity, retroperitoneal cavity, or both. A loss of integrity of any portion of the urinary tract can cause uroabdomen, the most common site of rupture in dogs being the urinary bladder and pelvic part of urethra. This pathology is an emergency because, urine accumulated in the abdomen is absorbed in the body and this fact produces serious metabolic imbalances (1, 4, 8, 13, 14, 16, 17, 26, 27, 29).

Materials and methods

The patient used in this study was a five-months-old intact male Yorkshire terrier, weighing 3 kg with abdominal distension. The study aimed to diagnose the focusing on surgical exploration of the abdominal cavity, identify the site of injury and restore the integrity of urinary tract.

The surgical procedure was performed at the Genesis Veterinary Clinic Timisoara.

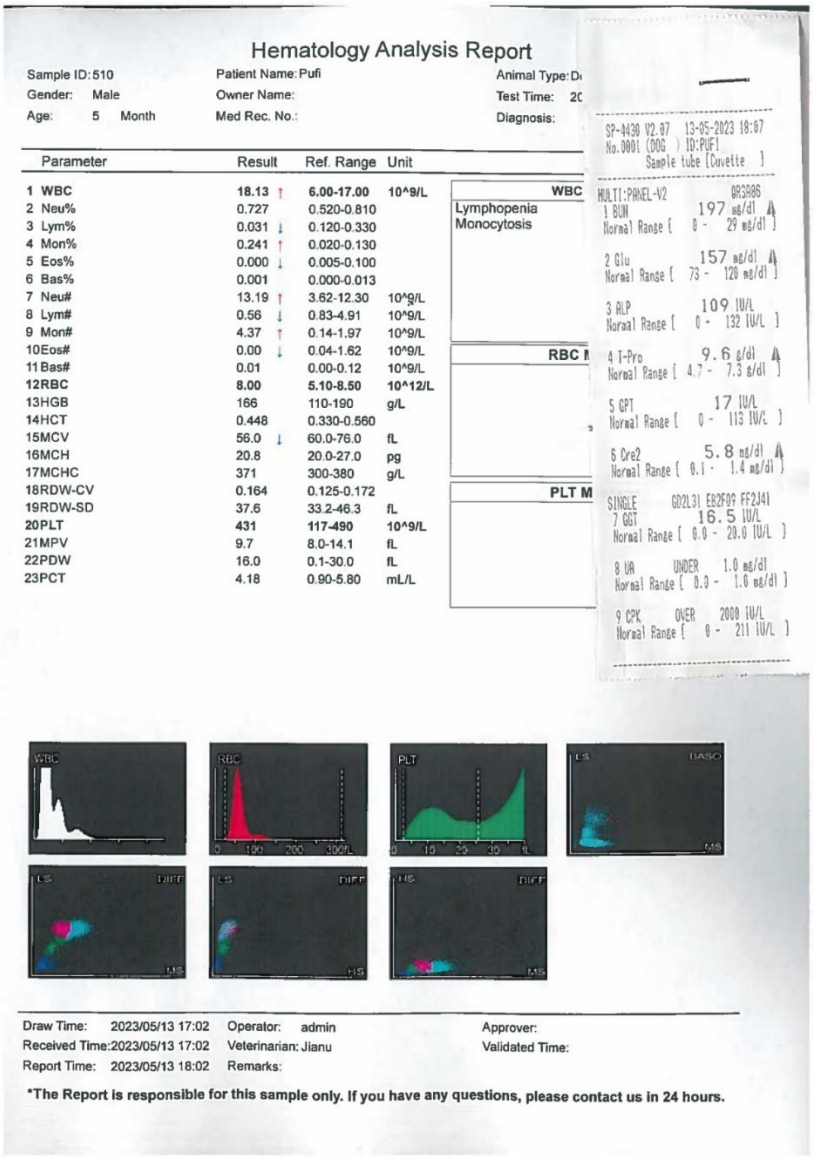
When a patient presented after vehicular accident for abdominal distension, abdominal pain and anuria can be difficult to initially differentiate based on history alone. Physical examination and imaging can be helpful to distinguish between hemoabdomen and uroabdomen (6).

The dog was seen at its primary care veterinarian, where a physical examination revealed an empty bladder that was not reported to be obstructed. The dog was prescribed an anti- inflammatory pain medication and a broad-spectrum antibiotic and then discharged on an outpatient basis to the owner. Later the following day, the dog was presented to emergency at another clinic for continued investigation because the patient was more lethargic and did not urinate from the day with vehicular accident. On physical examination, the patient was found to be approximately 10 per cent dehydrated. The abdomen was tense on palpation with a notable fluid wave and the bladder was empty. The animal was in a state of shock, the mucous membrane appears tacky and pallor, the pulse was weak, the eyes were enophthalmos and the body temperature was decreased, 37.3ºC. The urinary bladder catheterization can`t be perform because the catheter stops passing at the level of the pelvic portion of urethra (4, 5).

Haematological and biochemical analyses were performed on an in- house machine at presentation to the emergency facility (7). Serum biochemistry showed azotemia (blood urea nitrogen 197 mg/dl, reference range 9.0–29 mg/dl; creatinine 5.8 mg/dl, reference range 0.4–1.4 mg/dl), mildly elevated total protein 9,6 g/dl, reference range 4.7-7.3 and highly elevated creatine phosphokinase 2000 UI/L, reference range 0–211 UI/L (7). Complete blood count showed neutrophilic leukocytosis (white blood cells 18,13 x 103/µl, reference range 6.0–17.0 x 103/µl; neutrophils 13,19 x 103/µl, reference range 3.62–12.3 x 103/µl), monocytosis (monocytes 4.37 x 103/µl, reference range 0.14- 1.97 x 103/µl) and lymphopenia (lymphocytes 0.56 x 103/µl, reference range 0.83- 4.91 x 103/µl) (Fig. 1) (7).

Radiographs at presentation showed peritoneal effusion with loss a serosal contour and right pelvic fractures (Fig. 2-3) (3, 17).

An abdominal triage ultrasound confirmed a large amount of fluid present within the abdomen (9). A sample of abdominal effusion was obtained via abdominocentesis with ultrasound guidance (2). The sample was analyzed by visual and olfaction examination, showing the effusion is represented by urine, because the sample smells of ammonia.



Hematology Analysis Report

Sample ID: 510 Patient Name: Pufi Animal Type: D
Gender: Male Owner Name: Test Time: 2C
Age: 5 Month Med Rec. No.: Diagnosis:

Parameter	Result		Ref. Range	Unit
1 WBC	18.13	↑	6.00-17.00	10^9/L
2 Neu%	0.727		0.520-0.810	
3 Lym%	0.031	↓	0.120-0.330	
4 Mon%	0.241	↑	0.020-0.130	
5 Eos%	0.000	↓	0.005-0.100	
6 Bas%	0.001		0.000-0.013	
7 Neu#	13.19	↑	3.62-12.30	10^9/L
8 Lym#	0.56	↓	0.83-4.91	10^9/L
9 Mon#	4.37	↑	0.14-1.97	10^9/L
10Eos#	0.00	↓	0.04-1.62	10^9/L
11 Bas#	0.01		0.00-0.12	10^9/L
12RBC	8.00		5.10-8.50	10^12/L
13HGB	166		110-190	g/L
14HCT	0.448		0.330-0.560	
15MCV	56.0	↓	60.0-76.0	fL
16MCH	20.8		20.0-27.0	pg
17MCHC	371		300-380	g/L
18RDW-CV	0.164		0.125-0.172	
19RDW-SD	37.6		33.2-46.3	fL
20PLT	431		117-490	10^9/L
21MPV	9.7		8.0-14.1	fL
22PDW	16.0		0.1-30.0	fL
23PCT	4.18		0.90-5.80	mL/L

WBC
Lymphopenia
Monocytosis

RBC M

PLT M

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2 Glu 157 mg/dl A
Normal Range [73 - 128 mg/dl]
3 ALP 109 IU/L
Normal Range [0 - 132 IU/L]
4 T-Pro 9.6 g/dl A
Normal Range [4.7 - 7.3 g/dl]
5 GPT 17 IU/L
Normal Range [0 - 113 IU/L]
6 Cre2 5.8 mg/dl A
Normal Range [0.1 - 1.4 mg/dl]
SINGLE GD2L31 EB2F09 FF2J41
7 GGT 16.5 IU/L
Normal Range [0.0 - 28.0 IU/L]
8 UA UNDER 1.0 mg/dl
Normal Range [0.0 - 1.0 mg/dl]
9 CPK OVER 2000 IU/L
Normal Range [0 - 211 IU/L]

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Fig. 1. Haematological and biochemical analyses performed after four days from the vehicular accident (courtesy of the Genesis Veterinary Clinic Timisoara)

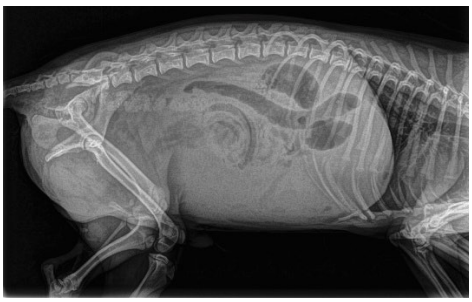


Fig. 2. Lateral radiograph of a 5-monts-old, intact male, Yorkshire terrier breed dog that reveals pelvic fractures and loss of serosal detail after four days of the car accident (courtesy of the Genesis Veterinary Clinic Arad)

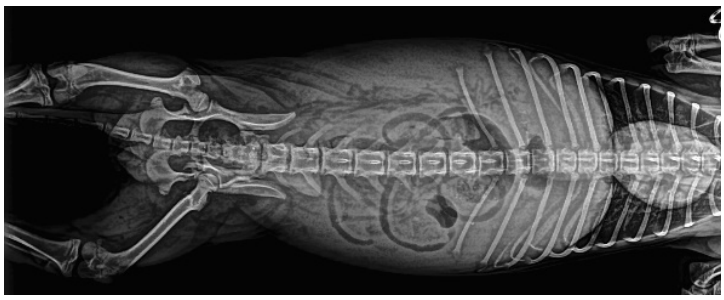


Fig. 3. Ventrodorsal radiograph of the same patient that reveals right pelvic fractures and loss of serosal detail after four days of the car accident (courtesy of the Genesis Veterinary Clinic Arad)

The diagnosis of uroabdomen was established based on clinical presentation and paraclinical investigations and the anatomic lesion was suspected after urethral

catheterization based on inability to perform bladder catheterization and empty bladder on ultrasound examination was confirmed after exploratory surgery (2).

The dog presented anuria, anorexia, abdominal pain and eventual diagnosis of abdominal effusion, both urogenital diseases should be considered.

It is important to consider physical examination findings as well as patient history and reported timing of clinical signs to direct differentials and diagnostic selection. Differentials for abdominal pain and distention could include but are not limited to a mass, volvulus of the gastrointestinal tract, constipation or abdominal effusion. After the diagnosis of a uroabdomen was established, more aggressive treatment and diagnostics were needed in order to ensure the most appropriate care which was provided for this patient, as delayed treatment or improper care could lead to significant complications and rapid decline. After non-invasive diagnostics proved inconclusive, the patient underwent abdominal exploratory celiotomy to evaluate the internal structures of the urinary tract and determine if there were defects that would require surgical repair (1, 4, 8, 13, 14, 16, 17, 26, 27, 29).

The prognosis was guarded due to urine absorption for several days (1).

Medical management consist of stabilization of the patient, in this case, the dog was given balanced crystalloid fluids intravenously at a calculated rate of maintenance plus 10 per cent dehydration (Ringer Lactat fluid; B. Braun) to correct electrolyte and acid-base disturbance, followed by analgesic medication represented by the butorphanol bolus (0.5 mg/kg intravenously, Alvegesic 10 mg/ml; Alvetra) in association with sodic metamizole (50 mg/kg intramusculary, Novasul 500 mg/ml; Richter pharma) and meloxicam 0.1 mg/kg subcutaneously, Melovem 5 mg/ml; Dopharma) for pain control (21, 25, 27, 29, 30).

Once the cause of the uroabdomen was not identified after numerous diagnostics, exploratory coeliotomy was elected. The dog was premedicated with a butorphanol bolus (0.5 mg/kg intravenously, Alvegesic 10 mg/ml; Alvetra) in association with sodic metamizole (50 mg/kg intramusculary, Novasul 500 mg/ml; Richter pharma) and meloxicam 0.1 mg/kg subcutaneously, Melovem 5 mg/ml; Dopharma) for pain control and induced with diazepam (0.35 mg/kg intravenously, Diazepam terapia 5 mg/ml; Terapia SA) followed by medetomidine (0.1 mg/10 kg intravenously, Domitor 1 mg/ml; Orion Pharma) in association with ketamine (8 mg/kg intramusculary, Ketamidor 100 mg/ml; Richter Pharma) and was intubated and maintained with inhalant anaesthesia via isoflurane (Isoflurane USP; Dechra) vapourised in oxygen and monitored throughout the procedure with stable vital signs recorded. The dog received amoxicillin- clavulanic acid (17.50 mg/kg equivalent with 1 ml sol Synulox RTU/ 10 kg, subcutaneously; Pfizer) (22, 25).

Surgical management consisted of exploratory celiotomy, identify the site of injury and restore the integrity of urinary tract (12, 26, 27, 29). Before make the exploratory celiotomy, the urine was drained from this space via abdominocentesis and performed the peritoneal lavage with saline solution to decreases the effects of uroabdomen (10, 18, 28).

After exploratory celiotomy has been identified accumulation of urine in the intraperitoneal cavity. Thus, the integrity of urogenital organs was controlled and observed the site of lesion indicated by the site of leakage urine and presences of hematoma around the pelvic portion of the urethra. For a better access at the site of lesion, has been made public symphysiotomy and once reached the leakage location the complete oblique section of the pelvic part of the urethra was identified (Fig. 4, 5) (11, 12, 19, 20, 23). The lesion of urethra was caused by the bone fragment results from the complete oblique fracture of the iliac bone (11, 12, 14, 15, 19, 24). The goal of treatment was represented by the repair of urethral rupture to stopping leakage of urine and then by the pelvic fracture repair, after the patient is stable (12, 16, 20, 24).

The restoration of the urinary way consisted in catheterization of urethra up to urinary bladder, excision devitalized free margins and apositioned the edges by simple interrupted sutures with absorbable threads and the knots placed extraluminal to prevent urethral stricture (Fig. 6) (8, 12, 14, 20).

After the urethral reconstruction, the pelvic canal and abdominal cavity was flushed with saline solution and finally closed by classic technique (10, 18, 28).

The pubic symphysis is closed by placed two wire cerclage and the medial muscles of the tights were apposite in a single plan by a continuous sutures pattern with absorbable threads (Fig. 7). The abdomen was closed by classic technique and the urinary catheter remained in place after surgery for five days to prevent urethral obstruction or stricture and urinary leakage in abdominal cavity (Fig. 8) (8, 12, 14, 20).

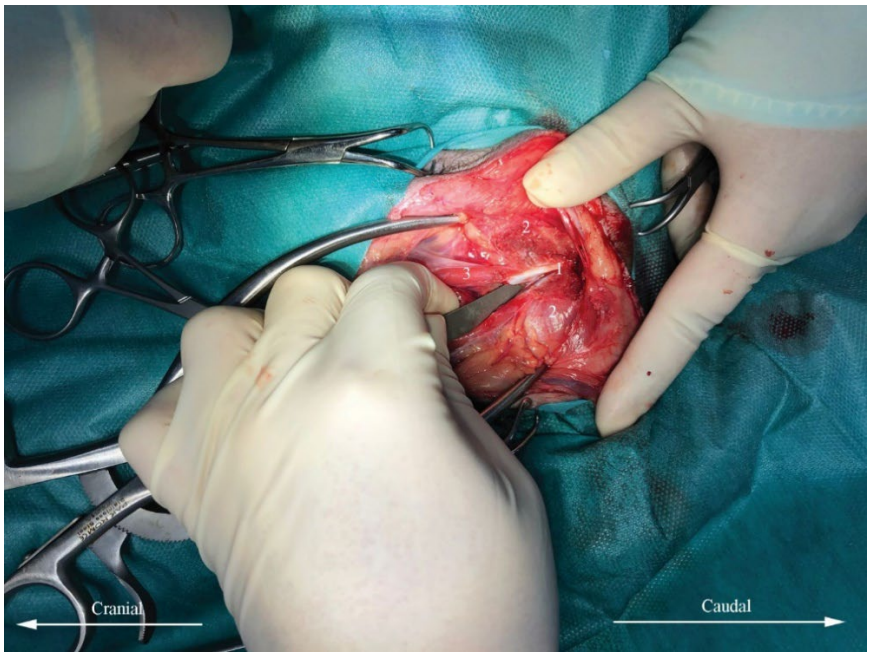


Fig. 4. Intraoperative image showing how to performing pubic symphysiotomy (courtesy of the Genesis Veterinary Clinic Timisoara)
1. Pelvic symphysis; 2. Medial muscles of the tight; 3. Ventral muscles of the abdominal wall

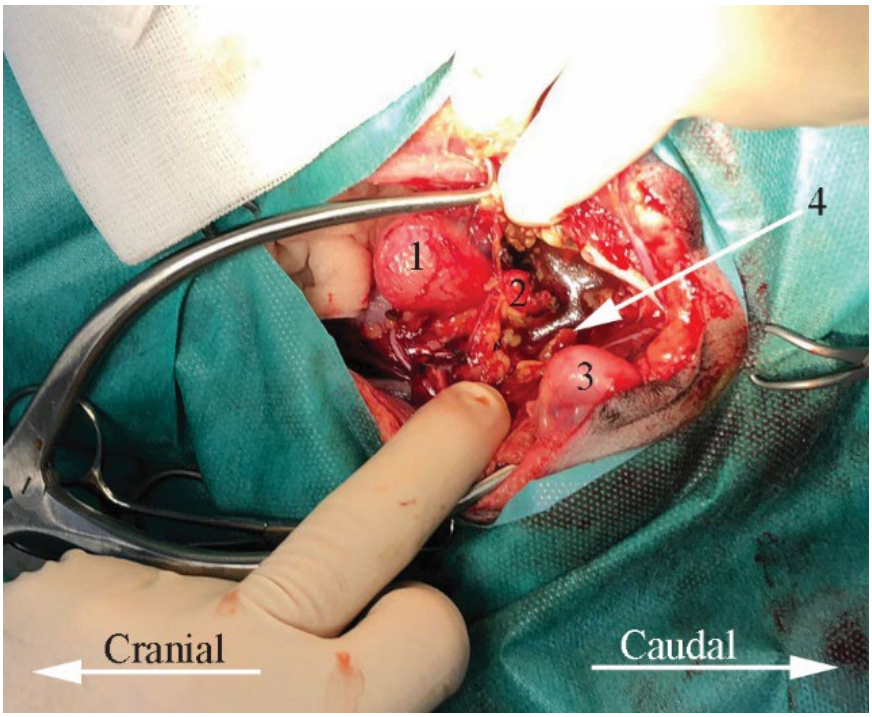


Fig. 5. Intraoperative image showing urethral lesion and leakage of urine (Image courtesy of the Genesis Veterinary Clinic Timisoara)
1. Urinary bladder; 2. Pelvic part of urethra; 3. Right testicle; 4. Fragment bone from pelvic fracture responsible for the urethral lesion.

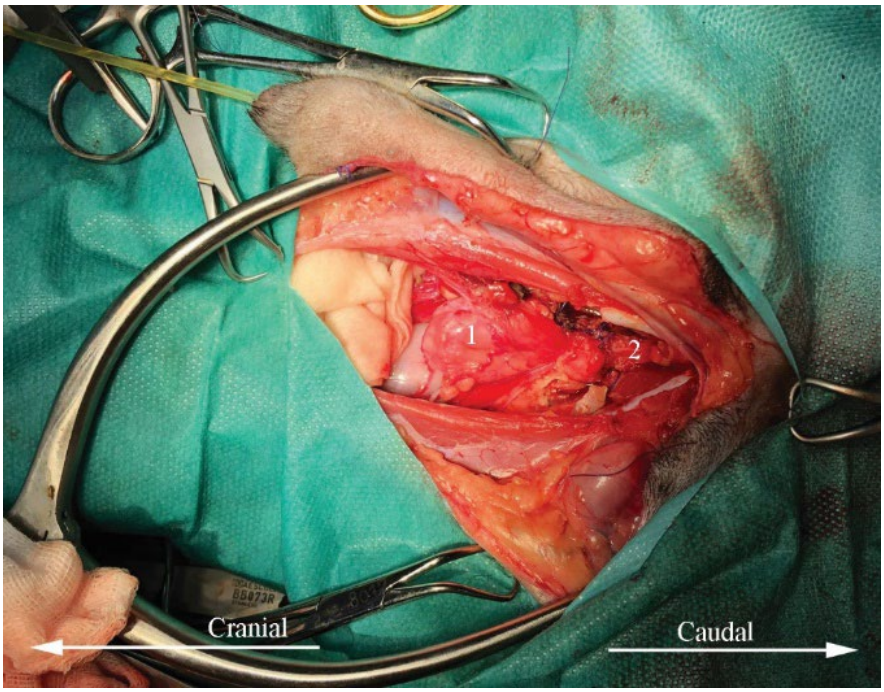


Fig. 6. Intraoperative aspect of urethral reconstruction (courtesy of the Genesis Veterinary Clinic Timisoara)
1. Urinary bladder; 2. Pelvic part of urethra after reconstruction.

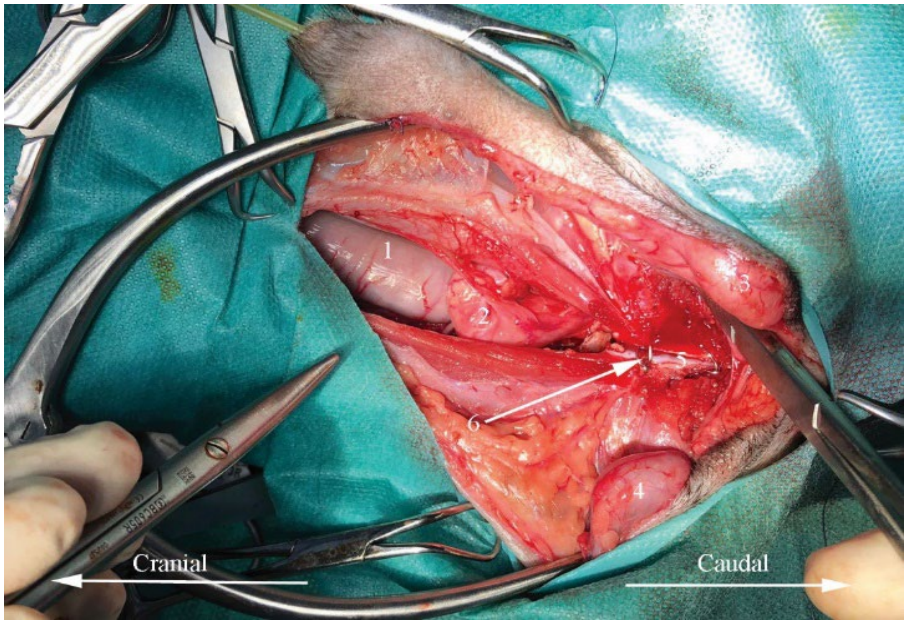


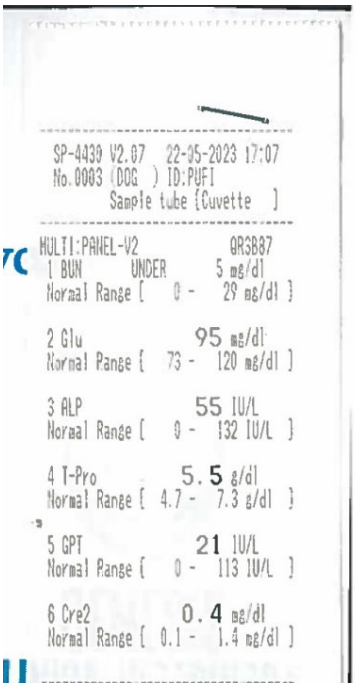
Fig. 7. Intraoperative image showing symphysioraphy with two wire cerclages (courtesy of the Genesis Veterinary Clinic Timisoara)
1. Descending colon; 2. Urinary bladder; 3. Left testicle; 4. Right testicle; 5. Pelvic symphysis; 6. The wire cerclage inserted for closed the pelvic cavity.

Fig. 8. Final aspect of the skin suture and technique of the fixation of the urinary catheter at the prepuce by performing a Chinese finger suture and skin by performing a simple interrupted pattern from place to place, and preventing self-removing the catheter and protecting the surgical wound an Elisabethan collar was applied (courtesy of the Genesis Veterinary Clinic Timisoara)

Results and discussions

On recovery from surgery, the patient's urinary catheter was removed after five days from surgery and it was able to urinate without any difficulties and normal posturing with a normal stream of urine was noted. The remainder of its stay in the intensive care until performing the next surgery for pelvic fracture repair, time in which the patient received for seventh days balanced crystalloid fluids intravenously at a calculated rate of maintenance plus per cent dehydration (Ringer Lactat fluid; B. Braun) to correct electrolyte and acid-base disturbance, followed by analgesic medication represented by the butorphanol bolus (0.3 mg/kg intravenously, Alvegesic 10 mg/ml; Alvetra) every eight hours in association with sodic metamizole (50 mg/kg intramusculary, Novasul 500 mg/ml; Richter pharma) and meloxicam 0.1 mg/kg subcutaneously, Melovem 5 mg/ml; Dopharma) every twenty-four hours for pain control and to prevent postoperatively infections the dog received amoxicillin- clavulanic acid (17.50 mg/kg equivalent with 1 ml sol Synulox RTU/ 10 kg, subcutaneously; Pfizer) every twenty-four hours (16, 21, 22, 25). Immediately after surgery, the patient had an appetite and zest for life. At the eight days after perform the surgery, serum biochemical analyze showed normal value of blood urea nitrogen and creatinine (Fig. 9).

At 14 days after surgery the threads of suture was removed and the physical examination was unremarkable. The owner reported that the patient continued to urinate without difficulties (5, 12, 20).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2 Glu 95 mg/dl
Normal Range [73 - 120 mg/dl]

3 ALP 55 IU/L
Normal Range [0 - 132 IU/L]

4 T-Pro 5.5 g/dl
Normal Range [4.7 - 7.3 g/dl]

5 GPT 21 IU/L
Normal Range [0 - 113 IU/L]

6 Cre2 0.4 mg/dl
Normal Range [0.1 - 1.4 mg/dl]

Fig. 9. Serum biochemical analyze at eight days after surgery that reveals the normal value of the blood urea nitrogen and creatinine (Analyze courtesy of the Genesis Veterinary Clinic Timisoara)

Conclusions

Uroabdomen is a life-threatening condition that requires rapid diagnosis and stabilization of electrolytes, acid-base disturbances, and azotemia before any consideration for

advanced diagnostics, anesthesia, and surgery. Once the patient is deemed stable, diagnostics to determine the location of urinary tract lesions followed by surgical repair can be performed.

Close monitoring in the postoperative period is important to document improvement or resolution of laboratory abnormalities and overall patient well-being.

In addition, it is important to monitor for postoperative complications such as ongoing urine leakage from dehiscence of the surgical site, stricture formation, or progression to urosepsis.

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URETHRAL OBSTRUCTION AND POSTRENAL AZOTEMIA IN A ONE YEAR OLD INTACT MALE CAT

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Abstract

Urethral obstruction and postrenal azotemia are potentially life-threatening conditions commonly seen in male cats. This case report describes a one-year-old intact male European cat presented to the Internal Medicine Clinic of Veterinary Faculty in Timișoara with a three-day history of inability to urinate, lethargy, and abdominal pain. Clinical examination revealed a distended urinary bladder, and postrenal azotemia was confirmed through blood and urine tests. The cat was immediately hospitalized, and a urinary catheter was placed to relieve the obstruction. Treatment included buprenorphine, fluid therapy, and a specialized supplements to improve the urinary and renal function. Bladder flushes were performed for two days. The cat's condition improved rapidly, with average urine output resuming and renal function returning to normal. The catheter was removed after five days, and the cat was discharged on a long-term urinary diet after seven days of hospitalization to prevent recurrence. This case highlights the importance of early diagnosis, catheterization, and supportive care in managing urethral obstruction and restoring normal renal function in cats.

Keywords: *urethral obstruction, postrenal, azotemia, cat, treatment.*

Postrenal azotemia is a serious and potentially life-threatening condition that arises as a consequence of urinary obstruction, often requiring immediate medical intervention (1, 2, 8). In feline medicine, urethral obstruction (UO), commonly associated with feline lower urinary tract disease (FLUTD), is a well-documented cause of acute renal dysfunction, electrolyte imbalances, and metabolic disturbances (3, 4). The obstruction leads to increased intravesical pressure, which in turn causes urine to backflow into the kidneys, increasing intratubular pressure and decreasing glomerular filtration rate (6, 7, 8). As a result, critical renal functions such as tubular reabsorption, sodium and water regulation, and potassium excretion are impaired, leading to uremia, metabolic acidosis, and hyperkalemia (1, 4, 12).

Although most cases of urethral obstruction in male cats result in mild electrolyte and acid-base disturbances, severe cases can lead to significant metabolic derangements (5, 10, 17).

This report presents a case study of a one-year-old, intact male European cat with postrenal azotemia due to urethral obstruction. The clinical course, diagnostic findings, and therapeutic management are discussed in detail, highlighting the critical interventions needed to restore renal function and correct the associated metabolic disturbances.

Materials and methods

A one-year-old intact male European cat presented to the Internal Medicine Clinic of the Veterinary Faculty in Timișoara with symptoms characteristic of urethral obstruction. The owners reported that the cat had been apathetic and unable to urinate despite frequently assuming a posture to urinate in the litter box for extended periods without success. These symptoms had persisted for three days prior to presentation at the clinic.

On clinical examination, the cat exhibited moderate abdominal pain upon palpation, mild dehydration, and a distended urinary bladder, suggesting significant urine retention. An abdominal ultrasound was performed, revealing a markedly enlarged urinary bladder, but no other abnormalities were detected. Post-void ultrasound evaluation revealed a thickened bladder wall (Fig. 1), suggesting cystitis progression. Blood samples were collected, and complete blood count and biochemical parameters of the renal profile (total protein, albumin, creatinine, blood urea nitrogen, and phosphorus) were determined.

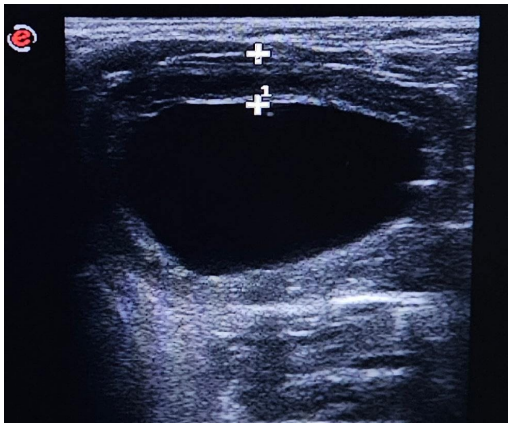

Fig. 1. Thickening of the bladder wall. First day of ultrasound (0.54 cm)

The cat was hospitalized immediately, and a urinary catheter was placed to relieve the obstruction and restore normal urine flow. Following catheterization, treatment was initiated, focusing on addressing the idiopathic cystitis and renal impairment. The treatment protocol consisted of buprenorphine for pain management and fluid therapy to stimulate renal function and correct electrolyte imbalances. Urinary bladder flushes with saline solution were performed for two consecutive days to further aid in clearing the obstruction.

Upon initial laboratory evaluation, the patient's blood test results revealed significantly elevated urea, creatinine, and phosphorus levels, all markedly above the normal reference ranges (Table 1). These abnormal values strongly indicated impaired excretion of these metabolites, which aligned with a clinical diagnosis of urethral obstruction. These findings suggested postrenal azotemia, a condition often associated with urinary tract obstruction that hinders the elimination of waste products through the kidneys.

Table 1

Biochemical values at the initial tests and throughout the treatment

Parameters	Before Treatment	3 days of treatment	7 days of treatment	Referance range
Ureea (mg/dl)	>133	72	22.69	13-37
Creatinine (mg/dl)	8.09	5	0.7	0.70-2.00
Phosphorus (mg/dl)	13.6	8.5	6	3.1-7.5
Total Proteins	5	6	6.7	5.7-8.9

The cat began to urinate normally throughout treatment, and renal function gradually returned to normal levels (Table 1). After five days of hospitalization, the urinary catheter was removed. The total duration of hospitalization was seven days, during which the cat was monitored closely for any recurrence of symptoms.

The laboratory results after 7 days of treatment indicate significant improvements across all parameters, suggesting a positive response to therapy (Table 1). The BUN value and the creatinine level have decreased to within the normal range. Laboratory values after 7 days of treatment show significant improvements, with BUN, creatinine, phosphorus, and total protein levels either within or close to normal ranges and also the reduction of bladder wall thickening (Fig. 2).

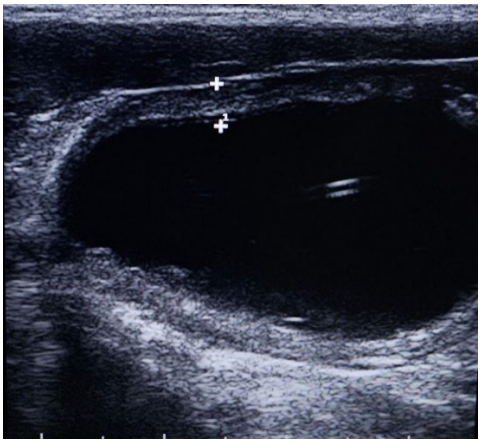

Fig. 2. A reduction in bladder wall thickening following treatment (0.3 cm)

These findings suggest a successful response to the treatment, with marked recovery in renal function and improved metabolic regulation. Continued monitoring and maintenance of current therapeutic strategies were recommended to ensure sustained recovery.

Results and discussions

This case report illustrates the critical role of early diagnosis and intervention in managing postrenal azotemia, particularly in male cats with urethral obstruction. Urethral obstruction, a common cause of feline lower urinary tract disease (FLUTD), can rapidly progress to severe metabolic derangements such as uremia, metabolic acidosis, and hyperkalemia, compromising renal function (11, 14, 16). Timely catheterization to relieve the obstruction is essential in preventing irreversible renal damage and restoring normal urinary flow. In this case, the use of analgesics, fluid therapy, and a specialized urinary diet facilitated the cat’s full recovery, with renal function returning to normal within days.

The elevated serum creatinine is a classic indicator of renal impairment. A value of 8.09 mg/dL suggests a substantial reduction in glomerular filtration rate (GFR), pointing to advanced kidney dysfunction. Creatinine is a reliable marker of renal function, and this significant increase implies that the kidneys are failing to eliminate creatinine efficiently (15, 16).

Hyperphosphatemia reflects the kidneys' inability to excrete phosphorus adequately, a common feature of acute and advanced chronic kidney disease (CKD). Elevated phosphorus

levels can lead to mineral and bone disorders associated with kidney disease, as well as cardiovascular complications due to vascular calcifications. Managing phosphorus levels is crucial to prevent such complications (2, 8, 10).

The successful resolution of this case underscores the importance of prompt and comprehensive therapeutic management in cats with postrenal azotemia. The combination of catheterization, bladder flushes, and targeted medication addressed both the immediate obstruction and the underlying idiopathic cystitis, preventing recurrence (9, 14, 15). Additionally, the adoption of a long-term urinary diet highlights the role of dietary management in preventing future episodes, showcasing a holistic approach to feline urethral obstruction and renal care.

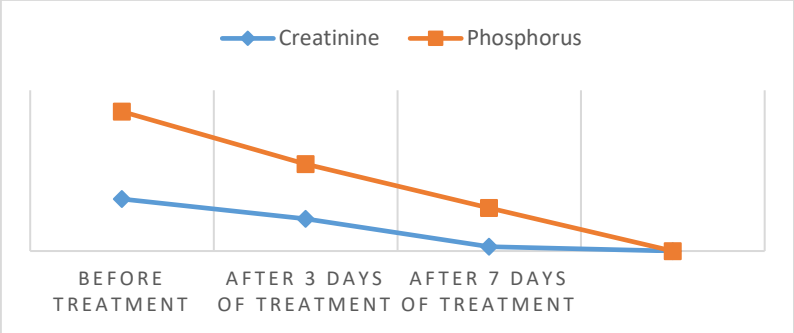

Fig. 3. Serum creatinine and phosphorus concentrations durring the treatment

The lower creatinine level and the ureea value (Fig. 3-4) indicate that kidney function has improved, and the kidneys are now able to excrete urea effectively. This is a strong indicator that kidney filtration has returned to near-normal levels. The improvement in creatinine suggests that the glomerular filtration rate (GFR) has increased, reflecting restored kidney function (13,15,16).

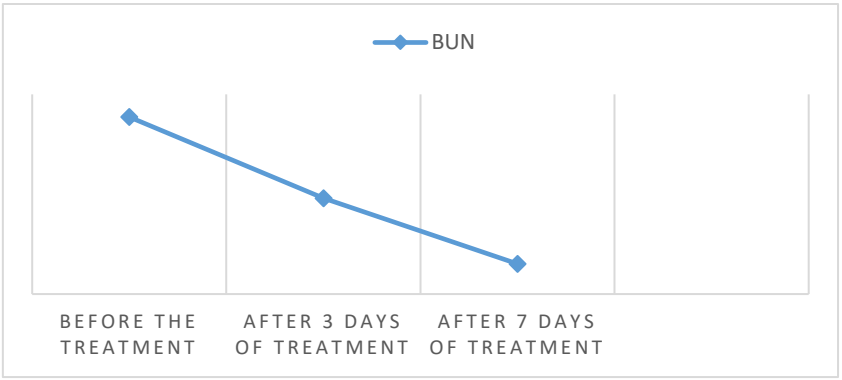

Fig. 4. BUN levels during the treatment

In a 2010 study, Francis et al. (7) investigated the prevalence of post-obstructive diuresis in cats following urethral obstruction. They revealed that 46% of cats developed diuresis within the first 6 hours after the resolution of obstruction, with the likelihood increasing over time. Notably, diuresis was significantly associated with acidemia, as cats with a venous pH lower than 7.35 on admission were five times more likely to experience diuresis. These findings highlight the importance of monitoring urine output and acid-base status in managing post-obstruction cases (7).

In a study by Ostroski et al. (13), the clinical features and outcomes of a cat exhibiting acute neurological signs after relief of urethral obstruction were discussed. The cat developed a grand mal seizure following a rapid decline in blood urea nitrogen and serum osmolality, indicating a process similar to dialysis disequilibrium syndrome. This case underlines the importance of monitoring for neurological complications in the post-obstructive period, especially when severe azotemia resolves quickly. Treatment with hypertonic saline and supportive care resolved the neurological symptoms, underscoring the need for vigilance in managing such complications (13).

A study from 2018 by Lamb et al. presented that cats with ureteral obstruction were often presented with non-specific clinical signs such

as lethargy, inappetence, vomiting, and weight loss. Despite these vague signs, azotemia was a common finding, often linked to acute kidney injury. Treatment options varied, including medical management, ureteral stenting, and subcutaneous ureteral bypass (SUB) devices. The study found that factors like high creatinine levels at discharge and fluid overload during hospitalization negatively impacted survival. Most cats were discharged after treatment, although 23% did not survive hospitalization (11).

A study by Burrows et al. presented that urinary obstruction of 24 to 48 hours in 23 cats caused marked azotemia, hyperphosphatemia, hyperkalemia, and severe metabolic acidosis (venous pH, 7.11 +/- 0.09). Glycosuria was noted in 74% of the cases. No significant differences in serum chemical values or electrolyte concentrations were found between cats obstructed for 24 hours and those obstructed for 48 hours or more. After relief of obstruction, metabolic acidosis was corrected within 8.4 hours using fluid replacement and sodium bicarbonate therapy, while serum creatinine and phosphorus levels improved at 19.5 hours. Postobstructive diuresis occasionally led to hypokalemia, emphasizing the need for careful fluid and electrolyte management (1).

Conclusions

This case report illustrates the critical role of early diagnosis and intervention in managing postrenal azotemia, particularly in male cats with urethral obstruction. Urethral obstruction can rapidly progress to severe metabolic derangements such as uremia, metabolic acidosis, and hyperkalemia, compromising renal function. Timely catheterization to relieve the obstruction is essential in preventing irreversible renal damage and restoring normal urinary flow. In this case, using analgesics, fluid therapy, and a specialized urinary diet facilitated the cat's complete recovery, with renal function returning to normal within days.

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STUDY ON THE INCIDENCE OF CYSTIC OVARIAN DISEASE IN DAIRY COWS IN TWO INTENSIVE FARMS IN NORTH-WEST ROMANIA

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Abstract

Cystic Ovarian Disease (COD) is commonly observed in high-yielding dairy cows and is a serious fertility problem that can lead to economic losses for farms as it affects the annual production of one calf. Although the etiology of this disease is not fully understood, factors such as endocrine disruption, stress, nutritional problems, genetics and other diseases have been associated with the risk of COD. This study was carried out from June to October on two intensive dairy farms in north-west Romania. The aim of the study was to determine the incidence of COD in lactating cows in relation to the number of lactations, the reproductive status of the female and the season. The cows included in the study were examined by transrectal ultrasonography using a portable ultrasound device (BCF Ultrasound) with a linear transducer operating at a frequency of 7 MHz, and their reproductive records were also analyzed. A total of 632 cows were examined between June and October, of which 66 (10.44%) were diagnosed with cysts. The highest incidence was observed in second lactation cows: 30.30% of cows with ovarian cysts. We found that 39.39% of cows with ovarian cysts were in the postpartum period. Most cysts were found on the right ovary (69.69%). In general, the increase in ambient temperature correlates with the gradual increase in COD incidence. In the summer season, 66.67% of cysts were diagnosed, with a peak in July (28.78%) when the annual maximum temperature (39.4°C) was recorded. Conversely, in the autumn season, with a maximum temperature of 20°C, 33.33% of cysts were diagnosed.

Keywords: *cystic ovarian disease, dairy cattle, ultrasound.*

Ovarian cyst (OC) is an important ovarian dysfunction and a major cause of reproductive failure in dairy cattle (9). It is associated with an increase in the number of open days in the postpartum period, leading to longer calving intervals; increased drug and treatment costs associated with its management; increased semen costs due to increased services per conception; and higher culling rates of affected animals (15). The prolongation of the calving interval and the impact on the annual production of a calf, as well as the treatment costs associated with this pathology, cause economic losses to the dairy industry.

Various authors define cysts as large follicular structures (25 mm or more in diameter) present for 10 days in the absence of a corpus luteum (10). Others define it as cystic ovarian follicular structures of at least 17 mm that persist for more than 6 days in the absence of corpus luteum (7). According to others, in dairy herd health programmes, “cysts” are often diagnosed in the absence of clear clinical signs. Therefore, the term “Cystic Ovarian Disease” no longer seems appropriate and should be replaced by the term “Cystic Ovarian Follicle(s)” (COF) which does not necessarily imply a state of disease. Many researchers showed that OC are actually dynamic can regress and be replaced by other news (18).

The cysts may be single or multiple on one or both ovaries. These cysts may persist, but during the course of the disease, additional cysts may be recruited, and some cysts undergo atresia (14).

Further subclassification of the COF condition into follicular or luteal cyst is based on the degree of luteinization and on progesterone levels in blood or milk. Follicular cysts are typically thin-walled structures that secrete varying amounts of estradiol. The thickness of the layers of granulosa cells dictates the amount of estradiol present in the intrafollicular fluid. Both cyst types are considered to be different forms of the same condition, with luteal cysts being a follicular cyst in which theca and granulosa cells have undergone some luteinization and are producing progesterone. Follicular cysts do not secrete progesterone, whereas luteal cysts secrete varying amounts of progesterone depending on the degree of luteinization. (15). A luteinised cyst develops when ovulation does not occur and the theca undergoes luteinisation. There is no ovulatory papilla and the luteal mass is smooth and rounded.

Cystic corpora lutea can be distinguished from luteinized cysts by the ovulation papilla that distorts the outline of the cyst at the point of ovulation. Additionally, their size is greater reflecting their development from cystic follicles (14).

The most accepted hypothesis to explain ovarian cyst formation is that the release of LH (luteinising hormone) by the hypothalamic-pituitary axis is altered (1). Because of the lack of negative feedback effects of progesterone in

such anovulatory situations, the pulsatile secretion of LH is higher than the level observed in the normal luteal phase. The relatively high LH pulses promote continued excessive growth of dominant follicles. During this prolonged growth of follicles, production of estradiol, as well as of inhibin A, is sustained for longer than with a normal dominant follicle. A combination of inhibin A and estradiol establishes long-term dominance of the cystic follicle by the suppression of FSH secretion. When the cystic follicle regresses and loses its ability to produce inhibin A and estradiol, increased FSH secretion induces a new follicular wave. However, the newly emerged dominant follicle also becomes cystic unless the functional abnormality in the hypothalamus is resolved (16).

There can be a spontaneous recovery from the condition in some cows, whereas in others there may be repeated follicular waves that exhibit cystic follicular growth. There are studies suggesting that the environment (feed, management, lactational stress) and genetic constitution may contribute towards the aetiology of the disease (6).

Stress plays an important role in the COD incidence. Cows with normal cyclicity may develop ovarian cysts in response to various conditions that cause stress or low plasma progesterone concentrations because stress blocks the estradiol-induced preovulatory surge in LH concentration (2).

High producing cows under such stressors are prone to various reproductive problems especially COD. The role of stress in the pathogenesis of COD is believed to be mediated by the release of endogenous cortisol through inhibition of LH release. Endogenous opioid peptides (produced in the hypophysis and brain) are believed to block the oestrogen-induced LH surge and the release of hypothalamic GnRH. Stress may mimic the action of these peptides in postpartum cows (11).

Materials and methods

The studies were carried out on two intensive dairy farms in north-west Romania during the warm season, June, July, August and also during 2 months of autumn, September and October.

The first farm, "Rotur" farm, is located in the village Turulung, a village crossed by the river Tur, in the north-west of Romania, with the following coordinates 47.92536854570446, 23.088256271776096.

The farm is quite large and intensively farmed and the animals are in good condition, Most are Holstein, but there are also some Red Holsteins. The second farm is located in the village of Odoreu, in Satu Mare County, situated in the northwest of Romania, with the following coordinates: 47.81108455560824, 22.99497204060387. The farm is not very large but operates intensively, with cows in free-stall housing system and access to a small pasture. The cows are milked in a milking parlour located at the end of the first barn, there is a parallel milking system. Cows are milked twice a day. Estrous cycles are monitored using special programmes; the cows are fitted with pedometers and a collar with a chip around their neck that measures various parameters that can indicate heat and are also monitored by the farm staff.

A total of 632 cows included in the study were examined by transrectal ultrasonography using a portable ultrasound device (BCF Ultrasound) with a linear transducer operating at a frequency of 7 MHz, and their reproductive records were also analyzed. The information was extracted from the reproductive registers of each farm, kept by the veterinarians and veterinary technicians, which record the registration number, the current reproductive status, the diagnosis and any treatment given. The diagnosis was made based on transrectal examination and ultrasound. The entire reproductive tract was examined, the contractility of the reproductive tract was monitored and the presence of formations on the ovary, especially follicles larger than 2.5 cm, while also considering the history of each cow. The thickness of the cyst wall and the contents were also considered. The diagnosis of COD was made by correlating all this data.

Results and discussions

From a total of 632 cows examined between June and October, 66 (10.44%) cows were diagnosed with ovarian cysts. The highest incidence was observed in second lactation cows: 30.30% of cows with ovarian cysts and the lowest incidence in non-lactating cows: 3.03% (Table 1 and Fig. 1). Of the total cows with COD, 43.93% (29 out of 66) are cows in their first (13.63%) and second lactation (30.30%). Most cysts were found on the right ovary: 69.69%. On the left ovary, the percentage was 25.75% and on both ovaries 4.54%.

Table 1

Incidence of COD according to the number of lactations and location on the right or left ovary

Number of lactations	COD (No/ %)	Cyst right ovary (No/ %)	Cyst left ovary (No/ %)	Cysts on both ovaries (No/ %)
0	2 / 3.03%	2 / 3.03%	0 / 0%	0
1	9 / 13.63%	7 / 10.60%	2 / 3.03%	0
2	20 / 30.30%	11 / 16.66%	9 / 13.63%	0
3	13 / 19.69%	9 / 13.63%	2 / 3.03%	2 / 3.03%
4	14 / 21.21%	13 / 19.69%	1 / 1.51%	0
≥5	8 / 12.12%	4 / 6.06%	3 / 4.54%	1 / 1.51%
Total number of cows with COD	66 =100%	46=69.69%	17=25.75%	3=4.54%

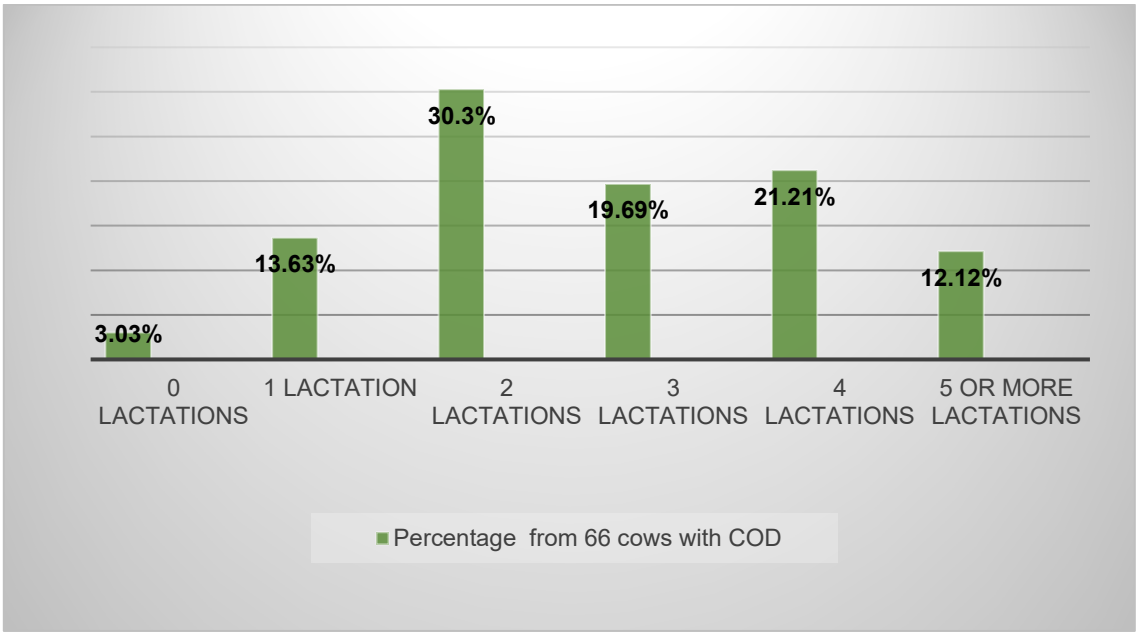

Fig. 1. Incidence of COD based on the number of lactations

Our findings are supported by other authors who report that is possible that in breeds with genetic predisposition to COD, selection to increase milk production can increase the incidence of COD. This condition has been observed more frequently in older cows during their second to fifth lactation (11). Cows in early lactation, which are trying to meet the increased requirements for milk production, are more susceptible to environmental changes with hormonal implications as a result". In other words, it is possible that the correlation between milk yield and COD incidence is based on the state of stress on the animal during early lactation rather than a genetic correlation (12).

Correlation with the reproductive status of cows revealed that COD most affected cows in the postpartum period (39.39%) and anestrous cows after artificial insemination AI (28.78%) (Table 2). It seems that after parturition, high yielding cows have a negative energy balance, which becomes more threatening to the cows' performance (13).

Table 2

Incidence of COD based on the cow reproductive status

Total number of cows with cysts	**COD in p**ost partum period **(No/ %)**	COD in anestrous after AI **(No/ %)**	**COD in r**ecently inseminated **cows** after anestrus **(No/ %)**	**COD in cows with r**epeat breeding **(No/ %)**
66	26 / 39.39%	19 / 28.78%	16 / 24.24%	5 / 7.57%

In our study, the increase in ambient temperature correlates with the gradual increase in COD incidence. In the summer season, 66.67% of cysts were diagnosed, with a peak in July (28.78%) when the annual maximum temperature (39.4°C) was recorded. Conversely, in the autumn season, with a maximum temperature of 20°C, 33.33% of cysts were diagnosed.

Irrespective of geography and husbandry, modern dairy cows experience heat stress (HS) effects leading to fertility declines, but it worsens in tropical climates. Lactating cows become hyperthermic even at 25-28.9°C. The principal biological mechanisms involved in adverse effects of HS on reproduction are reduced feed intake, negative energy balance, and endocrine disruptions. Under the eve of stress, cellular functions shift towards survival rather than their primary secretions. HS causing an increase in core body temperature has direct, adverse consequences on reproductive hormones. HS has been considered as a major contributing factor to the lower fertility rates of dairy cows inseminated in the late summer months. Imaging studies have shown that hyperthermia alters follicular dynamics, which are reflected in an increased ratio of large-sized follicles, double ovulations leading to twinning, early appearance of the pre-ovulatory follicle and elongated period of follicular dominance (13).

The study by Lopez-Gaitus et al. (8) carried out in warm (May-September) and cold (October-April) periods, showed that during the warm period, reproductive variables were significantly affected and a significant correlation was found between puerperal disorders and the subsequent development of ovarian cysts. The calving season influences COD with a higher incidence of cases associated with summer parturition and concluded that heat stress could be the principal responsible for such effect (4, 8)

Researchers from China confirm that the cumulative incidence rate of COD in cows on one farm was 35% (14/40), with a higher incidence between August and October, indicating the influence of heat stress (19).

Another study performed in North-East Spain (40°N) identified the season as a risk factor of high ovarian cysts incidence. This study demonstrated that cows calving in the summer were 2.6 times more likely to develop this disorder compared with the cows calving in the winter. The researche assigned the heat stress to the seasonal variation (3).

Based on these previously presented studies, several authors have highlighted the link between heat stress and increased incidence of COD during the hot season. Our observations provide new evidence in this regard, confirming the effect of heat on cow health. In the next section, we present the results of the study that support this hypothesis. Depending on the season, we analysed the hot season, summer, from June with maximum average temperatures in this north-western area of Romania of 25-35°C, July with 32-40°C, August with 24-28°C. We also analysed the autumn season, only with the months of September with 24-28°C and October with 18-22°C. We noticed that the incidence of COD increased according to the season, so that in the warm season we found 66.66% of the total number of cows with cysts, 44 out of 66, with the highest incidence in July with 28.78% (19 out of 66) and in August with 27.27%. These months were the hottest of the summer, with a maximum in July of 38.9°C in Satu Mare County and 41.7°C in Romania. In the autumn season, the incidence dropped to 33.33% (22 out of 66), but the temperatures also dropped (Table 3 and Fig. 2- 3).

Table 3

Incidence of COD based on the season

Summer season (June, July,August)	No / % 44 / 66.66%
June	7 / 10.60%
July	19 / 28.78%
August	18 / 27.27%
Autumn season (September, October)	22 / 33.33%
September	7 / 10.60%
October	15 /22.72%

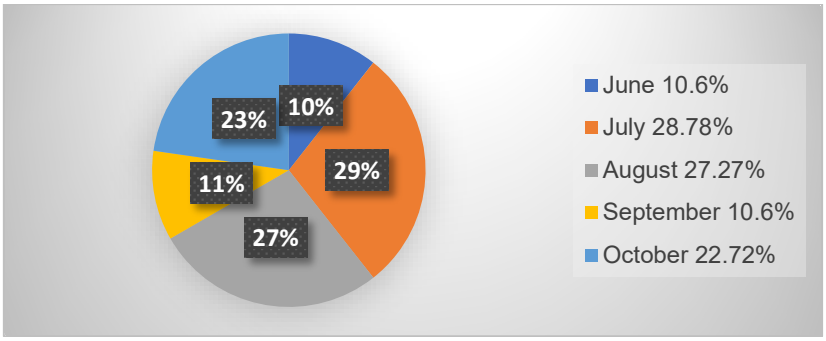

Fig. 2. Incidence of COD over a 5 months period

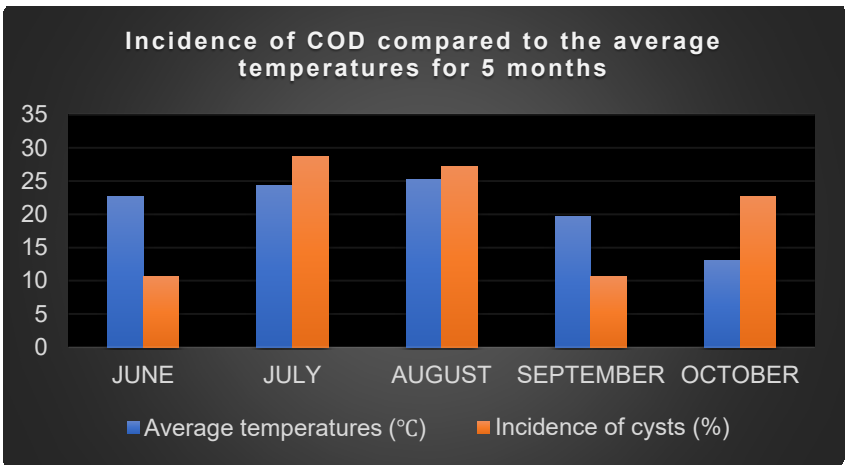

Fig. 3. Incidence of COD compared to the average temperatures

Other authors recognised the effect of high milk production on cyst incidence and found that older cows were more likely to develop a chronic cyst condition than those in earlier lactations (12).

Environmental factors such as nutrition, feed management and housing type are associated with the risk of developing ovarian cysts in dairy cows. Cow housing is a significant risk factor. Was identified a 5.7 greater risk of succumbing to ovarian cysts in dairy cattle when calving takes place indoors compared to pasture calving (3). Some researchers have also implied that higher milk yield in dairy cattle can increase the risk of developing ovarian cyst (3). Our study supports these statements as both farms have high milk production, cows are free range and calving takes place indoors rather than on pasture.

However, it has also been recognised that there is an association between high milk yield and increased incidence of COD, and that cows genetically selected to produce more milk will also be more likely to develop multiple follicular cysts during their lifetime. The selection for milk yield leads to an increase in COD incidence of 1.5% per 500 kg increase in milk yield (12).

For diagnosis, some authors say that in addition to the most common practice in the field for diagnosing cystic ovarian disease: examination by rectal palpation with or without the use of B-mode ultrasound. Colour Doppler ultrasound can be used, this technology allows the assessment of blood flow area measurements in the ovary, which has been proposed as a potential indirect measure of plasma progesterone (P4) concentrations (17).

Ultrasound examination of the ovaries of the cows included in this study revealed follicular cysts (Fig. 4), luteal cysts (Fig. 5) and polycystic ovaries (Fig. 6). Several studies report that follicular cysts are more common than luteal cysts; in one study, 30.5% of 1191 ovarian cysts were luteal (5).

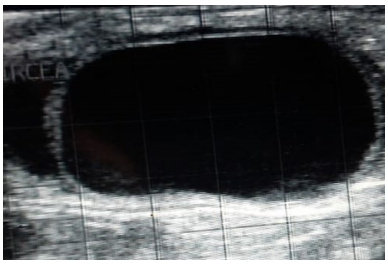

Fig. 4. Ultrasound image of a 4 cm follicular cyst (original image)

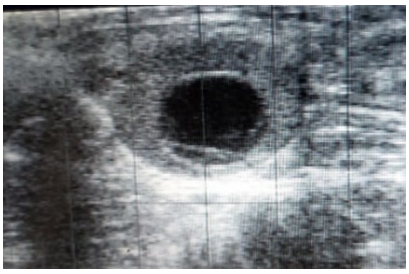

Fig. 5. Ultrasound image of a luteal cyst (original image)

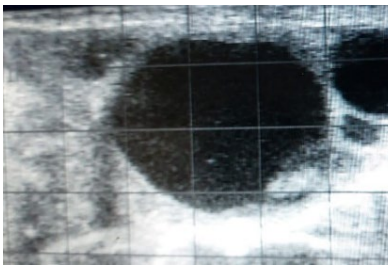

Fig. 6. Ultrasound image of a polycystic ovary (original image)

Similarly, the administration of compounds that induce LH release can stimulate luteinisation of the cysts and ovulation of mature follicles, thereby resolving the cystic condition. Manual rupture of the cysts is not recommended because of the risk of haemorrhage and the formation of adhesions to the mesovarium, which may compromise fertility. Administration of human chorionic gonadotropin (3,000 units) and GnRH analogues and agonists (gonadorelin diacetate (100 µg) or buserelin acetate (10 µg) have been used to treat ovarian cysts, but GnRH is preferred because it is a small, stable molecule and does not cause adverse effects or immune reactions (2).

The use of progesterone to treat COD is well documented. A progesterone releasing intravaginal device (PRID) has been used to synchronise estrus, treat postpartum anestrus, COD and other functional abnormalities in dairy cows. PRID has also been used in cases of COD not responding to GnRH. It seems that progesterone treatment appeared to cure endocrine lesions in cows with follicular cysts after restoration of LH response to oestradiol following 7 days of PRID treatment (20).

Conclusions

This study investigated the incidence of COD in Holstein Friesian cows, specialized in dairy production, from two intensive dairy farms in northwestern Romania, in correlation with reproductive status, lactation number and ambient temperature. Most of the cysts were diagnosed in the postpartum period, especially in early lactation, which highlights the need for targeted monitoring during this period. The introduction of regular ultrasound monitoring protocols and interventions as soon as possible in the first weeks after calving could significantly reduce the incidence of COD. Also, heat stress management through cooling systems, efficient fans especially in the summer months, is essential to reduce the impact of high temperatures on cow health. In addition, nutrition also requires special attention, especially in the antepartum and postpartum periods.

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PERCEPTION OF THE CAT AS A PET IN ROMANIAN AND MOROCCAN CULTURES

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Abstract

Given the worldwide distribution of cats, differences in attitudes and behaviour towards these animals are to be expected in different cultures, especially given the differences in levels of economic development and religious traditions in many countries. In Europe, the rise and spread of Christianity, produced a dramatic shift in attitudes towards cats; from being essentially benevolent symbols of female fertility and motherhood, they became, instead, the virtual antithesis - animals that embody evil and the companions of witches and necromancers. Despite its growing popularity among pet owners in Europe and America, many people still continue to regard the sudden appearance of a cat as a sign of bad luck, and others fear or dislike these animals, perceiving them as sneaky and untrustworthy. On the other hand, Morocco being a Muslim country plays in favour of the receptivity that people have towards cats, as in Islam the domestic cat is a revered. In order to determine if there are any differences in societal perception of cats between the Romanian society and the Moroccan one, a questionnaire of 21 questions, available in Romanian, English and French, was created by using Google forms. There was a total of 200 respondents, 100 from each country. In terms of societal perception, Morocco, as a country and a society, has proven to be a cat-loving nation, clearly preferring cats to dogs, while the opposite is observed in Romania, where the dog is preferred as a pet over the cat. Despite this contrast, there were no significant differences in the behaviour and empathy shown towards stray cats between respondents in the two countries.

Keywords: *cats, companion animals, cultural perception, Muslim society, European society.*

The earliest known evidence of cat domestication are those indicating the taming of an African wildcat (*F. lybica*) (22), when a cat remains were excavated close by a human Neolithic grave in Shillourokambos, southern Cyprus, dating to about 7500–7200 BC. Since there is no evidence of native mammalian fauna on Cyprus, the inhabitants of this Neolithic village most likely brought the cat and other wild mammals to the island from the Middle Eastern mainland (3, 21). Scientists therefore assume that African wildcats were attracted to early human settlements in the Fertile Crescent by rodents, in particular the house mouse (*Mus musculus*), and were tamed by Neolithic farmers. This mutual relationship between early farmers and tamed cats lasted thousands of years (8, 19). As agricultural practices spread, so did tame and domesticated cats. Wildcats of Egypt contributed to the maternal gene pool of the domestic cat at a later time (17, 21).

During domestication, cats have undergone only minor changes in anatomy and behaviour, and they are still capable of surviving in the wild, which explains way there tend to be many free-ranging cat colonies throughout the world (20). Several natural behaviours and characteristics of wildcats may have pre-adapted them for domestication as pets. These traits include their small size, social nature, obvious body language, love of play and relatively high intelligence. Captive *Leopardus* cats may also display affectionate behaviour toward humans but were not domesticated. House cats often mate with feral cats, producing hybrids such as the Kellas cat in Scotland. Hybridization between domestic and other *Felinae* species is also possible (13, 16, 18).

Development of cat breeds started in the mid-19th century. An analysis of the domestic cat genome revealed that the ancestral wildcat genome was significantly altered in the process of domestication, as specific mutations were selected to develop cat breeds. Most breeds are founded on random-bred domestic cats. Genetic diversity of these breeds varies between regions, and is lowest in purebred populations, which show more than 20 deleterious genetic disorders (14).

Despite the earliest evidence of its successful domestication were found in Cyprus, a European country, the importance of cats in ancient Egyptian culture where they were considered vessels that the gods chose to inhabit, and whose likeness gods chose to adopt, suggests that similar believes might have been part of other cultures throughout the continent (1, 2).

Despite their importance in different African cultures, one thing is sure is that their domestication and the human-cat relationship has been developing for far longer in this part of the world (11).

Morocco being a Muslim as well plays in favour of the receptivity that people have towards cats, as in Islam the domestic cat is a revered. Admired for their cleanliness, cats are considered "the quintessential pet" by Muslims. This stems as well from the belief that the prophet Muhammad was himself a cat lover and

devoted pet cat owner, as it is talked about and described in many hadiths; one of them stating "he would do without his cloak rather than disturb one that was sleeping on it" (4).

On the other hand, the image of cats in European society started taking a turn for the worse when in the Middle Ages, cats were often thought to be witches' familiars in England, and during festivities were sometimes burnt alive or thrown off buildings. This belief as since then spread far and wide, and to this day is present in many places with cats struggling to escape such prejudice. On top of that black cats receive even more hate as they are believed to be a bad luck or even death omen much like ravens (5, 6, 9, 15).

European folklore dating back to as early as 1607 tells that a cat will suffocate a new born infant by putting its nose to the child's mouth, sucking the breath out of the infant (7).

It would come as no surprise that people interact much differently with cats in Morocco and Romania, especially with stray ones as it is safe to state that pet cats have been carefully selected by their owners who already like cats (20). This can explain why Moroccan street cats are perceived to be much friendlier and easy to approach, as they are used to human contact and are most usually fed and cared for by people living in those neighbourhoods where the stray cat populations have settled. It is not uncommon either for people to take in pregnant queens or those that have just given birth to provide them with a safe environment until they deem the kittens old enough to fend of for themselves; or to take injured or sick cats to veterinary practitioners despite them not being their pets (12, 20).

In Romania street cats tend to be shier and hide when someone tries to initiate contact as people seem to be more indifferent to them and only care about their own pet. Unfortunately, there are many cases of people giving away or even throwing kittens so young that they should not be separated from their mother as they are still nursing. Ovariohysterectomies performed on queens sometimes even nearing the full development of the kittens are also something that can be encountered at many veterinary practices (10, 15).

This study aimed to determine if there are any differences in societal perception of cats between the Romanian society and the Moroccan one, by using an online survey.

Materials and methods

A questionnaire of 21 questions was created by using Google Forms and translated from Romanian into English and French, in order to fit the needs of each population and make it accessible to more people and not only to those that speak English, as it is not an official language in neither country. After their creation they were each shared as google drive link, with family and friends who were also asked to share it with furthermore acquaintances. The objective was to try to get varied opinions, not only sharing it with cat lovers. Age was a factor that we tried taking into consideration as well try to share it with people of different age categories to cover more generations.

The questions are listed below:

1. Sex: Man / Woman
2. Age:
3. Do you like cats? Yes / No / I feel indifferent
4. What do you prefer: Dogs / Cats
5. Do you see cats as clean animals? Agree / Neither agree nor disagree / Disagree
6. Have you ever owned a cat? Yes / No
7. Can you see yourself owning a cat someday? Yes / Maybe / No
8. Are there stray cats in your neighborhood? Yes / No
9. Do you ever feed stray cats? Always / Often / Occasionally / Rarely / Never
10. Do you interact with stray cats, pet them, play, etc? Always / Often / Occasionally / Rarely / Never
11. Would you help a hurt animal? Yes / No
12. If a queen gives birth in your home, what would you do? Keep the queen and the kittens until they become independent / Contact a rescue to take them / Put them on the street
13. If you see a cat being chased by another animal, would you intervene? Yes / No
14. Would you intervene if someone was mistreating a cat? Yes / No
15. Do you consider stray cats as non-desired? True / False
16. Do you consider that stray cats are a problem? Yes / No
17. How would you best describe the cats you interact with? Friendly / Shy / Furious / Scared
18. Does it even happen to you that stray cats demand your affection by meowing, following you, rubbing themselves against you etc? Often / Rarely / Never
19. Generally stray cats: Let you interact with them / Runaway and hide / Showcase aggressivity
20. Do consider that in your culture the cat is a beloved animal? Yes / No
21. In your country people would rather adopt: Cats / Dogs

For the statistical interpretation Chi square

test was used. The Yate's correction was performed when at least one of the expected values was smaller than 5, in order to prevent overestimation of the statistical significance. Where the data did not meet the requirements of Chi square test, we proceeded with the Fisher exact test.

Results and discussions

We started by seeing how the 200 (100 people in each country) people that answered our questionnaire personally felt about cats, asking them if they liked them or not. 85 (85%) people in Romania considered that they did like cats, while 7 (7%) did not like them and 8 (8%) people feel indifferent. The Moroccan demographic showed that 76 (76%) people like cats, 17 (17%) of people don't like cats and 7 (7%) are indifferent. (Fig. 1). No statistical difference was noted after comparison (p=0.06). Despite there not being a statistical difference, Moroccans expressed a stronger dislike towards cats than we had prior expected.

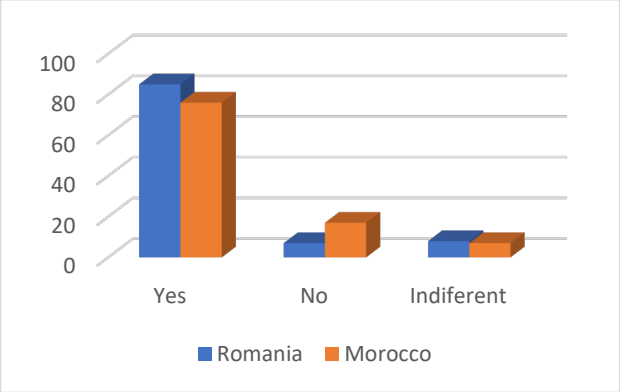

Fig. 1. Graphical representation of how the 200 survey respondents feel about cats per country, either they like them, feel indifferent or do not like them

Two of the questions aimed to highlight which is preferred in each country either dogs or cats, as they are the most common pets. But it was important to know not only if the people that answered our survey liked dogs more than cats or the other way around, the most important was to see which animal each society preferred. In Romania 42% of people preferred cats as a pet while the reminder 58% favoured dogs. But only 24% of people considered that Romanian society prefers cats as pets, while the majority, the 76% remaining people, agreed that Romanians would rather adopt dogs as their pets. In Morocco 66% of the people that participated in the survey favoured cats, meaning that only 34% people preferred dogs to cats. While on a societal level it was voted that cats were favoured as a pet by 60% people with 40% considering that in Moroccan society people would rather adopt dogs. (Fig. 2) Clear statistical difference for both preferred pet by individual (p=0.001) and when it came to societal preference (p=0.0001). As expected, Romanians prefers dogs to cats individually and societally, while Moroccans prefer cats to dogs both personally and societally.

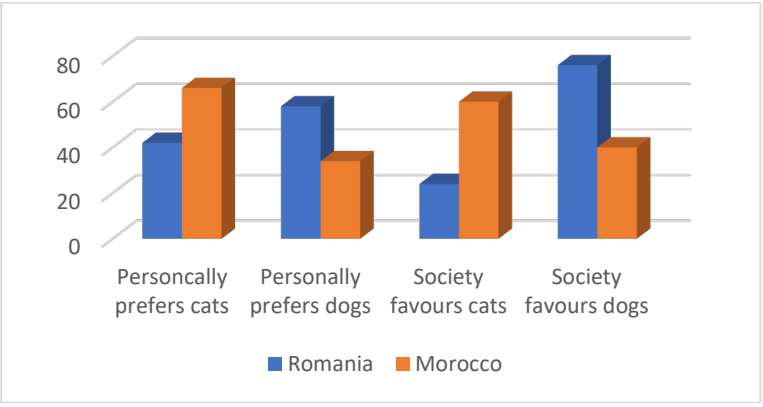

Fig. 2. Comparison of which pet is preferred personally, cats or dogs, by Romanian and Moroccan respondents; in contrast with which they believe is preferred in their society

People who have owned cats represent the majority in both countries, as shown in Fig. 3. In Romania 76 (76%) people already owned cats, and of those: 62 (82%) answered that yes, they would adopt again, 13 (17%) that they might adopt again, and only 1 (1%) person that as previously owned again noted that they would not be adopting again. In Morocco 64 (64%) surveyed people stated having already owned a cat and out of them: 37 (58%) people said that they would adopt again, 15 (23%) that might do so, and 12 (19%) stated that they would not be adopting cats again. So, no important difference was seen when it came to owning a cat. Interestingly, a significant statistical difference (p=0.0001) has been reported in the choice of people in both countries to stop adopting cats, with Moroccans being less likely to newly adopt a cat in comparison with Romanians, despite majority of them both having answered that they would adopt again.

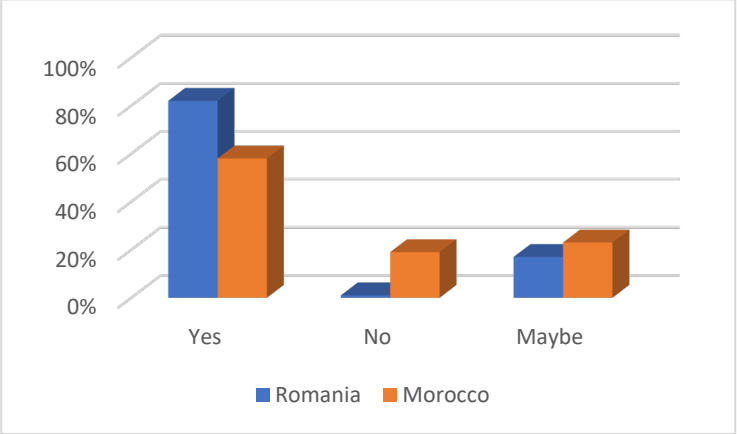

Fig. 3. Comparison of whether people that have owned cats in the past would be willing to do so in the future, by country

Cats are most often in comparison to dogs at least referred to as clean animals. We can here see that it is an idea that exist both in Romania, where 82% of people agree with this idea and in Morocco, with 79% people agreeing as well. In Romania only one (1%) person disagreed with the statement, while in Morocco 18% people disagree. Leaving 17% of people being torn on the matter in Romania and only 3% undecided people in Morocco (Fig. 4). Both Romanians and Moroccans vastly view cats as clean animals which matched our expectation, but Moroccans unexpectedly expressed a stronger disagreement with the statement. After data comparison we obtained a significant statistical difference (p=0.0009), representing a cultural difference when it comes to cats being perceived as clean animals.

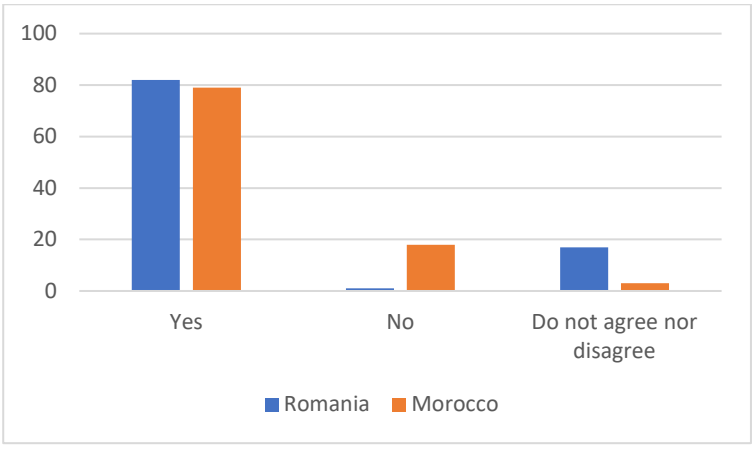

Fig. 4. Perception of cats as clean animals by Romanian and Moroccan respondents

When it comes to assessing if the cat is a beloved animal in each countries culture, in Romania 73% people think that cats are beloved in their cultures and 27% considering it a disliked animal. 85% Moroccans agreed on the fact that cats are liked in their culture and only 15% disagree (Fig. 5). After data comparison we were left with a significant statistical difference (p=0.03) expressing an expected cultural difference. Despite well over half of the submitted answers

people in both countries, significantly more Romanians believe that cats aren't beloved in their culture.

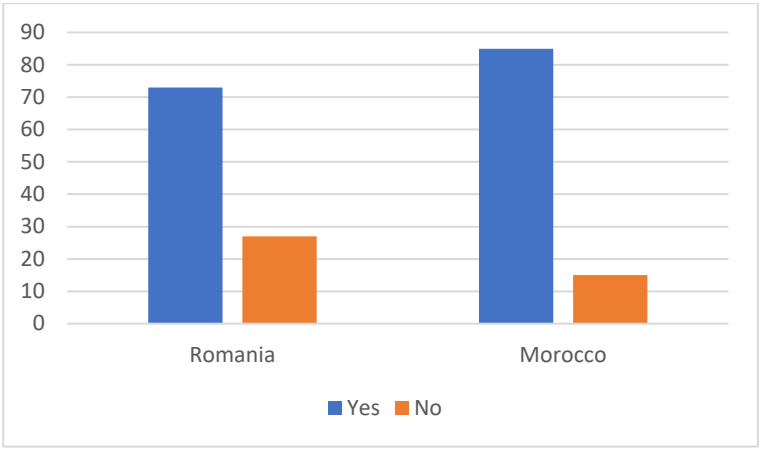

Fig. 5. Comparison whether Romanian and Moroccan respondents believe that cats are a loved animal in their own cultures

Before being able to assess the human-stray cat interaction and if there are any differences, we had to establish the existence of stray cats where our responders live. 88% Romanians reported the existence of stray cats in their neighborhoods with 12% people claiming that there are no stray cats where they live. A higher prevalence of stray cats in neighborhoods was noted in Morocco with 96% responders selecting yes as an answer, leaving only 4% people without stray cats in their neighborhoods. Statistically, a significant difference was noted (p=0.037), which confirmed the prior statement. Clearly in Romania more neighborhoods were noted to be stray-cat free; despite the vast majority of people in both countries stating the presence of stray cats in their neighborhoods (Fig. 6).

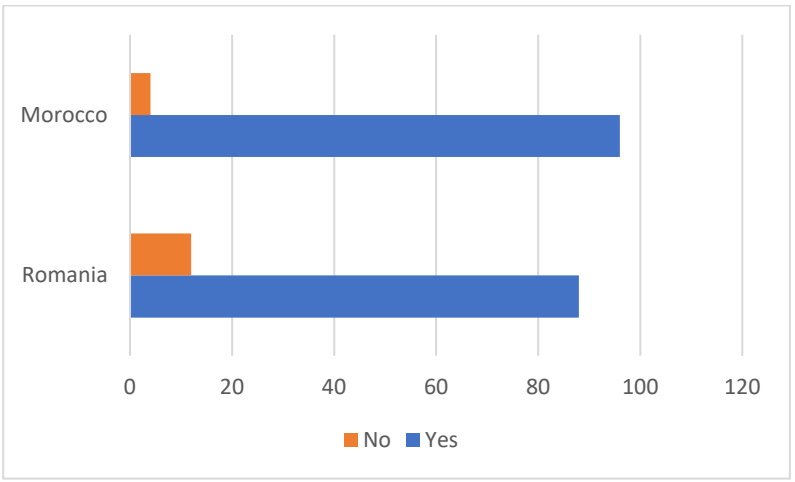

Fig. 6. Occurrence of stray cats in the neighborhood where the Romanian and Moroccan participants reside

In Romania 54% people noted that stray cats always came up to them demanding attention and affection, 37% people answered that this rarely occurred but that it was still something they have experienced and only 9% people selected that stray cat never came to them to ask for affection. In Morocco 43% of respondents agreed that stray cats always demand their attention, 47% of people selected that it rarely took place and 9% people have never had a stray cat come up to them to ask for attention (Fig. 7). After data comparison no significant statistical data was noted (p=0.11), as seemingly in both countries almost every person that has answered the survey has had stray cats coming to them to demand affection.

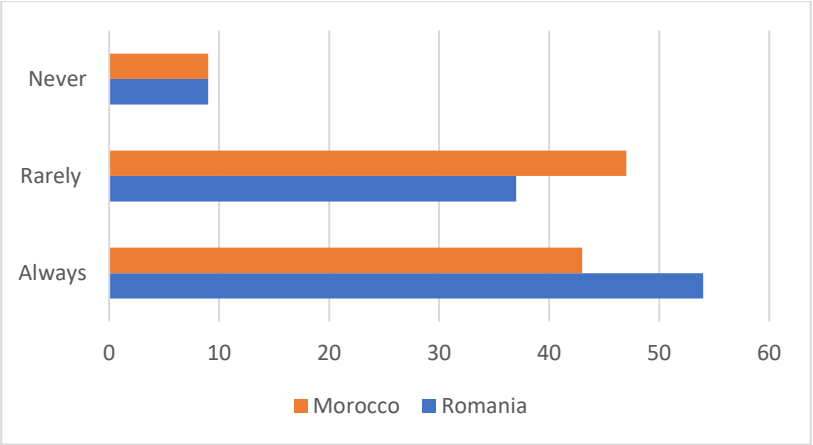

Fig. 7. Comparison between Romanian and Moroccan respondents, having been in a situation where stray cats demand their attention

When it comes to how stray cats react when the roles are reversed and humans are the ones initiating the contact, we had 3 options:

1. Let you interact with them: 64% of the Romanian responders and 48% of Moroccan responders agreed with this option.
2. Runaway and hide: 36% Romanians and 46% Moroccans selected this option.
3. Display aggressivity: It was not selected by anyone in Romania and just 6% of respondents selected this last option in Morocco (Fig. 8).

After data comparison we obtained a significant statistical difference ($p<0.05$) which matched our expectation of cultural differences. Unexpectedly, more Romanians than Moroccans stated that stray cats accept the interaction initiated by people, while more Moroccans than Romanians said that the stray cats runaway and hide. We expected the results to be the other way around, with Romanian cats to be shier. The result that did match our expectations is the fact that more (only) Moroccan cats described some of the stray cats they have interacted with as aggressive, as we do expect them to act bolder than their Romanian counterpart.

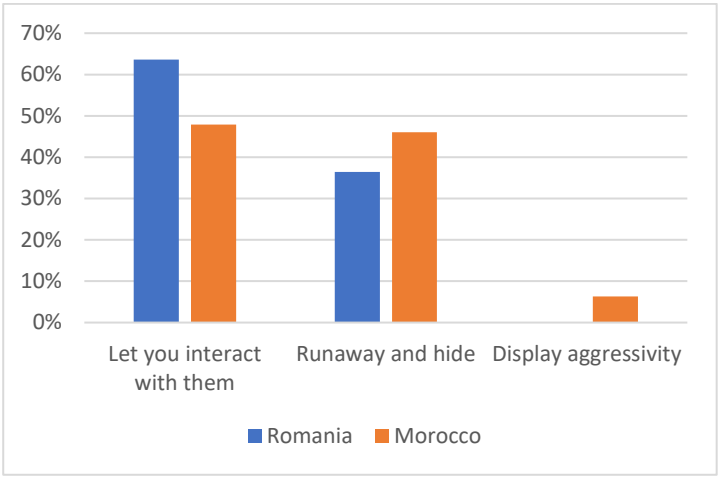

Fig. 8. Comparison of how stray cats respond in Romania and Morocco, when the participants have tried to initiate contact

Contrary to the prior questions referring to interactions this one englobes all kinds of cats people have interacted with: cats with owners (even being their own) and stray. Majority of cats in Romania and Morocco are perceived as friendly, with 45% Romanian responders selecting it as an answer and 52% Moroccans doing the same. For the other attitudes the results are: scared cats come in second place in Romania with 29% votes, leaving shy cats in third place with 25% of votes and no Romanian responder considering the cats they have ever interacted with being aggressive. In Morocco, scared cats come as well in second place, with 21% votes, close by in third place shy cats with a difference of only 1 vote. The main difference being that contrary to no Romanian

considering that the cats they have encountered as aggressive, 3% of Moroccan considered it to be the best way to describe their attitude out of the four given options (Fig. 9). No significant statistical difference was noted (p>0.05). Despite there being no cultural differences and results being similar, Moroccan cats come out as bolder (friendly and aggressive), while the Romanian ones were described as less bold (shy and scared), matching our expectations.

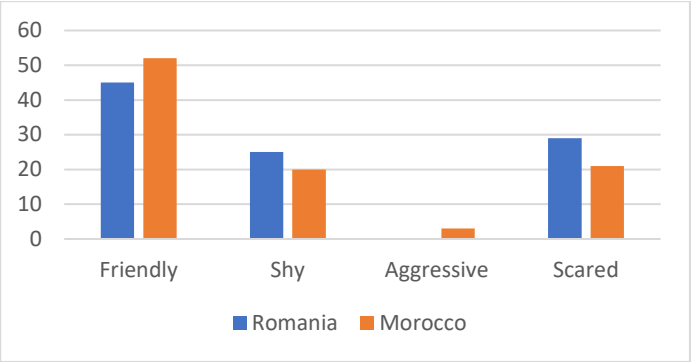

Fig. 9. How Romania and Moroccan participants perceive the cats they interact with

When posed the question of what they would do if a queen gave birth in their home, people selected the following answers: Romania: 34% people said that they would keep the queen and kittens until they were old enough to be independent, 62% people would relinquish them to a rescue and only 4% people selected that they would put them on the street; Morocco: 62% people selected that they would keep the queen and kittens until they become independent, 28% that they would contact a rescue to take over and 10% people (considerably more than in Romania) selected that they would put them on the street (Fig. 10). Significant statistical difference (p<0.001) and cultural difference as well, when it comes to what to do with them; majority of people in both countries wanted to make sure they are provided for, with a very small group agreeing with putting them on the street. While Moroccans would mostly keep them at home until they are independent, Romanians prefer to give them to a rescue that can take care of them. This can also be explained by the fact that there is more of a rescue and shelter culture in Romania than in Morocco. As well as that in Morocco, stray cats giving birth in someone home happens frequently.

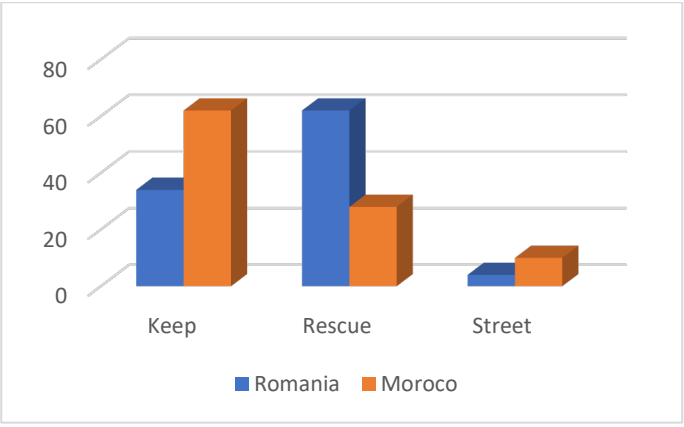

Fig. 10. How would the Romanian and Moroccan respondents react in the eventuality of a stray queen giving birth in their homes

When it comes to people's willingness to partake into the feeding of stray cats we obtained the following results from our survey: Romanians - 14% of them selected that they always feed strays, 22% that it's a practice they often partake in, 35% selected occasionally, 18% people selected rarely and the remaining 11% people that they never feed stray cats; Moroccans - 11% people said that they always feed the strays, 31% selected that they often feed them, 31% that occasionally partake in feeding them, 16% people that they rarely proceed to feed stray cats and the last 11% that they never do. No significant statistical difference (p=0.69) nor a cultural one were found

when it came to the willingness of people to feed stray-cats (Table 1).

As for if people choose to interact with the stray cats that they might see: in Romania - 18% people said they always interact with them, 29% that they do so often, 24% people only interact with them occasionally, 16% only rarely and 13% never choosing to do so; in Morocco - only 6% people said that they always do so, 23% interacting often with them, 20% only occasionally, 18% rarely and 33% people selected that they never interact with stray cats. Clear statistically significant difference (p=0.0032) expressing cultural difference when it comes to people interacting with stray cats (Table 1). We expected the contrary. Romanians were showed to have a higher tendency to interact with cats independently of the frequency, while considerably more Moroccans choose not to interact with strays.

Table 1

Frequency at which Romanians and Moroccans proceed to feed and interact with stray cats

	Feed cats				Interact			
	Romania		Morocco		Romania		Morocco	
	Nb	%	Nb	%	Nb	%	Nb	%
Always	14	14%	11	11%	18	18%	6	6%
Often	22	22%	31	31%	29	29%	23	23%
Occasionnally	35	35%	31	31%	24	24%	20	20%
Rarely	18	18%	16	16%	16	16%	18	18%
Never	11	11%	11	11%	13	13%	33	33%
		Statistical results				Statistical results		
		X	p			X	p	
		0.0224	0.69			0.1586	0.0032	

Table 2 shows people's willingness to help a stray cat in distress. First, we compared if they would help a hurt cat: Romania - 90% of people answered with yes and only 10% with no; Morocco - 79% selected yes as their answer and 21% selected no. A greater proportion of Moroccans said that they would not help a hurt cat if they saw one than expected, this can be explained by the great prevalence of animals needing help in Moroccan streets. A significant statistical difference was found (p=0.031).

The second comparison was made to see if they would intervene if a cat were being chased by another animal: in Romania we obtained 98/100 for this question - 82 (83.7%) of respondents said that they would intervene while 16 (16.3%) said that they would not; in Morocco we obtained very similar answers to those in Romania, with 82 (82%) people having said that they would intervene and 18 (18%) of people saying that they would not. The answers for both countries matched our expectations, and no significant statistical difference was found (p=0.75).

The last question we had for this assessment was if they would intervene if a cat was being mistreated. We obtained the same results for both countries with 97% Romanians and Moroccans having selected that they would intervene and the remaining 3% people having selected no (Table 2).

Table 2

Romanian and Moroccan people willing or not to assist distressed stray cats

	Help a hurt cat				Intervene if cat is chased				Intervene if cat is mistreated			
	Romania		Morocco		Romania		Morocco		Romania		Morocco	
	Nb	%	Nb	%	Nb	%	Nb	%	Nb	%	Nb	%
Yes	90	90%	79	79%	82	83'7%	82	82%	97	97%	97	97%
No	10	10%	21	21%	16	16'3%	18	18%	3	3%	3	3%
		Statistical results				Statistical results				Statistical results		
		X	p			X	p			X	p	
		4.61	0.031			0.09	0.75			0	1	

Conclusions

For societal perception it is very clear that Morocco as a country and society is a cat loving nation, clearly preferring cats to dogs. While the contrary is observed in Romania, where the dog is preferred as a pet to cats. Despite this reef, no significant differences when it comes to the treatment and empathy shown towards stray cats are worth discussing, which suggests that

European society's perception of cats as pets is beginning to change for the better, with cats gaining more and more ground in people's homes and hearts.

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PRE-APPOINTMENT MEDICATIONS ADDRESSING FEAR AND ANXIETY RELATED TO VETERINARY VISITS, IN CATS – A REVIEW

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Abstract

This review concentrates on currently available pre-appointment medications used to prevent fear and anxiety associated with veterinary visits, in cats. A literature review identified seven potentially effective drugs in alleviating acute situational fear and anxiety in cats: tranquillisers/neuroleptics/antipsychotics class – acepromazine, anxiolytics class – alprazolam, atypical antidepressants class – trazodone and mirtazapine, antiepileptic/anticonvulsant class – gabapentin and pregabalin, and sympatholytic class – dexmedetomidine. The available data on the mechanism of action, interactions with other drugs, side effects, recommended use and dosage were reviewed and presented.

Keywords: *pre-appointment medication, cats, fear, anxiety, veterinary settings.*

There is no doubt that cats have special needs when it comes to veterinary visits, and in most cases fear and anxiety set in. Some are stressed just by the presence of the transport cage, but for most the main stress is the transportation itself, followed by the consultation. This is why owners avoid regular check-ups, which can jeopardise the cat's health. Fortunately, there are ways to change this, by improving the experience for both the cat and the owner (19).

Psychological well-being is as important as physical health and should be addressed in every feline patient. The physical and social environment in the home and in the clinic should meet the cat's needs to optimize emotional and physical health. Cats have long-term memory and learn from both positive and negative experiences. In some cases, learning from a single experience can substantially influence subsequent behavior (18).

In addition to enriching the clinic environment for the cat's needs, which is very important to reduce stress, pre-appointment medications have been studied and developed. These can be very helpful in ensuring a smooth check-up, reducing the risk of injury to the cat and staff, facilitating the diagnosis and developing a relationship of trust between owner, cat and vet (19).

This review concentrates on currently available pre-appointment medications used to prevent fear and anxiety associated with veterinary visits, in cats. Seven medications were found to be potentially useful in reducing acute situational fear and anxiety in cats. These medications include the anxiolytics class (alprazolam), the tranquillisers/neuroleptics/antipsychotics class (acepromazine), the atypical antidepressants class (trazodone and mirtazapine), the antiepileptic/anticonvulsant class (gabapentin and pregabalin), and the sympatholytic class (dexmedetomidine). We reviewed and presented the information that was available on the dosage, suggested use, side effects, interactions with other medications, and mechanism of action.

Results and discussions

Following the decision to use psychoactive medications, the clinician must decide which agent to use with the aim of preventing side effects and improving behavior. A thorough understanding of the available options, pharmacological and pharmacokinetic properties, and potential side effects is necessary in addition to making an accurate diagnosis and ruling out physical illness (4). The available information about the commercial product, class, dosage, administration, and use of seven medications used to lessen cats' anxiety and fear during veterinary visits is shown in the table below (Table 1).

Acepromazine

The only psychoactive medication for a long time has been acepromazine (ACP), which was first authorized in several nations as an aid to control intractable animals and as an antiemetic agent to control vomiting due to motion sickness in dogs and cats (12).

Mechanism of action: Phenothiazine's depressant action on the central nervous system results in sedation and a decrease in spontaneous activity (1). The main mechanism by which phenothiazines produce their sedative effects is through blocking dopamine receptors, particularly D2 receptors. Decreases in cyclic adenosine monophosphate (cAMP) and adenylate cyclase activity, changes in postsynaptic potassium conductance, and calcium conductance are the results of this pre- and postsynaptic G-protein-coupled receptor

blockade. Sedation may also result from blocking central muscarinic, histaminic (H1), and α1-adrenergic receptors. Along with serotonin blockade, α1-Adrenergic receptor antagonism facilitates the blood pressure reduction observed with this class of drugs and may contribute to the decline in thermoregulatory control (9).

Interactions with other drugs: When used with ACP, central nervous system (CNS) depressants such as barbiturates, propofol, alfaxalone, and volatile anesthetics will result in additive CNS depression. When using ACP as a premedication, the dosage of other anesthetic medications should be decreased (14).

Side effects: The effects of phenothiazines on vessel tone (vasodilation) make them contraindicated in patients who are dehydrated, hypovolemic, bleeding, or in shock. Due to their impact on platelet aggregation, phenothiazines should be used with caution in patients who have thrombocytopenia or coagulopathies (3, 7).

Clinical studies: Authors now concur that phenothiazines are not the best medication for treating phobic or fearful behaviors, either temporarily or permanently, because they have poor anxiolytic activity, cause severe sedation, and even increase the sensitivity of the animals to stimuli. Acepromazine is not only not linked to long-term improvement of the emotional and behavioral signs in cats that exhibit signs of distress during particular situations (e.g., car transport, sudden noises), but it may even jeopardize the animal's welfare. Over time, acepromazine has also been used frequently in a variety of circumstances that made the animal more reactive or were linked to anxiety and fear symptoms, such as being near thunderstorms or fireworks. Even though ACP is used so frequently, it does not make animals feel less afraid or anxious. For these reasons, the authors concur that ACP should not be used as a preventative or only medication, to treat animals exhibiting symptoms of distress related to particular situations, like travel, loud noise exposure, or veterinary examinations (12).

Alprazolam

Alprazolam is a benzodiazepine with high potency. Cats with anxiety disorders and inappropriate urination behaviors can benefit from its use (5, 14).

Mechanism of action: Alprazolam produces anxiolysis and a variety of cognitive effects, such as memory inhibition, by increasing GABA activity in the central nervous system. As a short-acting drug with a two- to three-hour half-life, it is used to treat anxiety and fear-related disorders in cats, particularly when there are symptoms of panic. It is also used as an adjuvant to clomipramine or certain serotonin reuptake inhibitors to manage phobic reactions (3, 4).

Interactions with other drugs: Antifungals that block its metabolism, like itraconazole, should be used with caution. Ataxia or severe sedation may result from taking benzodiazepines with other sedatives, gabaergic drugs, or trazodone (1, 3).

Side effects: Sedation, paradoxical excitation, ataxia, muscle relaxation, and increased appetite are typical benzodiazepine side effects (3). Apart from alprazolam, the majority of drugs in this class are metabolized by the liver's glucuronidation pathway and are incriminated for some cases of hepatic necrosis in cats; as a result, alprazolam is regarded as a safe choice for cats (4). Although it seems to be less hepatotoxic than diazepam or clorazepate, it can cause glaucoma, severe liver or kidney disease, and hypersensitivity to benzodiazepines. It is not recommended to use in pregnant or lactating animals (1).

Clinical studies: A study conducted in Greece compared the anxiolytic effects of alprazolam and gabapentin and found that both were effective after 90 minutes of administration. Three groups of cats were prospectively and randomly assigned. Group A was given alprazolam (0.125 mg/cat), group P was given no medication (placebo), and group G was given gabapentin (100 mg/cat) 90 minutes prior to evaluation. The lowest dosage that was thought to be effective in causing anxiolysis in cats (0.125 mg/cat) was used in this investigation, and the results of this study indicate that alprazolam, when given to cats 90 minutes prior to their examination, is an effective anxiolytic drug (11).

Recommended use and dosage: Although absorption properties for cats and dogs have not been reported, it is best to use it about half an hour to 90 minutes before a frightening event. Initial doses for anxiolysis should be between 0.125 and 0.25 mg/kg p.o. as needed up to twice a day; however, reports have shown doses as low as 0.25 mg/cat p.o. q8–12h and as high as 0.6 mg/kg p.o. It is necessary to titrate the dosage to the lowest effective level after starting the first medication (1, 11, 14).

Table 1

Summary of commercial products, class, dosage, administration and use available for 7 drugs used to reduce fear and anxiety in cats at veterinary visits.

	Commercial product	Class	Dosage	Administration	Use	Reference
Acepromazine	Sedam	Tranquillizers/ neuroleptics/ anti-psychotics	1-3 mg/kg	Oral 15-30 min before a fear-inducing event	Recent studies no longer recommend using it.	1, 3, 9, 12
Alprazolam	Xanax, Niravam, CF-Flavazolam Oral Solution	Anxiolytics Benzo-diazepine	0.125–0.25 mg/cat /12h	Oral 30-90 min before a fear-inducing event	Most medications from this class are metabolised by the liver, except for alprazolam, therefore, is considered a safe option for cats	1, 11, 14
Trazadone	Desyrel, Oleptro, Trazodone	Atypical anti-depressants	for short-term use 50 mg/cat po or 150 mg/cat transderma l	Oral/ Transdermal one hour before travel	A single use of transdermal trazodone is effective and is easier for the owner to manage. The medication is applied to the hairless part of the inner pinna of both ears.	1, 10, 15, 18
Mirtazapine	Remeron, Miratаz	Atypical anti-depressants	1.9 mg/cat / 48h po or 2 mg/cat trans-dermal	Oral/ Transdermal 1-2 days before	The medication is applied to the hairless part of the inner pinna of both ears.	1, 6, 14, 21
Dexmedetomidine	Sileo(gel), Dexdomitor, Sedadex	Sympatholytic	2-10 mg/kg	IV/IM/SC	It is not an advantageous choice because of the injectable formulations and sedative effects. The commercial product Sileo is specifically designed for dogs, and	1, 3, 9, 14

					studies show that its effectiveness is insignificant in cats.	
Gabapentin	Gabapentin, Gabavet	Antiepileptic/ anticonvulsant	100 mg/ cat single use	Oral 90 minutes prior to placing the cat into a carrier and transporting it to the veterinary	Oral administration reduces stress-related behaviors during transportation and Examination, decreases aggression and increases compliance of cats during veterinary examination.	1, 12, 18
Pregabalin	Bonqat	Antiepileptic/ anticonvulsant	5 mg/kg	Oral 90 min before placing them into the carrier and transporting them in a car for at least 20 min to a veterinary clinic	FDA approved pregabalin oral solution (Bonqat) in 2023 for alleviating acute anxiety and fear associated with transportation and veterinary visits in cats. This therapy is the first for use in animals containing pregabalin to receive the FDA's approval	1, 8, 22

Trazodone

Trazodone is categorized as a serotonin antagonist and reuptake inhibitor (SARI) and is used to treat obsessive-compulsive disorders (OCD), depression, and anxiety in humans and animals (5).

Mechanism of action: Trazodone is a triazolopyridine antidepressant agent. By inhibiting the serotonin transporter (SERT or 5-HTT), 5HT2A/AC antagonism acts at moderate to high dosages. Apart from its potent 5HT2A antagonistic effects, trazodone also functions as an H1 histaminic and α1 adrenergic receptor antagonist at low dosages (3, 4, 12)

Interactions with other drugs: Use of monoamine oxidase inhibitors or medications

that are metabolized by cytochrome P450 2D6 (such as cimetidine and chlorphenamine) should be avoided. When combined with other serotonergic substances, there is a risk of serotonin syndrome; however, adjunctive therapy is occasionally employed. While carbamazepine will have the opposite effect, ketoconazole will prevent trazodone from being broken down, which will raise blood levels; itraconazole may have a similar effect, though there is no clinical evidence to support this (1, 14).

Side effects: Third eyelid protrusion, dry mouth, excitability, vomiting, and sedation. For patients with glaucoma, severe cardiac disease, and hepatic and/or renal disease, it should be used cautiously (3).

Clinical studies: A study from 2016 set out to assess the safety and effectiveness of oral trazodone as a single-dose sedative for cats. Randomized treatments were administered to six lab cats in doses of 50, 75, and 100 mg of trazodone and a placebo. Administration of trazodone did not result in any negative side effects or changes in laboratory results or physical examinations. Accelerometer data showed trazodone 50, 75 and 100 mg caused sedation as measured by activity reduction (83%, 46% and 66%, respectively) and there was a 14% activity increase after placebo. There was a significant reduction in video observation scores when cats were given trazodone 100 mg compared with placebo. Scores for behavioral response to examination, performed at 90 mins post-treatment, were not significantly different between cats receiving trazodone 100 mg and placebo (10).

Ten healthy client-owned cats, ages two to twelve, who had a history of anxiety during transportation or veterinary examinations were the subject of another study conducted in 2016. At random, each cat was given either 50 mg of trazodone hydrochloride or a placebo PO. After the treatment was given, the cats were put in carriers and driven to a veterinary clinic for a structured veterinary examination. Trazadone significantly reduced the cats' anxiety symptoms during transportation when compared to a placebo. Additionally, both the owner and the veterinarian gave it higher marks for handling ease during a veterinary examination. Signs of sleepiness were the most frequent adverse event associated with trazodone administration; no significant differences were found between treatments in heart rate or other physiological variables (18).

In a recent 2024 study, the effectiveness and safety of transdermal trazodone in cats were assessed in reducing stress and anxiety prior to a veterinary visit. For each dose, 150 mg of trazodone hydrochloride was mixed with 0.3 ml of cream base to create the transdermal trazodone. One hour before leaving the house, 0.15 ml of transdermal trazodone was applied evenly to the hairless part of the inner pinna of both ears. The benefit of using transdermal cream instead of oral trazodone is that less force is used to administer the medication, which reduces stress and improves drug response. Additionally, 84.6% of cats had a more seamless examination following administration. Applying the cream to the ear pinna was more convenient for both the cats and their owners. According to the current findings, cats may benefit more from transdermal trazodone in lowering transportation-related stress and anxiety, as evidenced by a significant decrease in vocalization and struggling. Transdermal trazodone may lessen stress hyperglycemia in cats, according to the results of the first visit is serum glucose test, which was also noticeably higher than the second visit's (15).

Recommended use and dosage: One hour prior to travel, 50 mg/cat is given orally to treat anxiety related to transportation and examinations, or 150 mg/cat is applied transdermal to the hairless area of the inner pinna of both ears (1, 15).

Mirtazapine

Mirtazapine is most commonly used to increase appetite in cats, especially those with chronic kidney disease (14). It is occasionally used to treat behavioral issues (21).

Mechanism of action: Noradrenaline levels in the brain are raised by this tricyclic antidepressant's action on central alpha-2 receptors and inhibit a variety of histamine (H1) and serotonin receptor types (1). These characteristics stimulate appetite in addition to having an anxiolytic effect (4).

Interactions with other drugs: Tricyclic antidepressants like clomipramine, serotonin reuptake inhibitors (SSRIs) like fluoxetine, and amitraz or selegiline may interact with mirtazapine and result in adverse effects. Diazepam, cimetidine, tramadol, and other painkillers are among the other medications that may interact with mirtazapine and should be used with caution. Patients who already have a haematological condition should not use this medication (21).

Side effects: Adverse reactions include increased vocalization and social interaction, sedation, which is common and can have profound effects on behavior (1).

Clinical studies: The effects of transdermal mirtazapine on the quantity of food containing gabapentin consumed as well as the impact of gabapentin on the symptoms of fear and anxiety

in cats when they were handled for examination were evaluated in a double-blind, placebo-controlled study. 94 healthy shelter cats were divided into five treatment groups at random. Transdermal placebo-treated cats ate substantially less food containing gabapentin than transdermal mirtazapine-treated cats. Although heart rate and respiratory rate were significantly inversely correlated with the estimated gabapentin dose taken, these effects were reversed by the addition of transdermal mirtazapine. The stimulating effects of pre-treatment transdermal mirtazapine may have offset the anxiolytic effects of gabapentin, even though it made gabapentin administration easier (16).

Finding the most frequently reported side effects and the dosage linked to these symptoms was the aim of another study. Vocalization, agitation, vomiting, ataxia/abnormal gait, restlessness, tremors/trembling, hypersalivation, tachypnea, lethargy, tachycardia, anorexia, disorientation, dyspnea, hypothermia, mouth breathing/panting, mydriasis, altered behavior, depression/sedation, fasciculations, hyperactivity, hypertension, pacing, dysphoria, inappropriate elimination, polyphagia, circling, discomfort, concealment, inappetence, seizures, and weakness were among the side effects linked to the administration of mirtazapine, listed from most to least common. The following dosages were linked to negative effects: 15 mg (40 cats), 3.75 mg (25 cats), 7.50 mg (4 cats), 30.00 mg (one cat), 18.75 mg (one cat), 11.25 mg (one cat), 5.80 mg (one cat), and 1.88 mg (one cat) (6).

Recommended use and dosage: 2 mg/cat PO or applied topically to the inner ear pinna 1-2 days prior (1, 21).

Dexmedetomidine

For dogs and cats, dexmedetomidine is FDA-approved for sedation and pain relief (13). Dexmedetomidine is medetomidine's pure dextroenantiomer (1). Dexmedetomidine was also frequently used to treat anxiety and other phobias in cats and dogs (13).

Mechanism of action: Acting as an agonist at both central and peripheral alpha-2 adrenoreceptors, it produces analgesia, muscle relaxation, and dose-dependent sedation (1).

Interactions with other drugs: No information is available regarding drug interactions (1).

Side effects: Side effects include mydriasis, decreased intraocular pressure, a brief rise in blood sugar due to a decrease in endogenous insulin secretion, and diuresis due to arginine vasopressin secretion suppression. When vomiting is contraindicated, dexmedetomidine should be avoided because it is common to vomit after intramuscular administration (1, 3).

Clinical studies: A medication called dexmedetomidine oro-transmucosal gel (Sileo®) has been approved by the FDA and the European Medicines Agency to help dogs who are noise averse feel less anxious and afraid (13). Dexmedetomidine has been administered orally through the transmucosal route in cats at a dose of 40 µg/kg and has been found to be both safe and effective. The sedative and antinociceptive effects were comparable to those of the parenteral administration (14). However, in a more recent study on the effectiveness of dexmedetomidine oromucosal gel in reducing cats' anxiety, there was no discernible difference between the placebo and the cats' anxiety levels during travel or veterinary exams (23).

Recommended use and dosage: For cats, an FDA-approved injection for preanesthesia, sedation, and analgesia (3). It is not an advantageous choice because of the injectable formulations and sedative effects in cats (1).

Gabapentin

In the literature, gabapentin has recently been mentioned as a safe and efficient way to help cats feel less stressed during veterinary examinations, transportation, and trap-and-release operations in feline colonies (12).

Mechanism of action: Gabapentin does not act on GABA receptors, despite having a structural resemblance to GABA. Rather, its effects seem to result from the amygdala's decreased glutamate release. This reduces the fear response, which is partially caused by glutamate (17). Although its exact mechanism of action is unknown, gabapentin's anxiolytic effect is thought to be connected to its impact on calcium channels in the brain (5, 12).

Interactions with other drugs: Antacids that contain magnesium and aluminium decrease the absorption of gabapentin from the GI tract; gabapentin should be taken at least two hours after taking such antacids. Cimetidine has been shown to decrease gabapentin's renal clearance; however, this is not deemed clinically significant in the product information (1, 17).

Side effects: When gabapentin is used regularly in animals, no significant safety issues have been documented. The only notable side effects are ataxia and sedation. Abrupt discontinuation has been known to cause a withdrawal syndrome in humans, but not in animals (14).

Clinical studies: Numerous studies have demonstrated gabapentin's efficacy. In one study, 20 healthy pet cats with a history of fractious behavior or signs of stress during a

veterinary examination were shown to benefit from a single dose administered before a visit. The cats were randomized to receive either a placebo or a capsule containing 100 mg of gabapentin before the first visit, and the opposite treatment before the second visit. The cats were scheduled for two veterinary visits, one week apart. When cats were given gabapentin, their stress levels during transportation and veterinary examination, as well as their compliance scores, were significantly lower than those of cats given a placebo. The most common side effect of gabapentin was sedation; ataxia, hypersalivation, and vomiting were also reported, but all of these side effects went away eight hours after gabapentin was administered. The study's findings indicated that gabapentin is a safe and efficient way to help cats avoid stress and aggression while also improving their compliance with veterinary exams and transportation (2, 18).

Recommended use and dosage: 100 mg taken orally once per cat, 90 minutes prior to placing the cat into a carrier and transporting it to the veterinary or for behavior issues, a starting dose of 5–10 mg/kg p.o. every 8–12 hours is recommended; gradual dose increases are encouraged (1, 18).

Pregabalin

Pregabalin oral solution (Bonqat) was the first to be approved by the FDA for use in animals containing pregabalin, and it was approved in 2023 for the treatment of acute anxiety and fear related to transportation and veterinary visits in cats (22).

Mechanism of action: Pregabalin is similar in mode of action to gabapentin. It functions by binding to the α2 delta subunits of the voltage-dependent calcium channels found in presynaptic neurons; this reduces the calcium influx via decreased release of glutamate and substance P at the synapse. It is structurally related to GABA but inactive at GABA receptors and does not seem to physiologically mimic GABA (3).

Interactions with other drugs: There is no information available (1).

Side effects: In cats, sedation, ataxia, and vomiting are common side effects (affecting 1–10%); other side effects, such as muscle tremor, are acknowledged but rare (1).

Clinical studies: In 2023, a clinical field study, conducted at 22 veterinary clinics in five European countries, was published to determine whether pregabalin reduces cats' anxiety and fear during veterinary visits and transportation. At a dosage of 5 mg/kg, 209 client-owned cats were given either a flavored pregabalin oral solution or an identical placebo about 90 minutes prior to being loaded into a carrier and transported to a veterinary clinic for at least 20 minutes. Only a small number of cats experienced temporary lack of coordination and fatigue, and the treatment was well tolerated. The flavored oral solution formulation was deemed user-friendly by the owners, and the cats enjoyed it (8).

This study showed that a single oral dosage of the new pregabalin oral solution reduces cats' anxiety and fear associated with travel and vet visits, offering a useful tool for both cat owners and veterinarians to facilitate cat-friendly handling and enhance the welfare of cats in circumstances that they frequently find extremely stressful (8).

Recommended use and dosage: The suggested dosage for anxiolytic properties is 5 mg/kg, about 90 minutes prior to the event (about 50% of cats respond well or better) (1, 8).

Conclusions

The most effective pre-appointment medications for cats that are also the easiest for owners to administer are Trazodone, Mirtazapine (because they can be administered transdermal), Alprazolam and Pregabalin (both being available in the form of oral solutions). Acepromazine and Dexmedetomidine are not recommended, as their effectiveness is insignificant.

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FUNGAL SPECIES ISOLATED FROM UNHATCHED HEN EGGS

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Abstract

This study investigated the fungal load in unhatched eggs as well as the presence and proportion of fungal species known to contaminate eggs and chicken embryos. The hatching percentage of three series of 60 chicken eggs subjected to incubation was very low. 136 (n=180) samples collected from unhatched chicken eggs, used in the incubation of 3 series consisting of 60 fresh eggs, were analyzed. For primary inoculation and colony isolation, the decimal dilution technique was used. From the last dilution, inoculations were made using Potato-Dextrose-Agar (PDA) medium. After incubation at 25ºC for 7 days, each colony was reinoculated to obtain pure cultures, which were characterized macroscopically and microscopically in order to identify the species of microscopic fungi. The proportion of isolated fungal species was different in the three series of unhatched eggs, but in all of them the *Aspergillus* genus prevailed (44%, series 1, 43.7%, series 2 and 37.1%, series 3). Other fungal species in a large proportion belonged to the genus *Penicillium* (20%, 15.6%, 29.6%) and *Fusarium* (12%, 28.1%, 29.6%). The species with pathogenic potential, most frequently identified according to macroscopic and microscopic characteristics were: *Aspergillus niger* (13/35), *Aspergillus fumigatus* (12/25), *Aspergillus flavus* (10/35) and *Penicillium crysogenum* (10/18) and *Fusarium oxysporum* (12/20).

Keywords: *fungi, hen eggs, low hatching.*

So far, the issue of mycobiota has been studied mostly on table eggs and less on hatching eggs, as research has focused on other factors that influence embryogenesis and especially hatching. The latest studies highlight a threat caused by contamination of the eggshell with microscopic fungi (5, 13).

Incubation conditions (high air humidity and diffusion of vapors from the contents of the eggs) favor the development of microscopic fungi on the eggshells. Consequently, the embryos may be contaminated with mycelia and die (4, 6).

Egg production conditions such as quality of straw and feed, presence of dust, temperature and humidity can cause fungal contamination of eggshells. It is known that eggs that are produced inside hens kept on litter are most susceptible to contamination by microscopic fungi (18).

Contamination in indoor, permanent bedding systems is usually caused by wet bedding and dirty nests (14, 19). Researchers examined different types of litter used in poultry farming and found that they contained genera of fungi such as *Aspergillus, Cladosporium, Drechslera, Penicillium, Stemphylium* and *Fusarium* (17, 22).

The fungal load (CFU/g) in the litter was the highest (28.49 log CFU/g) in straw chaff, being considered to have the highest risk of contamination for eggs. In addition, in this type of litter, a high concentration of ergosterol, a chemical compound present in the fungal wall, of 605.74 mg/kg was demonstrated, which means that this would be a good chemical indicator of microscopic fungal contamination of the litter material (22).

The spores present in the air can also cause numerous diseases (10, 20).

The hatchability of the fertile egg of hens can be adversely affected by factors such as temperature, humidity, ventilation, sanitary management of birds and inadequate facilities as well as bacterial and fungal infections (15). Rodríguez-Moya et al. (15), state that the ideal temperature for good embryo development is between 37 and 38 degrees Celsius. If the embryos are exposed to 47 degrees Celsius, they die immediately. Embryos, on the other hand, can withstand low temperatures for a short period of time, but cold reduces the yolk consumption of embryos, which prevents them from surviving (7, 16).

Mortality and embryonic development are significantly affected by the proportion of humidity inside the incubators. Van der Pole et al. (24), found that a relative humidity of 30 to 35 percent increased embryonic mortality. It has also been shown that a low relative humidity in the incubator increases the evaporation of water from the eggs, which leads to embryonic dehydration and as a result, the live chick weight is lower compared to the egg weight (12).

In addition, bacteria and fungi help reduce hatching. During incubation, both pathogens enter the egg through the pores of the eggshell. One of the most widespread bacteria in eggshells is *Salmonella spp.* (21). *Escherichia coli* is another species of pathogenic bacteria that causes embryonic death, if it enters the egg, passing through the pores of the eggshell (9).

Fungi such as *Aspergillus fumigatus, Fusarium culmorum* and *Fusarium equiseti*

could penetrate in eggs and contaminate the embryos, developing the mycelium that leads to embryonic death (23).

Knowing these aspects, the research carried out in this study sought to highlight the fungal load in unhatched eggs as well as the presence and proportion of fungal species known to contaminate eggs and chicken embryos (*Aspergillus, Penicillium, Fusarium, Alternaria, Cladosporium*).

Materials and methods

To carry out the study, the samples collected from the unhatched eggs obtained from the incubation of 3 series consisting of 60 fresh eggs from a farm where hens are raised for reproduction, the Ross308 hybrid, were analyzed. Their maintenance is done on the ground, with litter. The eggs are laid in nests equipped with a sloping floor and a place for collecting the egg. A harvesting belt runs under the nest so that the eggs are transported to the sorting room where staff pick out the broken, chipped, cracked eggs and eggshells and then each egg passes over a scale, discarding those smaller than 53g and the largest large of 74g.

The eggs received for the purpose of the experiment were subjected to fumigation, on the farm, with the Fumagri product, in a hermetically sealed room, with fumigants, for 1 hour. They were transported for the experiment, in egg formwork, at a temperature of 20°C.

The eggs were incubated in an individual system, using a Cleo 5 incubator, at a temperature of 37.8 - 38.4°C and a relative air humidity of 55-65% during the incubation period, and in the last 3 days of 65-75% (Fig. 1).

Fig. 1. Series of 30 eggs placed in Cleo 5 incubator

The eggs were turned twice a day, daily, from day 4 to day 18 inclusive. In addition, at exact time intervals (5 days, 12 days, and 19 days) the mirage of the eggs was realized in order to assess the viability of the embryos. On day 21, the hatched chicks were counted, the hatching percentage was calculated, and the unhatched eggs were subjected to mycological examination.

Before introducing the next series of eggs to the incubator, this was washed and decontaminated with *Ecocid solution* (1%), and then it was rinsed very well with water and left to dry. The hatched eggs were not subjected to any other disinfectant treatment.

For the mycological examination, samples were collected from the inside of the 136 (n=180) unhatched eggs, in the microbiological niche. Each egg was wiped with a swab soaked in 70° alcohol solution, after which, with the help of a Pasteur pipette, the egg shell was slightly punctured and the contents were collected from the inside (Fig. 2).

Fig. 2. Preparation of eggs for sample collection

From each sample, decimal dilutions were made in order to obtain a lower concentration of fungi to ensure the development of isolated colonies. From the last dilution (x106), 1 ml was seeded each in two sterile Petri plates, after which 10 ml of culture medium, agar with potato extract and dextrose (PDA medium – Potato-Dextrose-Agar) was poured, after it had previously been liquefied and allowed to cool down to a temperature of 45ºC.

After homogenization of the contents and solidification of the medium, the plates were placed for incubation at 25 ºC. Incubation was carried out for 7 days, to reach the maturity of the colonies, but they were examined daily macroscopically.

To identify the genus and species of fungi developed after seeding the samples with contents from unhatched eggs, primary examination of the macroscopic and microscopic characters of each colony grown on the seeded plates was made. Then secondary seeding was done to obtain isolated

colonies (subculturing) and final examination of morpho-structural characters (2, 11).

The primary examination of the colonies was carried out macroscopically and microscopically, starting from the third day. Macroscopically, the appearance and structure of the colony, the appearance of the edges of the colonies, the color on the obverse and reverse, the presence of pigment that diffuses into the environment were noted. Microscopically, the component structures were examined: hyphae and the gear of characteristic structures. For this, microscopic preparations were made from each isolated colony between the slide and the slide, using the solution with aniline blue to color the structures. Each slide was examined with two magnification objectives, 10x and 40x (Fig. 3).

For the purpose of seeding for subculturing, each colony grown on the primary inoculated and examined plates was inoculated onto Petri dishes containing the culture medium, potato extract agar and dextrose (PDA). The results obtained were carefully noted and monitored in order to establish the frequency of the isolated species.

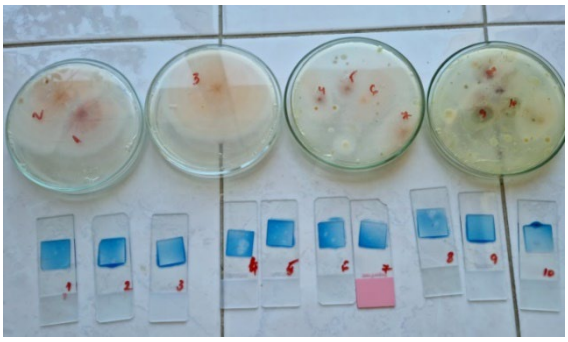

Fig. 3. Microscopic preparations between slide and coverslip, with aniline blue solution, prepared for microscopic examination of the morphological structure of fungi

Results and discussions

The fertility of the eggs, at each series (nr=60) and at each mirage, at 5 days, at 12 days, at 19 days is shown in Table 1.

Table 1

Fertility of eggs from the three incubation series, at reading intervals

No. series	No. fertile eggs / % at:		
	5 days	12 days	19 days
Series 1	58 / 96.6	36 / 60	17 / 28.3
Series 2	56 / 93.3	34 / 56.6	15 / 25
Series 3	57 / 95	35 / 58.3	17 / 28.3

From the data obtained, it was found that after 5 days of incubation, the percentage of viable eggs was over 90%, in all three series of chickens, with the specification that in the second series, a lower percentage was found, of 93.3 compared to the third series, where the percentage was 95 and compared to series one, where the percentage of fertile eggs was the best, 96.6. After 12 days of incubation, the proportion of viable eggs dropped drastically in all three egg series by about 1.6-fold. Thus, in series one, only 60% of the eggs were viable, in series two, only 56.6%, and in series three, the percentage was 58.3. After 19 days of incubation, the percentage of viable eggs was 3.5 times lower, so that in series one only 28.3% of eggs were viable, in series two, only 25%, and in series three only 28 .3%.

The number of hatched chicks and the hatching percentage, in each series of hatched eggs, after 21 days of incubation, are shown in Table 2.

Table 2

Percentages of chicks hatched per incubation series

No. series	Number of chicks hatched / unhatched eggs	Hatching percentage (%)
Series 1	15/45	25
Series 2	14/46	23.3
Series 3	15/45	25

The number of chicks hatched from each series was very low, resulting in a hatch percentage of 25% in the first series, 23.3% in the second series and 25% in the third series. In series one there were 15 viable chicks, 1 incompletely developed, non-viable and 1 fully developed non-viable that did not hatch either. In series two there were 14 viable chicks and 1 non-viable, incompletely developed. In series 3 there were 15 viable pups, 2 non-viable,

incompletely developed pups that did not even hatch.

The type of fungi and their proportion isolated from the unhatched egg samples is shown in table 3.

In the first series of unhatched eggs (n=25), fungi of the genus Aspergillus predominated (44%), followed by those of the genus *Penicillium* (20%) and those of the genus *Fusarium* (12%). Species from the genus *Alternaria* and from the genus *Cladosporium* were also highlighted, in small proportions.

In the second series of unhatched eggs, species from the genus *Asperillus* were also isolated in high proportion (43.7%), but the proportion of species from the genus *Fusarium* was much higher (28.1), compared to those from the genus *Penicillium* (15.6%).

From the third series of unhatched eggs, a very high proportion, 37.1%, of *Aspergillus* species were isolated. Also, in similar proportions, species of the genus *Penicillium* (29.6%) and species of the genus *Fusarium* (29.6%) were isolated.

Table 3

The genus of fungi isolated from unhatched eggs and their proportion

Hatched Egg Series	The genus or species of fungi isolated	Number of developed colonies	Proportion of total colonies (%)
Series 1 (number of colonies=25)	*Alternaria*	**2**	**8**
	Aspergillus	**11**	**44**
	Cladosporium	**7**	**28**
	Penicillium	**5**	**20**
	Fusarium	**3**	**12**
Series 2 (no. colonies=32)	*Alternaria*	**1**	**3.1**
	Aspergillus	**14**	**43.7**
	Cladosporium	**3**	**9.3**
	Penicillium	**5**	**15.6**
	Fusarium	**9**	**28.1**
Series 3 (number of colonies=27)	*Alternaria*	**0**	**0**
	Aspergillus	**10**	**37.1**
	Cladosporium	**1**	**3.7**
	Penicillium	**8**	**29.6**
	Fusarium	**8**	**29.6**

The potentially pathogenic species, most frequently identified according to the macroscopic and microscopic characteristics were: 13/35 - *Aspergillus niger* (Fig. 4), 12/25 - *Aspergillus fumigatus* (Fig. 5), 10/35 - *Aspergillus flavus* (Fig. 6) and 10/18 - *Penicillium crysogenum* (Fig. 7) and 12/20 - *Fusarium oxysporum* (Fig. 8).

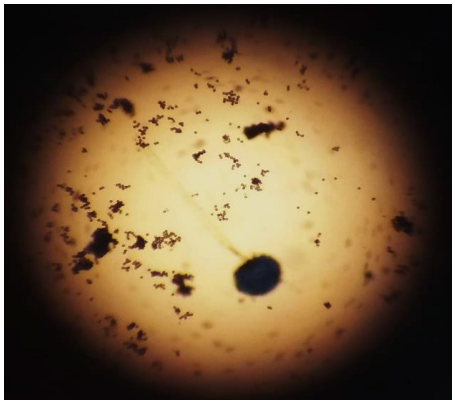

Fig. 4. *Aspergillus niger*

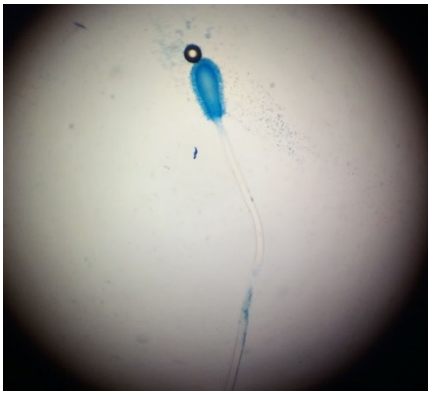

Fig. 5. *Aspergillus fumigatus*

It is known that the hatchability of the fertile egg of chickens can be reduced by factors such as temperature, humidity, ventilation, etc. (16). Thus, French (3), stated that the ideal temperature for good embryo development is between 37°C and 38°C. If the embryos are exposed to 47°C, they die immediately. Embryos, on the other hand, can withstand low temperatures for a short period of time, but cold reduces the yolk consumption of embryos, which prevents them from surviving (16).

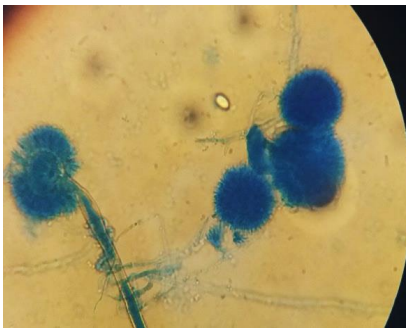

Fig. 6. *Aspergillus flavus*

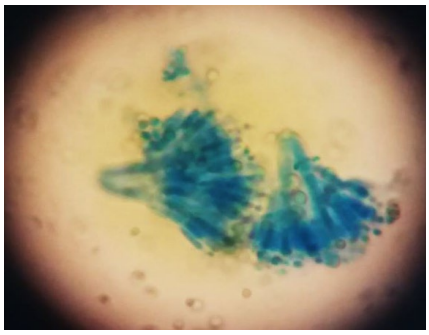

Fig. 7. *Penicillium crysogenum*

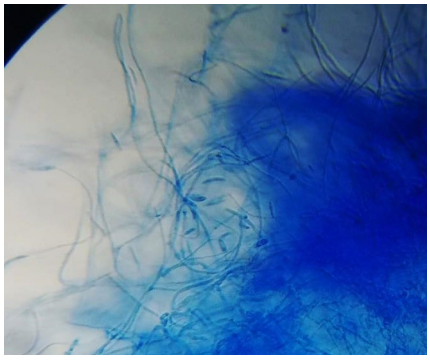

Fig. 8. *Fusarium oxysporum*

Mortality and embryo development are significantly affected by the percentage of humidity inside the incubators. Van der Polet et al. (24) in 2013 found that a relative humidity of 30 to 35 percent increased both hatchability and embryonic mortality. They also found that a low relative humidity in the incubator increases the evaporation of water from the eggs, which leads to embryonic dehydration (24).

In addition, bacteria and fungi help reduce avian hatch. Nests, floors, bedding and airborne dust particles are all sources of fungal and bacterial contamination of eggs on any farm. During incubation, both categories of pathogens can enter the egg through the pores of the eggshell (1).

Scientists Jacobsen et al. (8) found that *Aspergillus fumigatus* and *Aspergillus flavus* were the most common contaminants of eggs prepared for hatching. In the eggs, this species develops the vegetative apparatus, the hyphae, which give the appearance of green mold and which produces embryonic mortality (8).

In a study by Tomczyk et al. (23), fungi belonging to the genera *Alternaria, Penicillium* and *Chaetomium* were most frequently isolated from the eggshell surface. The Alternaria species isolated in this experiment are mainly found in cereals. These fungal species have the potential to generate such toxins as *alternariol* and *tenuazonic* acid. It was also found that *Penicillium* species, isolated in the experiment, accelerated the process of egg rotting and caused qualitative changes in the egg content (23).

Conclusions

The research carried out revealed the presence of an extremely diverse mycotic population in the contents of unhatched eggs.

Among the species isolated, the species of the genus *Aspergillus* (*A. niger, A. flavus, A. fumigatus*) had the highest frequency.

Other fungal species isolated in this study, which also have pathogenic and toxic potential, belong to the genera *Alternaria, Penicillium and Fusarium*.

A low egg hatch percentage must also account for primary fungal contamination, which occurs on the farm. In the incubator, fungi find excellent conditions for development that can induce indirect embryo mortality.

It is recommended to use a product, which, applied by fumigation, will also act effectively on fungi, not only on bacteria and viruses.

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WELFARE ASSESSMENT IN TWO SHEEP FARMS

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Abstract

This study investigates the reproductive performance and welfare assessment of sheep across two farms in Romania, Farm A and Farm B, each representing distinct climatic conditions and management practices. Data were systematically collected through structured questionnaires, field observations, and laboratory analyses of dietary components. Key reproductive metrics such as fertility rates, prolificacy, neonatal mortality, and inter-lambing intervals were analysed alongside environmental factors, veterinary management practices, and stress impacts from handling and transport. Results indicated that Farm A, utilizing a free-stall system and a diverse natural diet for Tsigai sheep, reported a fertility rate of 90% and prolificacy of 140%. Conversely, Farm B, employing a closed-stall system with controlled feeding for Assaf sheep, demonstrated slightly superior fertility (95%) and prolificacy (180%). Welfare assessments between 2021 and 2024 revealed an improvement in practices at both farms, with Farm A achieving a score of 89/100 in 2024 and Farm B rising to 85,25/100. Despite these advancements, areas for further improvement remained, particularly in biosecurity, animal handling, and environmental management. Overall, this comparative analysis underscores the influence of management systems and environmental conditions on the reproductive performance and welfare of sheep, providing insights for optimizing practices in similar agricultural contexts.

***Keywords*:** *welfare quality assessment, sheep evaluation, improvement of management*.

Sheep welfare can vary based on several factors, such as the management system (3), each type having both challenges and opportunities that must be analysed to ensure the enhancement of animal welfare (8). For example, some researchers (7) emphasize the environmental challenges that come with extensive practices due to seasonal changes amongst others. Others (14) that looked more in depth at the effects of intensive systems, are highlighting the impact of housing types and production strategies while some (19) are focusing their research on behavioural responses and indicators as a way to constantly improve the sheep welfare. Thus, all current data in the research field is proving that no matter what type of system is preferred, there is indeed a need to assess the welfare in a continuous form (4, 5, 11, 13, 17). At the beginning the main focus was on ensuring sheep welfare inside the slaughterhouses (6, 9, 10, 15), but now there is solid proof that the quality of meat is affected by the in-farm management and welfare, on more than one area (17, 21). Understanding the socioeconomic constraints that influence farmers' ability to implement welfare-friendly practices is crucial for developing effective interventions (3, 7, 8). Support programs that offer financial aid or resources can help bridge the gap in welfare practices (13). Consumers are increasingly interested in the origins and conditions under which their food is produced. Studies, such as those by Nielsen (15), show that informed consumers are more likely to prefer products that adhere to strict animal welfare standards. The key points, when assessing sheep welfare, are based on the Welfare Quality assessment protocol made by AWIN (18), which was formulated as a part of the European Programme in the 7th Framework. This includes the same 4 principles that are found in other farm animals which are complying to the 5 freedoms of animals (2), concept established and recognized worldwide. The principles are evaluating the housing, feeding, health and behaviour (2), each having their own set of indicators specifically selected based on number of animals evaluated, the type of management and dual approach, meaning there are both qualitative and quantitative indicators (2). This approach ensures the validity of the evaluation and can outline specific areas that need to be improved. Good feeding addresses the nutritional needs of sheep, utilizing indicators such as body condition score and lamb mortality to assess the effectiveness of feeding practices and growth outcomes (2, 9, 21). Good housing focuses on the comfort of the animals, evaluating factors such as fleece cleanliness and stocking density to ensure that sheep have a suitable environment (3, 7, 12, 14). Good health emphasizes the importance of monitoring the health status of the flock (2, 18). This principle involves the observation of indicators related to the absence of disease and injury, including signs of lameness, body lesions, and overall health metrics, ensuring that sheep remain healthy and free from suffering (2, 8, 11, 18). Appropriate behaviour assesses the animals behavioural expressions and their social interactions. This principle documents important behaviours, including social withdrawal and stereotypy, to evaluate

how sheep respond to their environment and manage stress (3, 16, 19). The most issues are seen in the health department, farmers stating the presence of several health conditions among their flock, such as: lameness, skin lesions, and dermatophilosis, which are often associated with poor body condition scores and insufficient nutrition (4, 9, 10). Regular health monitoring and immediate treatment are essential to address these concerns (1, 2). When looking at housing conditions, the extensive system, confront specific environmental difficulties, such as heat stress and limited food resources, which can exceed their natural coping abilities and ultimately compromise their welfare (14, 18). Research indicates that sheep under stress from heat may show increased aggression, lower feed intake, and decreased productivity, highlighting the need for appropriate environmental management (1, 16). Practices like short tail docking and castration are widespread but raise significant welfare concerns (12). Lastly, educating farmers and stockpersons about the significance of welfare and appropriate management strategies is vital, the findings from Munoz (13) indicate that improving farmer engagement through education and outreach programs can significantly enhance sheep welfare practices on farms. Farmers are more likely to adopt management practices that benefit sheep, if they have the means to access good monitoring systems and technologies (1, 20).

Materials and methods

The study was conducted in two farms located in different climatic regions of Romania between years 2021 and 2023 with general resolution in 2024, Farm A and Farm B, selected based on their different management practices and environmental conditions. Data on nutrition, environmental conditions, veterinary management practices, and animal handling were gathered using several methods (Table 1). Structured questionnaires were administered to farmers to collect information regarding dietary practices, health management protocols, and handling methods.

The main focus was to review the difference between the two farms and the relevant indicators for each of the farms studied to ensure proper method of evaluation.

Table 1

Welfare Assessment Results according to Welfare Quality® Criteria (adapted model)

EVALUATION CRITERION	SUB-CRITERION	FARM
Feed Quality	Quality of feed	Good quality
		Adequate provision
	Access to water and mineral supplements	Good quality water, adequate mineral supplements
Shelter	Quality of shelter	Well-maintained facilities
	Bedding and ventilation	Good bedding and ventilation
Health	Disease prevention and treatment protocols	Separate health indicators
	Health monitoring and record keeping	Good health monitoring and records
Water Quality	Availability and access to water	Adequate availability and access to water
	Water quality parameters	Good quality parameters
Comfort	Density of livestock and available space	N/A
	Rest areas, social interaction opportunities	No enrichment activities
Animal Handling and Movement	Handling practices	Appropriate handling practices
	Transport and movement practices (N/A)	Transport and movement practices (N/A)

Additionally, field observations were conducted to assess the conditions in which the sheep were raised, including housing and grazing practices. Nutritional components of the

diets were analyzed to identify the essential nutrients present in the feed offered to the sheep.

The reproductive performance of the sheep was evaluated, including fertility rates (percentage of ewes that conceived), prolificacy (number of lambs born per ewe), neonatal mortality rates (percentage of lambs not surviving to weaning), and inter-lambing interval (the time between successive births). Environmental factors, including temperature and humidity levels, were monitored using standard meteorological equipment.

Each farm was assessed based on the 4 principles and was given a score from 2021 until 2024, along with recommendations of improvement, to see the difficulty of changing, the impact of changes made for each lacking area and yearly improvement rate.

Results and discussions

The implementation of WQS assessment on sheep farms involves the use of a set of standardized indicators and criteria, specifically tailored to reflect the specific needs and particularities of sheep (18). We established periodic and systematic evaluations a monitor progress and to identify areas needing improvement in animal management and care (4). Farm A, located in Constanța County, Romania, uses an intensive free-stall growth method and has 1600 sheep, including 1200 ewes and 60 rams with average weights of 60 kg for ewes and 110 kg for rams, while Farm B, in Arad County, practices an intensive permanent closed-stall growth method with a smaller flock of 190 sheep, consisting of 160 ewes and 10 rams, who weigh an average of 50 kg for ewes and 90 kg for rams; both farms are using natural mating, with Farm A introducing rams in August for its mating. Lambing season, which occurs from August to September and January to February, whereas Farm B introduces rams in March for its mating and lambing season, running from March to July. Farm A reports a fertility rate of 90% and a prolificacy of 140%, resulting in approximately 80-90 lambs daily during the lambing season, Farm B has a slightly higher fertility rate of 95% and a greater prolificacy of 180%, yielding about 30 lambs per day.

Table 2

General descriptors of the farms

CATEGORY	FARM A	FARM B
Location	Constanța County	Arad County,
Area	Plain	Plain
Growth Method	Intensive, Free-stall	Intensive, Permanent closed-stall
Breed	Tsigai	Assaf
Total Sheep Population	1,600	190
- Ewes	1,200	160
- Rams	60	10
- Lambs	350	20
Age	1.3-6 years	1-4 years
Average Weight	Ewes: 60 kg Rams: 110 kg	Ewes: 50 kg Rams: 90 kg
Age Of First Insemination	1 year	7 months
Insemination Method	Natural mating	Dual
Number Of Rams Per Ewe	1 ram for every 20 ewes	1 ram for every 8 ewes
Month Of Introducing Rams	August	March
Mating/Lambing Season	August-September / January-February	March / July
Number Of Lambings Per Day	80-90	30
Fertility Rate	90%	95%
Prolificacy	140%	180%

The yearly global scoring (Fig. 1) has shown differences between the two farms, as well as a great improvement over the years when looking individually. We should note that the global scoring is resulted from the average scoring of the 4 principles included in the assessment that were individually scored each year based on the indicators included. Farm A, when first evaluated, received a total score of 82.5/100 which is considered to be Good Welfare. After the first assessment, recommendations of were given resulting in the improvement of welfare over the study period, by 6.5 points (89/100). The yearly improvement resulted in a 8.79% increase of the score.

Farm B, had an initial score of 65/100 which can be considered to be on the lower end of the Adequate Welfare. By the end of the study, the score increased up to 85.25/100, meaning a 20.25 (31.54%) increase. Comparing these two data sets we can see that from the beginning, Farm A had areas that were slightly lacking and needed to be improved, but they weren't severely impacting the welfare of the sheep. Farm B, had a larger area that needed improvement and following the yearly recommendations, it managed to tighten the scoring gap between Farm A and B. The recommendations were based on the issues revealed during the evaluation period and known and researched in the field.

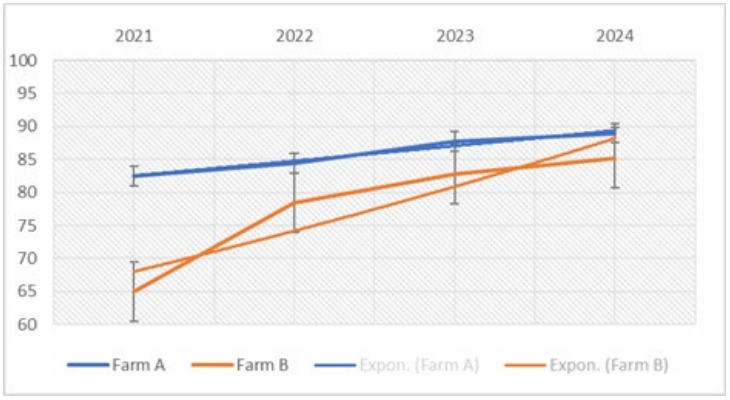

Fig. 1. Welfare yearly scoring for both farms

This improvement can be attributed to its enhanced commitment to animal welfare, characterized by a more reliable disease monitoring system and comprehensive record-keeping practices capturing essential information on health, vaccination, treatment, breeding, and nutrition (20). Despite these advancements, the evaluation identified remaining gaps, particularly in biosecurity measures requiring attention to ventilation improvements during colder months and a more efficient waste management system (7). Looking at the principles scoring we can explain better the areas that were impacting the overall score (Fig. 2, Fig. 3).

In 2021, the baseline health score was 76.5, revealing significant gaps in health monitoring. To address this, the farm implemented regular veterinary assessments and improved helth protocols in 2022, which resulted in an increase in its score to 79.5. However, some staff members needed additional training to effectively assess health needs. The positive trend continued into 2023, as Farm A refined its health management practices, raising its score to 85. Despite this progress, issues with record-keeping persisted, highlighting areas for further improvement. By 2024, the farm concentrated on targeted health interventions, achieving a score of 86.

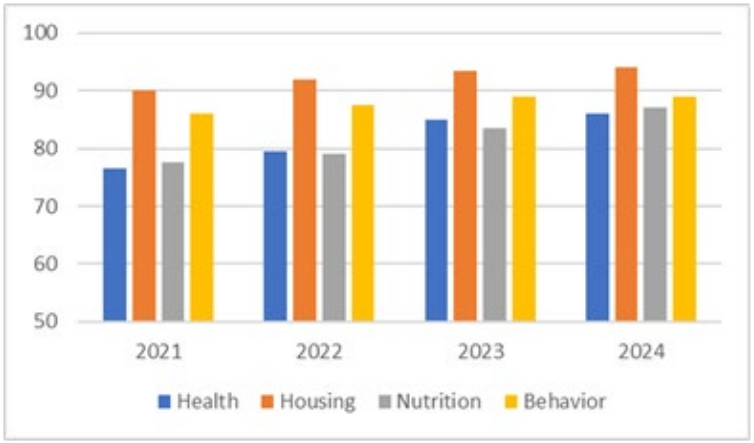

Fig. 2. Welfare scoring on each category for Farm A

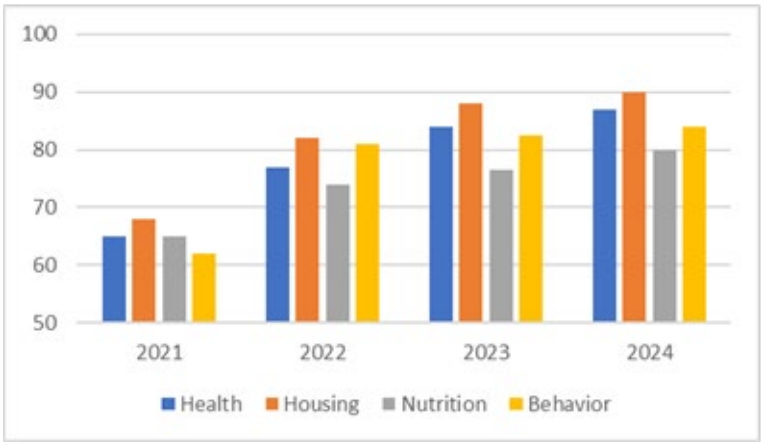

Fig. 3. Welfare scoring on each category for Farm B

In terms of housing, Farm A began had an initial score of 90, indicating good living conditions. Nevertheless, assessments revealed that ventilation and lighting required enhancement. The farm addressed these issues in 2022, resulting in a slight rise in the housing score to 92. Although these upgrades were beneficial, they did not substantially influence the overall score. Continuous efforts in 2023, including regular maintenance and minor improvements, led to a small increase to 93.5. By 2024, further refinements raised the score to 94.

Regarding nutrition (Good Feed), Farm A had a score of 77.5 in 2021. After implementing more balanced feeding programs in 2022, the score increased to 79, reflecting moderate progress. Despite this, issues with underweight and overweight animals persisted. Farm A refined its dietary programs in 2023, resulting in a notable increase to 83.5. By 2024, the nutrition score reached 87, due to proper feed for all the animals, although additional staff training for calculation of individualized feeding remained necessary.

The score for the last principle, was 86 in 2021, the evaluations showed a lack of enrichment activities and social interactions. In 2022, improved handling techniques resulted in a slight score increase to 87.5, yet limited enrichment continued to affect the overall behaviour. The introduction of minor enrichment strategies in 2023 raised the score to 89, indicating steady progress, but by 2024, the score remained stable.

Farm B had a lower health score of 65 in 2021, which prompted a thorough evaluation of its health management systems. In 2022, updates on health monitoring and additional staff training were implemented, resulting in an increase of the score (77), despite initial resistance from some staff. The trend continued in 2023, with enhancements in health management leading to a further increase (score 84/100). In 2024 the final scoring was 87, due to the continuous improvements.

Farm B's initial housing score was 68, revealing an urgent need for improvements. After significant renovations in 2022, to enhance ventilation and lighting amongst space adequacy, the given score was 82. While these changes were beneficial, renovation costs and time delays caused temporary discomfort for the animals. Further enhancements in bedding and cleanliness raised the housing score to 88 in 2023, and it reached 90 by 2024. Although the total improvement in housing quality was substantial, some facilities still did not meet optimal welfare standards.

In terms of nutrition, Farm B had a score of 65 in 2021. After revising the nutrition programs and feeding techniques in 2022, the score increased to 74, reflecting significant improvement, although consistency remained a challenge. In 2023 there was a change when it came to water availability and proper feeders and the total score given was 76.5, and by 2024, it reached 80, while continuing to require attention to access to clean water and minerals.

Finally, Farm B's behaviour score was 62 in 2021, highlighting the need for better handling practices and socialization. Training in handling techniques resulted in an increase of the score up to 81 by 2022, though the transition caused temporary stress for the animals. In 2023, efforts to improve interactions and social environments increased the score to 82.5, with a slight rise to 84 by 2024 due to reliance on similar strategies that raised concerns.

Conclusions

The implementation of standardized welfare assessments on sheep farms has led to substantial improvements in animal welfare scores over time; Farm A displayed a consistent

upward trend in areas such as health, housing, nutrition, and behavior, from 82.5 to 89, while Farm B, starting from a lower baseline of 65, implemented targeted enhancements and changed management practices to raise its score significantly to 85.25. This progress underscores the effectiveness of systematic evaluations in identifying areas for improvement and highlights the vital role of dedicated animal welfare practices in achieving better outcomes for sheep management across varying farming methods.

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ASSESSMENT OF AGGRESSIVE BEHAVIOUR OF CATS IN ROMANIAN AND MOROCCAN VETERINARY PRACTICES

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Abstract

A questionnaire containing 30 questions and pictures of different body postures was used to assess the emotional state and aggressiveness of cats during the consultation in veterinary practices, in Morocco and Romania. The cats were presented to the clinics for routine check-ups or various medical conditions and not for aggression or other behavioural problems. A total of 60 cat were evaluated, 30 in each country. Moroccan cats expressed higher levels of fear related aggressivity, in comparison with their Romanian counterpart. This difference could be the result of Moroccan cats being usually taken to the veterinary practice only when needed (emergency). This makes them unfamiliar with such environments and procedures, which understandably can be a great source of stress and fear. Moroccan cats showed also a tendency to bolder behaviour, displaying not only more aggressivity, but also outgoing friendly behaviour. In terms of cooperation and the need to use restraint, there were no differences between cats from the two countries, demonstrating the strong influence of following standard veterinary protocols for approaching and restraining feline patients, at the expense of personalized and emotional status-centered approaches to each individual.

Keywords: *cats, aggressive behaviour, body postures, veterinary practices, Muslim country, European country.*

After house soiling, cat aggression is the second most common behavioural problem that cats are presented with a referral to the veterinary practice. Depending on the type, degree, and expression of aggressivity, aggressive cats can present a danger at home, and it is primordial to advise owners of aggressive cats on how to handle them and cohabitate with them while trying to rehabilitate them and solve the root cause. Treatment cannot always be guaranteed and even in cases where it is both possible and successful, it doesn't mean that the problem will be solved or that the aggressivity will completely disappear (3, 13).

Aggressivity can be explained as harmful or threatening behaviour towards an individual or group thereof. It can be expressed with a wide variety and range of behaviours: subtle body posture changes, facial expressions, vocalization, all the way to full-blown vicious attacks. Taking into account these factors, aggression can be split into different categories depending as well on what triggers the cat (10).

Play aggression is the most common type of aggressivity that cats exhibit towards their owners, being displayed mostly by kittens and young cats. Although a natural expression and phase of development, it can still result in a variety of injuries still needing to be controlled as to reduce the danger posed to family members and other pets in the same household.

Predatory-type play behaviour can be more intense, often resulting in more serious injuries that are the result of biting and clawing that are done without appropriate inhibition. To people that are not familiar with such behaviour it can result in anguish as it often leads them to think that have a mean pet living at home (2, 11, 12, 16).

Predatory behaviour is a highly motivated and instinctive behaviour for cats as they naturally hunt and kill prey animals. The earliest signs of such behaviour are silent stalking, body is held close to the ground during the approach and attack is devised as to kill the prey. Predation is not preceded by any vocalization or postural threats as it is the natural instinct to hunt and kill, which makes any kind of warning counterproductive to the goal. There is also a learned component to predatory behaviour on top of it being a natural instinct; kittens kept with their mothers for a longer period of time grow up to become better hunters and as such make up for better cats that are kept for rodent control. Unfortunately, this behaviour can at times be directed towards family members or other pets, causing problems and inducing fear, creating the need for its correction. It is very difficult to keep predatory behaviour from developing but it is important to manage it by discouraging wildlife from visiting the yard (1, 8, 14, 19).

Fear aggression happens when a cat is exposed to something he perceives as a threat, and it escalates as there is no way to escape. It can also arise from inanimate stimuli such as sound and the aggression can be redirected at the nearest by person or animal, even if the sound does not emanate from them. The fear response intensity is directly proportional to the intensity of the stimuli and how much fear it induces in the cat. It is usually seen when an animal is: touched, looked at, approached, or even simply stared at. Fear aggression is also often referred to as defensive aggression, the

cats do believe that they are in imminent danger leading them to react. Genetics can contribute to this problem, but environmental factors such as inadequate socialization and punishment are the biggest contributors (3, 4, 10, 17).

Petting-induced aggression. Some seemingly social cats, that might even be attention seeking and go to the extent of requesting their owner's attention and affection by: rubbing against them, jumping into their laps, or even by crying; will allow petting and physical touch but they seem to have a threshold, as after some time they will bite and run away from the same person that moments ago was petting them and whose attention they asked for in the beginning. Some owners are able to limit contact to that agreed period of time because their pet will always exhibit certain warning signs before resulting into aggressions. Such behaviour is potential dangerous for children, as not like adults they fail to read the cats body posture not knowing when to stop and continuing to pet it despite it reaching its threshold (10).

Status aggression. Is displayed by some cats in situations when other pets or even the owners are displaying assertiveness, they tend to attack or bite them in order to take control over the situation. Often seen when an owner attempts to move the cat away from where it is, during stroking, lifted or approached. Other cats if that request attention or play biting might also display assertiveness. It is hard to recognize this behaviour as it is poorly described in literature and isn't displayed by any specific marks. Any type of pushy behaviour should always be ignored and never rewarded (1, 6, 18).

Redirected aggression is seen mostly in adult cats. Intermale, territorial, fear-induced and defensive aggression are the types that are most likely to be redirected; meaning that the target of the aggression is not the cause of the stimulus that triggered the reaction. The aggression usually takes place when a person or animal approach or touch a cat that's already aroused following a prior stimulus. But it is not a requirement for a person to initiate contact as often the cat will attack anyone that is in a range of 3-5m despite them not provoking them. These attacks leave the owners surprised and shocked as they cannot make sense of what has caused them failing to realize that the cat was already aroused, leaving them shaken (9, 15, 20).

Pain-induced and irritable aggression. Some metabolic disorders such as renal or liver disease, endocrine disorders, sensory decline, CNS disorders or even lack of sleep, are conditions that do not cause any pain to the patient but increase irritability and therefore aggression. As it is hard to properly assess pain in cats, it is also very hard to separate irritable aggression from pain aggression thus grouping them as one. Handling, or palpation of a painful area can be triggers to such behaviour, making it frequently seen in the veterinary scene, leading to attacks from the patient (13).

Pathophysiological aggression. Arise from a very complex root of both medical condition of chronic or acute duration, drug-responsivity, and behavioural modification techniques. Their expression is hard to fit within any other type of aggression. Neurological disorders, infections, trauma and parasitic infestation can all lead to modified behaviour (13).

Learned aggression is suspected to be a component with many types of feline aggression. This behaviour might arise from one aversive event or from exposure to multiple mildly threatening stimuli. Unfortunately, such aggression can be willingly provoked by some people but can also happen in pets that are punished, threatened or even unintentionally rewarded by the owner after displaying aggressivity, making them progressively more aggressive. Thus, oblivious owners unintentionally condition aggressivity. Behavioural modification can be explored as a treatment (7, 10, 13).

Idiopathic aggression groups all kinds of aggression with an unknown causative agent and appears unpredictably. Can only be diagnosed in cats in which a full medical work up has been run ruling any other medical root cause, and no stimuli have been found by a competent behaviour consultant. Pertinent information might not be all available to the consultant and some stimuli and information is most probably overlooked by the owners, leading to fear or misdirected aggression falling into this category. Owners must always be advised to be cautious around their pet if it behaves aggressively when the stimuli are unknown thus unavoidable. Unfortunately, euthanasia is often recommended in such cases as it is almost impossible to correct this behaviour, as we ignore the cause (5, 13).

This study aimed to determine if there are any differences in the emotional state and aggressiveness of cats during consultations in veterinary practices in Morocco and Romania.

Materials and methods

A questionnaire containing 30 questions and pictures of different body postures (eye aperture, pupil size, position of the tail, facial expressions and overall body posture) was used to assess the

emotional state and aggressiveness of cats during the consultation in veterinary practices, in Morocco and Romania. The cats were presented to the clinics for routine check-ups or various medical conditions and not for aggression or other behavioural problems. A total of 60 cats were evaluated, 30 in each country. The evaluation of the cats and completion of the questionnaire was carried out by a veterinarian or by a practising student.

For the statistical interpretation, Chi square test was used. The Yate's correction was performed when at least one of the expected values was smaller than 5, to prevent the statistical significance's overestimation. Where the data did not meet the requirements of Chi square test, we proceeded with the Fisher exact test.

Results and discussions

The questionnaires were filled on a "first come first served basis", meaning the objective was to survey 30 cats as soon as possible, disregarding sex, age, or else. By some coincidence, in both countries, the first 30 cats happened to be 15 (50% of total cases) female and 15 (50% of total cases) male cats.

The cats were grouped in the following age categories: young cats (< 2 years) - 10 (33%) cats in Romania and 18 (60%), young and mature adults - 18 (60%) of Romanian cats and 12 (40%) of Moroccan cats, senior cats - 2 (7%) cats in Romania and none in Morocco aged 13 years or more. There was a notable difference ($p = 0.01$) between the ages of Romanian and Moroccan cats, the latter being much younger. This most probably is due to two main factors. The first reason might be the fact that the sterilization rates in Morocco tend to be very low, meaning that there is a constant supply and renewal of the stray cat population. Many people end up adopting a kitten simply because they found it on its own in the street and could not leave it out there. In the second place, when it comes to Moroccan cats who live in houses, they always have access to outdoors, which leads to car accidents, getting killed by dogs or even being picked up by people who think they are stray. It is very rare for owners to put collars or microchips on their cats, so it is quite hard at times to differentiate between an owned cat and a friendly stray one when they are roaming around on the street.

While in Romania 18 (60%) of cats happened to be spayed (3 of the intact cats were also brought into the practice to undergo a spaying procedure) in Morocco only 5 (17%) of the surveyed cats happened to be spayed. This leaves 12 (40%) intact cats in Romania and 25 (83%) intact cats in Morocco. After comparison of data, we obtained a significant statistical difference ($p=0.0014$).

In both countries, owners tend to spay their cats much less than dogs, but spaying rates for both animals are lower in Morocco, often being described as a cruel practice, with people being less educated about the importance and advantages of spaying.

We could differentiate and group the reasons for which our cats were presented and taken into the veterinary practices and clinics from which the questionnaires were filled into 3 groups – routine examination, acute illness and grooming. Routine examination refers to procedures such as deworming, vaccination, treatment for chronic illness, etc. Acute illness means the patient presented with symptoms dating back no further than a few days, illness that had not been diagnosed prior to that visit, fractures, etc. Grooming includes cats that were brought in for beautifying procedures such as bathing and haircuts. The results were as follows: routine examination - 14 cats in Romania and 9 (30%) cats in Morocco, acute illness - 12 (40%) cats in Romania and 17 (57%) in Morocco, grooming - 4 (13%) cats in each country. No significant statistical differences were found after comparing the data we obtained about the reason for which the surveyed cats were brought into the veterinary practice ($p=0.4$).

In Morocco visits for routine examinations excluding vaccination, happens rarely. Owners would much rather come by quickly and buy products that they would need for deworming and always prefer treatment options that they can themselves at home. In Romania, it is more common for owners to keep up with routine procedures and bring their pets into the practice and even often request to bring them in to receive treatment instead of wanting to do them themselves at home.

These differences can also be explained by the fact that Casablanca and Timisoara which are the two cities the veterinarians were located in are of very different sizes. Commuting in Casablanca can take hours as it is a very big city with far more traffic than the one that happens in Timisoara. This could explain the owner's preference of not having to take their pet to the veterinarian unless it is unavoidable.

Out of all the cats that were brought in, 16 (53%) cats in Romania were clients that had already been to that veterinary practice before and the remaining 14 (47%) were new to the practice and had never visited it. In Morocco, we observed the contrary, 14 (47%) of the cats were clients while 16 (53%) of cats had visited the veterinary clinic for the first time. This only reflects if they had ever been to that particular practice, not if they had ever been to a veterinarian or not.

During the consultation both in Romania and Morocco, owners were allowed to be there but for

different reasons, such as time constraints or personal preference. Some decided to wait for their pet outside of the treatment room not being by their side while the veterinarian or veterinary staff was tending to their needs. In Morocco 23 (77%) of cats were accompanied by their owner while being tended to while 7 (23%) of cats were left in the capable hands of the veterinarian while the owners were tending to other things. In Romania, 26 (87%) of the cats had their owner with them for the duration of their veterinary visit, while only 4 (13%) out of the total cats were attended without the owners being present.

Cooperation was another one of the criteria that we evaluated, finding that in Romania 20 (67%) of cats were cooperative and easy to work with and 10 (33%) of the surveyed cats were noncooperative and hard to treat. For Moroccan cats 18 (60%) of cats were cooperative and 12 (40%) cats were hard to work with, hence deemed uncooperative. After data comparison, no significant statistical difference was found (p=0.78) (Fig. 1).

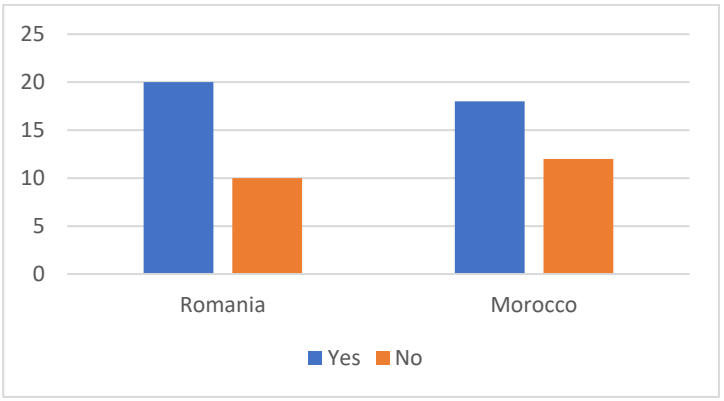

Fig. 1. Degree of cooperation with the veterinarian, without the need for contention, in both countries

The cooperative cats did not demand any means of contention, but some of the noncooperative cats could not have received the appropriate care without being contained. We have observed that in both countries veterinarians try to avoid as much as possible the use of contention tools, even in the case of cats that have displayed aggressive behaviour by vocalization or even scratching and biting them, meaning that the level of compassion and tolerance shown for these animals is closely tied to their profession and far less to their culture.

In Romania 6 (60%) of the noncooperative cats did receive care without the need for contention, while the remainder 4 (40%) noncooperative cats did require being contained to be taken care of: 3 (30%) cats needed to be wrapped in towels and 1 (10%) cat needed to be put in a contention cage and be given anaesthetics to be attended (Fig. 2). For Moroccan cats no contention ended up being used for 8 (67%) of the noncooperative cats and only 4 (33%) noncooperative cats had to be contained: 1 (8%) cat was wrapped in a towel and 3 (25%) of cats had to be put in a contention cage before the administration of anaesthetics (Fig. 3).

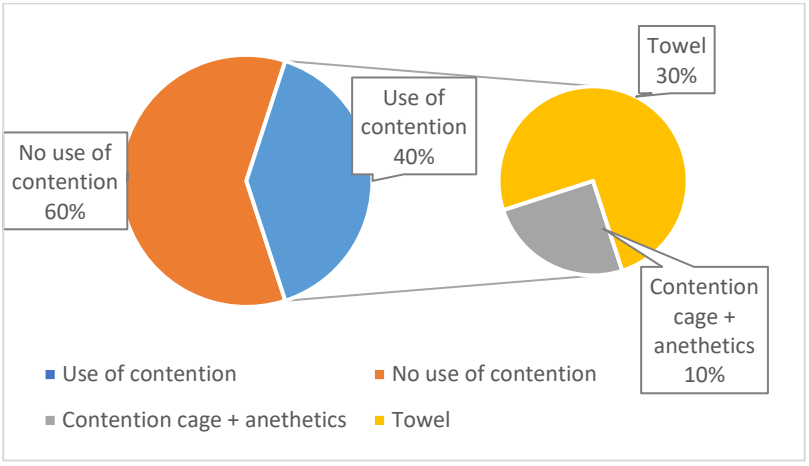

Fig. 2. How often was contention used with non-cooperative Romanian cats and which type of contention was used

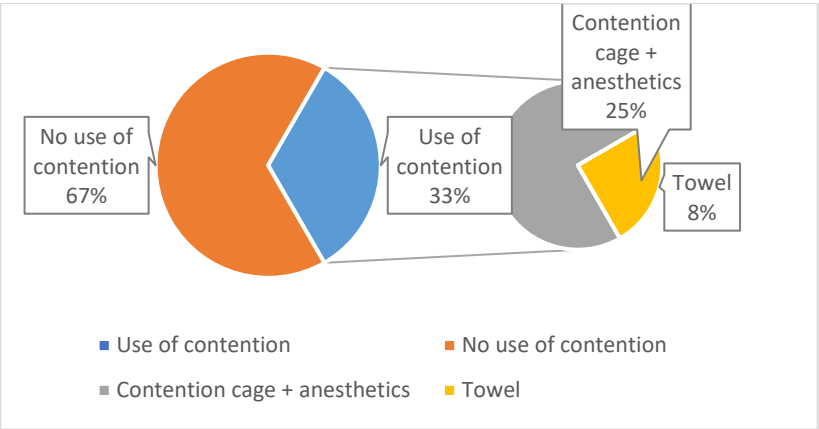

Fig. 3. How often was contention used with non-cooperative Moroccan cats and which type of contention was used

As previously explained in the methods all of our results were split accordingly to what state the conveyed into 3 categories: calm, scared and aggressive (Table 1).

The body postures parameters that were taken into account are the following:

Body posture, view of the all the body as a whole. In Romania 9 (30%) cats were described as calm, 8 (27%) cats as scared and the majority 13 (43%) cats as scared. Moroccan cats were found to be 6 (20%) calm, 9 (30%) scared and half 15 (50%) of the cats as aggressive. No statistical difference was noted ($p = 0.066$).

Ear position: 14 (47%) cats were reported as calm, 10 (33%) cats as scared, and 6 (20%) cats as aggressive in Romania. In Morocco 8 (27%) cats described as calm, 12 (40%) cats as aggressive, and the remaining 10 (33%) as aggressive. No statistical difference was noted between the 2 countries ($p = 0.24$).

Whisker's position: Romanian cats were reported as: 13 (43%) calm, 7 (23%) scared, and 10 (33%) as aggressive. While the Moroccan ones as: 9 (30%) calm, 7 (27%) scared, and 14 (47%) as aggressive. Once again, no statistical difference was found ($p= 0.49$).

Tail position: in Romania 15 (50%) cats were reported as calm, 10 (33%) cats as scared and the remaining 5 (17%) as aggressive. In Morocco 10 (33%) cats were reported as calm, 14 (47%) as scared and the last 6 (20%) as aggressive cats. After comparison no statistical difference was noted ($p= 0.41$).

Pupil size: Romanian cats were noted as: 2 (7%) cats as calm, 14 (47%) cats as scared and 14 (47%) as aggressive. In Morocco 2 (7%) cats were reported as calm, 19 (63%) cats as scared and 9 (30%) cats as aggressive. No statistical difference was noted ($p= 0.65$).

Eye aperture: in Romania 19 (63%) cats were reported as calm, 4 (13%) cats as scared and 7 (23%) as aggressive. In Morocco 21 (70%) cats were reported to be calm, 4 (13%) cats as scared and 5 (17%) cats as aggressive. Once again after comparing these values we didn't obtain any statistical difference ($p=0.8$).

Muzzle tension: Romanian cats were reported as 7 (23%) cats calm, 15 (50%) cats as scared and the remaining 8 (27%) cats as aggressive. The Moroccan cats were reported to be 8 (27%) cats calm, 17 (57%) cats scared and 5 (17%) cats as aggressive. No statistical difference was found ($p=0.64$).

Head position: in Romania 18 (60%) cats were noted as calm, 2 (7%) cats as scared and 10 (33%) cats as aggressive. In Morocco 21 (70%) cats were reported as calm, 4 (13%) cats as scared and 5 (17%) cats as aggressive. After comparing the values, no statistical difference was noted ($p=0.27$).

Overall state, describes how the person who filled the questionnaire perceived the cat. The results for this parameter are as follows: Romanian cats were perceived as: 9 (30%) cats calm, 18 (60%) cats scared and 3 (10%) cats aggressive. In Morocco cats were perceived to have been: 13 (43%) cats as calm, 12 (40%) cats as scared and 5 (17%) cats as aggressive. No statistical difference was found ($p=0.29$) (Table 1).

The results regarding the vocalization and defence mechanisms displayed by cats in each country, were as follows:

Bite: out of the Romanian cats, 3 (10%) bit the veterinary staff or veterinarian during the consultation, while 27 (90%) did not proceed to bite. In Morocco 6 (20%) cats did bite and the remaining 24 (80%) did not. No statistical difference was noted ($p=0.47$).

Scratch: in Romania 10 (33%) cats did scratch their handler while 20 (67%) did not scratch anyone. For Moroccan cats, 9 (30%) scratched the person handling them and 21 (70%) did not. No statistical difference was noted ($p=1$).

Hiss: 5 (17%) Romanian cats hissed and 25 (83%) did not vocalize in such a manner. In Morocco 8 (27%) cats did hiss while 22 (73%) did not. No statistical difference was noted ($p=0.53$).

Table 1

Body language parameters used to evaluate aggressivity display and results by country

BLP	Calm				Scared				Aggressive				Statistics	
	R		M		R		M		R		M			
	No	%	No	%	No	%	No	%	No	%	No	%	X	p
Body posture	9	30	6	20	8	27	9	30	13	43	15	50	0.8	0.06
Ear position	14	47	8	27	10	33	12	40	6	20	10	33	2.81	0.24
Whisker position	13	43	9	30	7	23	7	23	10	33	14	47	1.39	0.49
Tail position	15	50	10	33	10	3	14	47	5	17	6	20	1.75	0.41
Pupil size	2	7	2	7	14	47	19	63	14	47	9	30	0.2	0.65
Eye aperture	19	63	21	70	4	13	4	13	7	23	5	17	0.43	0.8
Muzzle tension	7	23	8	27	15	50	17	57	8	27	5	17	0.88	0.64
Head position	18	60	21	70	2	7	4	13	10	33	5	17	2.56	0.27
Overall state	9	30	13	43	18	60	12	40	3	10	5	17	2.42	0.29

Growl: 5 (17%) of the Romanian cats growled during their consultation, and the remaining 25 (83%) cats did not. For Moroccan cats, we observed the exact same results as those in Romania with 5 (17%) cats having growled and 25 (83%) not. No statistical differences were noted (p=1).

Spit: no Romanian cat spat during their visit 30 (100%) cats did not spit. In Morocco 7 (23%) cats did spit and 23 (77%) did not spit. A significant statistical difference was noted in this case (p=0.01).

Howl: 1 (3%) Romanian cat howled when he was at the veterinary practice and 29 (97%) cats did not howl. 11 (37%) Moroccan cats did howl while the remaining 19 (63%) cats did not howl during their consultation. A significant statistical difference was noted after data comparison (p=0.01).

Yowl: in Romania 5 (17%) cats yowled when they were at the veterinary practice while 25 (83%) cats did not proceed to yowl. When it came to Moroccan cats 16 (53%) of cats did yowl during their consultation and the remaining 14 (47%) cats did not. A significant statistical difference was noted (p=0.006) (Table 2).

Table 2

Vocalization and defence mechanisms displayed by cats in each country

VOCALIZATION	YES				NO				RESULTS
	Romania		Morocco		Romania		Morocco		Statistics
	No	%	No	%	No	%	No	%	p
BITE	3	10	6	20	27	90	24	80	0.47
SCRATCH	10	33	9	30	20	67	21	70	1
HISS	5	17	8	27	25	83	22	73	0.53
GROWL	5	17	5	17	25	83	25	83	1
SPIT	0	0	7	23	30	100	23	77	0.01
HOWL	1	3	11	37	29	97	19	63	0.01
YOWL	5	17	16	53	25	83	14	47	0.006

Conclusions

After the assessment of the behaviour and aggressivity, we can conclude that based on the differences observed in some of the vocalization parameters, Moroccan cats express higher levels of fear aggressivity, in comparison with their Romanian counterpart. This difference could be the result of Moroccan cats being usually taken to the veterinary practice only when needed (emergency). Making them unfamiliar with such environments and procedures, which understandably can be a great source of stress and fear, leading them to protect themselves from something they perceive as harmful.

Despite there not being any significant statistical difference in the body language parameters we can observe in most of them, a tendency of higher cases of Moroccan cats expressing aggressivity.

When it comes to cooperation and use of contention our results were almost identical for both of the countries, demonstrating the strong influence of veterinary knowledge more than other possible personal or cultural influences.

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MORPHOLOGICAL ASPECTS OF THE APPENDICULAR SKELETON IN THE COMMON SHELDUCK (*TADORNA TADORNA*)

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Abstract

From a taxonomic point of view, *Anseriforme*s are a highly differentiated avian order which includes over 150 species. At the same time, it is one of the most studied flying groups due to its historical importance in domestication and the poultry industry. The species that is the object of the study, the common shelduck (*Tadorna tadorna*), is classified in the subfamily *Tadorninae*, which consists of birds with intermediate characteristics between geese and ducks. In the specialised literature, there is interest in identifying valuable morphological aspects of this species from a taxonomic point of view. We conducted a thorough morphological analysis of the skeleton of the *Tadorna tadorna* species. In addition, we compared specific results with those of some authors who analysed the mallard (*Anas platyrhynchos*) from a morphological point of view. Some significant differences have been described for the first time, such as the appearance of the articular humeral head, the deltoid crest and the brachial fossa. At a femoral level, we identified a developed impression, the iliotrochanteric muscles insertion surface, and we described the topography of the ischiofemoral and caudofemoral muscles insertion rugosity.

Keywords: *morphology, shelduck, appendicular skeleton.*

The shelducks belong to a genus of wading birds (*Tadorna*) in the order *Anseriformes*, family *Anatidae*. Two species are found in Romania: the common shelduck (*Tadorna tadorna*) and the ruddy shelduck (*Tadorna ferruginea*). These birds realise the transition from ducks to geese but are still morphologically closer to geese (16, 17). The body length of the common shelduck is 58-67 cm, the wingspan is 100-120 cm, and the weight is 830-1500 g in the male and 562-1250 g in the female.

The common shelduck has an extensive range and does not meet the “Vulnerable” species criteria according to the Berne Convention, which has been effective since 1982 (6). In Romania, the common shelduck nests in the Romanian Plain, Dobrogea and isolated in Moldova and the Western Plain. It has been protected since 1955 by HCM 1625.

The species of the *Anatidae* family, to which the common shelduck belongs, are some of the most studied species. The morphological aspects were studied due to this group’s historical importance for hunting, domestication and poultry farming (7). An important issue, still debated today, is related to the taxonomic classification of the species in this family (8, 11). Numerous authors have relied on a series of morphological elements to achieve this objective; from this category, the skeleton-related features are of considerable importance (3, 4, 13, 14). In his work, Livezey (7) analysed 120 characteristics appreciated by the authors as criteria for taxonomic classification; 112 represented bone cavities and eminences (7). In the fossil category, bones are the most numerous, and they can provide helpful information on which phylogenetic investigations can be conducted, including those of extinct species (2, 5, 12, 15). Lastly, osteological studies are essential in understanding avian locomotor dynamics (1, 9, 10, 11). Since we have not found a similar study in the specialised literature, our investigations bring data into the bone morphology field in anatids. Having the opportunity to analyse the skeleton of Tadorna tadorna morphologically, we made a detailed study of the limb bones. In addition, we compared specific results with those of some studies done in the same anatomical area in the mallard.

Materials and methods

Bones from four specimens: two female common shelducks (*Tadorna tadorna*) and two female mallards (*Anas plathyrinchos*) represented the study material. The shelduck’s bones belong to Grigore Antipa Museum of Natural Sciences, with inventory numbers 15485 and 15486. The mallard bones were prepared using the maceration method from two specimens harvested in the 2023-2024 season in the Glodeanu region, county Buzau. The measurements were made with the calliper, and the images were taken with a Nikon Coolpix L820 camera. The values described are the averages for each category of birds. The terminology used follows Nomina Anatomica Avium -1993.

Results and discussions

The humerus shows the characteristics of the slender bone typical of palmipedes, with a solid articular head, a wide pneumatic foramen and a cylindrical diaphysis, slightly curved dorsally at the distal extremity. In both species,

it represents the longest skeleton bone. In the shelduck specimens, the average length is 110.4 mm, and the average thickness in the middle of the diaphysis is 80 mm. The ratio between the two dimensions was 1.38. In the mallard, the mean values of the humerus were 94.7 mm in length and 74 mm in width, with a ratio of 1.27, so the common shelduck humerus is relatively thinner than the mallard.

At the proximal extremity, the articular head (*Caput humeri*) is widened, similar from a conformational point of view in the two species, but relative to the bone dimensions, it is smaller in the shelduck compared to the mallard, as evidenced by the value of the humerus length / articular head width ratio, which is 7.03 in common shelduck and 5.22 in mallard. In dorsal view, the deltopectoral crest (*Crista deltopectoralis)* was more prominent in mallard, forming an angle of about 130°, while in common shelduck, it was curved, with the appearance of a semicircular arch (Fig. 1).

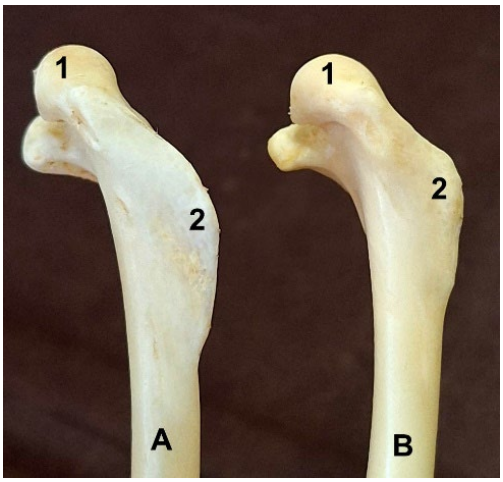

Fig. 1. Proximal extremity of right humerus in common shelduck (A) and mallard (B) in dorsal view (original)
1-humeral head; 2-deltopectoral crest

The pneumatic foramen (*Foramen pneumaticum*) appears wide in both species, limited ventrally by a bicipital crest (*Crista bicipitalis*) better represented in common shelduck.

In both cases, the vascular foramen was located on the caudal margin of the diaphysis approximately midway through the diaphysis.

In the dorsal view, it was observed that the distal epiphysis of the common shelduck was displaced dorsally and much more accentuated than that of the mallard (Fig. 2). The brachial muscle fossa (*Fossa m. brachialis*), in common shelduck, is wide and extends proximally on the diaphysis, measuring approximately 13-14 mm, starting from the level of the proximal edge of the ventral condyle. In mallard, it is represented by two short fossets, exceeding only 7.5-8 mm at the edge of the ventral condyle (Fig. 3).

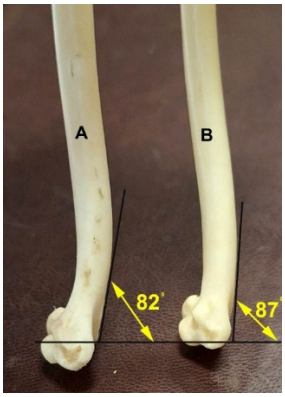

Fig. 2. Distal extremity of right humerus in common shelduck (A) and mallard (B) in ventral view (original)

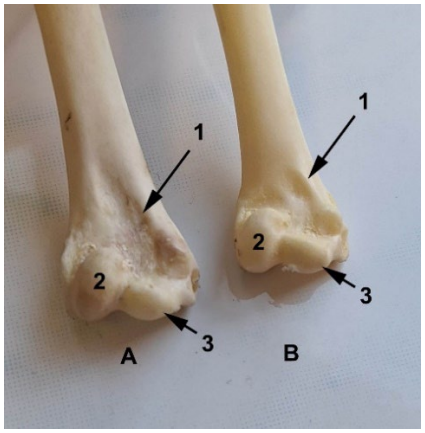

Fig. 3. Distal extremity of right humerus in common shelduck (A) and mallard (B) in cranial view (original)
1-fossa of the brachial muscle; 2-dorsal condyle; 3-ventral condyle.

In the case of the forearm bones**, the ulna** in common shelduck was less curved than in the mallard, forming a narrower radioulnar space. The length of the bone in the first case was 105.3 mm, and the maximum width of the interosseous space was 8.3 mm. In the case of the mallard, the length was 83 mm and the width 9 mm. As a result, the ratio of the two sizes is 12.6 in the common shelduck and only 9.2 in the mallard. **The radius** was approximately rectilinear in both species.

In the common shelduck, the alular digit consists of a single **phalanx** (*Phalanx digiti alulae*), and the major digit has two **phalanges** (*Phalanx proximalis digiti majoris*, *Phalanx distalis digiti majoris*), with the distal one being morphometrically similar to that of the alular digit (Fig. 4).

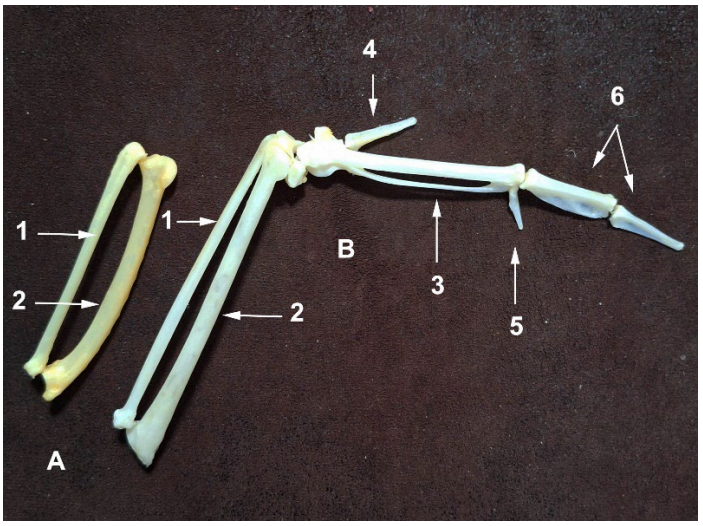

Fig. 4. Forearm bones in mallard (A) and bones of the forearm and thoracic autopodium in common shelduck (B), right limb dorsal view (original)
1-radius; 2-ulna; 3-metacarpal bones; 4-phalanx of the minor digit; 5-phalanx of the alular digit; 6-phalanges of the major digit.

The femur in the two species had very similar anatomical characteristics. The length of the bone was 53 mm in the shelduck and 52.2 mm in the mallard, and the thickness in the craniocaudal direction, in the middle of the bone, was 6 mm in the shelduck and 5.8 mm in the mallard. As a result, the value of the two ratios is very similar (8.83 and 9) (Fig. 5).

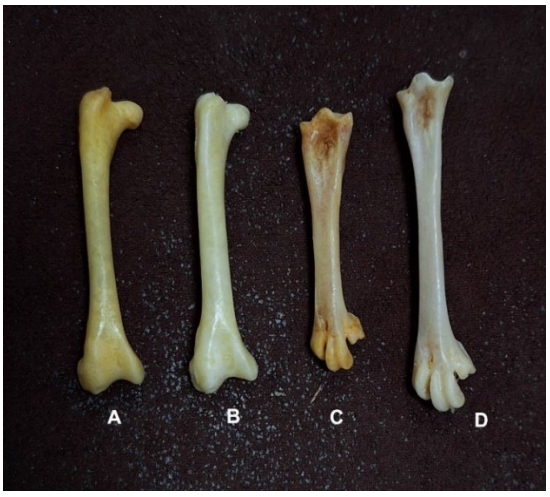

Fig. 5. The femur of mallard (A), common shelduck (B) and tarsometatarsus of mallard (C) and common shelduck (D), right limb, cranial view (original)
In mallards, the tarsometatarsus is shorter than the femur, while the situation is reversed in common shelducks.

A close examination of the femoral lateral surface in the proximal extremity revealed an important difference: the intertrochanteric impression (*Impressiones mm. trochanteris*) for the insertion of the cranial and caudal iliotrochanteric muscles is oblique in the shelduck and parallel to the axis of the bone in mallard. The ischiofemoral and caudofemoral muscles have different insertion locations, which correspond to the prominence of the caudal intermuscular line (Linea intermuscularis caudalis), which is slightly different in the two species. While in mallard, this impression is located precisely in the middle of the caudal face of the bone, in the shelduck, it appears in the proximal part of the distal half.

The intermuscular line (*Linea intermuscularis caudalis*), corresponding to the insertions of the schiofemoral and caudofemoral muscles, has different localisations in the two species. While in mallard, this impression is located precisely in the middle of the caudal surface of the bone, in the shelduck, it appears in the proximal part of the distal half.

At the distal extremity of the femur, the height difference between the two femoral condyles is more significant in shelduck. Positioned with the axis perfectly vertical, the tangent line of the ventral edge of the two condyles forms an angle of 17° with the horizontal line in shelduck and only 10° in mallard.

The tibiotarsus was perfectly straight in both species. The average length was 103.8 mm in the common shelduck and 89 mm in the mallard. In shelduck, it was shorter than the radius, representing the third longest bone in the skeleton. In mallard, the tibiotarsus is 5 mm longer than the ulna, thus being the second longest bone in the skeleton. The cranial cnemial crest (*Crista cnemialis cranialis*) is more developed in mallard, and the tibial notch

(*Incisura tibialis)* is deeper (Fig. 6).

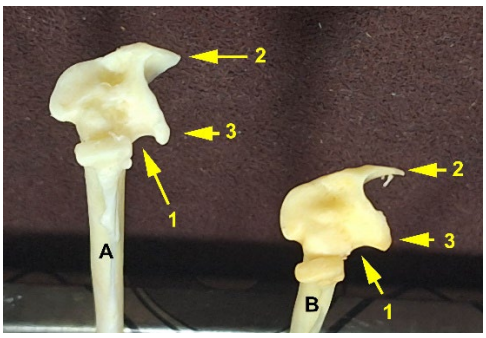

Fig. 6. The proximal extremity of the right tibiotarsus in the common shelduck (A) and mallard (B), dorsolateral view (original)
1- tibial notch; 2-cranial cnemial crest; 3- lateral cnemial crest

The proximal extremity of the fibula in the common shelduck is placed at a higher level than the medial condyle of the tibiotarsus, while in mallard, it does not exceed this condyle. The imprint of the medial collateral ligament is much more pronounced in mallard. An essential differentiating element was observed at the distal end of the bone. The transverse axis of this extremity is almost perpendicular to the axis of the diaphysis in the shelduck. At the same time, the mallard is oblique to the transverse axis at an angle of about 4-5 degrees (Fig. 7).

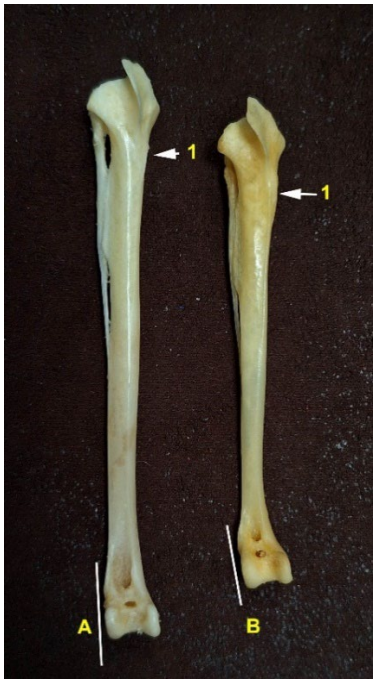

Fig. 7. Right tibiotarsus in common shelduck (A) and mallard (B), cranial view (original)
1-the insertion surface of the medial collateral ligament; The white line indicates the axis of the distal extremity

One of the most important differences between **the tarsometatarsal** of the two species was that the bone is longer and thinner in common shelduck than in mallard. Considering the length of the femur, which is approximately equal in the studied specimens, it was found that the tarsometatarsus of the shelduck is longer than the femur, 62 mm versus 53 mm. In mallard, the length of the tarsometatarsus does not exceed the length of the femur, 47.2 mm versus 52.2 mm.

No particularities of the pedal digits skeleton were observed.

Conclusions

The common shelduck's appendicular skeleton is similar to the mallard's in terms of the number of bones and morphological characteristics. However, indisputable anatomical differences exist between the bones of the two species.

The most significant differences are found at the level of long bones. In the forelimb, absolute differences are found within the deltopectoral crest, the bicipital fossa of the humerus and the angle between the distal diaphysis and the humeral epiphysis in the two species. In the pelvic limb, in the case of the femur, the topography of the ilio-trochanteric crest, the position of the two condyles correlated with the vertical axis, and in the case of the tibia, the value of the angle between the distal extremity and the axis of the bone are significantly different in between the two species.

In conclusion, the differential elements identified are described for the first time, supporting the establishment of this species' morphological profile and, if necessary, differentiating the bones from those of the mallard.

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COMPARISON BETWEEN THE USE OF LARYNGEAL AND ENDOTRACHEAL TUBE IN QUEENS FOR ELECTIVE OVARIOHYSTERECTOMY

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Abstract

The supraglottic airway devices in comparison with the endotracheal tube have been designed to ensure the comfort of the patient and to make the procedure easier for the doctor, while preserving the safety of the airways. Eighteen cats have been considered for this prospective randomized study and divided equally in two groups. Group A consisted of animals who have benefited of laryngeal mask placement during the maintenance of anaesthesia, and group B includes cats who have been intubated. The aim of this study was to compare the use of laryngeal mask versus endotracheal tube in cats brought in for elective spaying. The methods have been compared in order to assess the ease of the manoeuvres and to quantify the associated risks. Anaesthesia was provided with medetomidine, buprenorphine and ketamine, and during the procedure respiratory rate (RR), end-tidal CO2 (EtCO2), heart rate (HR), non-invasive blood pressure (NBIP) and oxygen saturation (SpO2) have been monitored. None of the groups benefitted of lidocaine local anaesthesia of the larynx prior to securing the airways in order to assess the difficulty of the manoeuvre and the comfort of the patient. In group A, two of the patients encountered complications due to the inability to properly secure and to attain proper seal of the laryngeal mask. In group B, three cats encountered bronchospasm and the intubation time was prolonged. We noticed EtCO2 variations between the two groups depending on the weight of the patient. This study concludes that the values of EtCO2 measured using side stream capnography vary significantly among the two groups.

Keywords**:** *Anaesthesia, Capnography, Endotracheal intubation, Laryngeal mask, Supraglottic airway device.*

Cat spaying is a common surgery that can be performed by using a wide variety of anesthesia protocols.

The objective of this prospective, randomised study is to evaluate the safety of using a supraglottic airway device for securing the airways in cats in comparison with endotracheal intubation.

In comparison to dogs, cats have a small and frail trachea, and the laryngeal spasm has a common ocurrence (4). For these reasons, endotracheal intubation may sometimes be a riskier procedure especially for common procedures like spaying. Reported risks of intubation in cats are associated with overinflation of the endotracheal tube (ET), trauma to the trachea due to the usage of a stylet or changing position of the patient while the ET baloon is inflated (9, 11, 4).

Because of this risk, we evaluated an alternative way for securing the airways. Airway management using supraglottic mask seems to be a good alternative to endotracheal intubation (15). A laryngeal mask (LM) or supraglottic devide (SD) can be used in patients where an endotracheal tube cannot be used (14). The laryngeal mask is fitted faster than the endotracheal tube (1, 11) and therefore it can be a suitable alternative for the inexperienced practitioners. Another advantage is that it the anaesthesia plane can be more superficial in comparison to performing the endotracheal intubation (13, 18). The ease of the procedure has bee documented on most of the species, emphasizing that for larger species securing the airways by using a SD can be performed by a single doctor, in comparison with the endotracheal intubation that usually requires two people (5, 7). For the reasons stated above, inexperienced practitioners have better success rate in placing a laryngeal mask than when placing an endotracheal tube (2).

On the other hand, the laryngeal mask has the disadvantage that it can easily move during procedures, leading to an improper delivery of respiratory gases or even obstructing the upper airways (7, 12). Selecting the proper size of the laryngeal mask can sometimes be a challenge (16, 19), but we considered the manufacturer's indications.

The proper positioning of the endotracheal tube can be assessed by direct visualization of the probe between the arytenoid cartilages, whereas the correct placement of the larynx must be checked using a capnograph (7).

Materials and methods

Eighteen cats presented for elective ovariohysterectomy have been included in this study. The inclusion criteria consisted of the following: age between 6 and 36 months, bodyweight between 2 and 4.5 kg, ASA Physical Status I. The patients have been randomly divided into two equal groups as following: group A consisted of patients in which a laryngeal mask has been used and group B, cats who have been intubated.

Group A included nine domestic shorthair (DSH) cats with an average age of 8.66±3.87

months (mean±SD) and an average body weight of 3.26±0.6 kg (mean±SD). Gropu B included eight DSH cats and a Scottish Fold cat, with an average age of 13±9.34 months (mean±SD) and an average body weight of 3.08±0.68 kg (mean±SD). All patients were included on the ASA Physical Status I based on the medical history and preanaesthetic clinical examination.

All patients have been anesthetized after a 12h fasting period. The anesthesia protocol comprised of the „Kitty magic" combination as following 40 µg/kg medetomidine, 0.012 mg/kg buprenorfine and 4 mg/kg ketamine administered via intramuscular route (6). None of the groups benefitted of lidocaine local anesthesia of the larynx prior to securing the airways in order to assess the difficulty of the maneuver and the comfort of the patient (20).

The venous catheter was placed 5 minutes after administering all the drugs. Ten to fifteen minutes after the administration of the anesthetics, the securement of the upper airways has been performed as indicated for the two groups. The management of the airways for the cats in group A has been performed blindly after pulling out the tongue (Fig. 1), whereas for the cats in group B a laryngoscope was required to visualize the arytenoid cartilages.

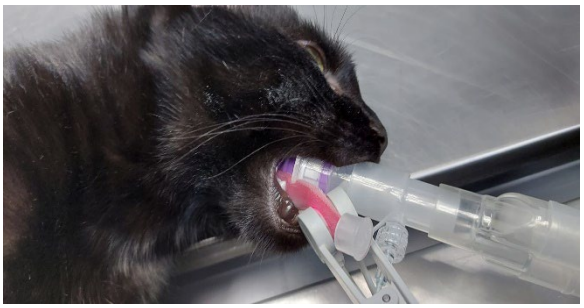

Fig. 1. Upper airway management using a laryngeal mask during anesthesia (group A)

In group A, the LM used were size 1 or size 2, depending on the weight of the patient. Size 1 was used for cats up to 3 kg and size 2 was used for cats over 3 kg. In group B, the ET used had variable sizes between 2.5-3.5 mm, according to the ideal body weight of the patient and the estimation of the size once the larynx was visualized (17). The patients have been connected to a rebreathing circuit, the flowmeter was set to 2 liters/minute, using a ventilation bag with a volume of 0.25L and the adjustable pressure-limiting valve (APL) was set to 10 mm H_2O for 10 seconds to check for the sealing of the endotracheal tube and the laryngeal mask. After that, the APL was opened. No inhalant agent has been administered to any of the patients.

During the anesthesia, the patient monitoring comprised of assessing his electrocardiography, the non-invasive blood pressure, the oxygen saturation and using the vital signs monitor Mindray ePM 12M Vet. The end-tidal CO2 and respiratory rate have been assessed using a side-stream capnograph incorporated in the anaesthesia machine Mindray Veta5.

The surgical procedure was standard, with an average duration of 25 minutes. We performed linea alba laparatomy, making a 1-2 cm incision. We used a spay hook to identify the uterus. The patients have been monitored until the sensors have not been tolerated anymore in the recovery period. The monitorisation of patient's reflexes continued until the patient adopted recumbency.

The extubation and the removing of the laryngeal mask were performed when all cats had positive mandibular reflexes.

The data has been exported from the monitorization devices, and the values for respiratory rate (RR), end-tidal CO2 (EtCO2), heart rate (HR), and mean arterial pressure (MAP) have been assessed every 5 minutes. The statistical analysis of data was performed by calculating the 95% confidence interval (CI) and mean for each parameter, using Statistical Package for the Social Sciences (SPSS) software.

Results and discussions

The oxigen saturation is a direct indicator of the efficiency of ventilation. The pulse oximeter sensor has been placed on the tongue in all patients included in this study. Figure 2 shows the comparison between the two groups and the trend of SpO2 assessed every 5 minutes during the surgery. Over time, group B (ET) shows greater stability, maintaining a higher SpO_2 level than group A (LM) leading to the conclusion that the oxygen delivery (DO2) is more efficient when an endotracheal tube is placed in comparison to supraglottic airway devices.

By comparing the SpO_2 trends in both groups, there is a significant difference, which can be derived from the inability to maintain the supraglottic device in a fix position for longer periods of time and also due to the measurement artefacts due to misspositioning the pulse oximeter. Two cats in group A showed lower readings for SpO_2 that were back to over 90 % after adjusting the position of the sensor. The LM is bigger and does not always leave proper room for placement of the pletismograph sensor on the tongue. Also, in this group the LM did not secure the airways in all patients. These 2 cats are not the same cats that had the LM dislocated.

The heart rate has been monitored using 3-lead EKG and during the surgery we evaluated lead II in all the patients. No haemodinamically

significant cardiac arrythmias have been detected during the interventions. Heart rate monitoring in Figure 3 was done every 5 minutes and shows a trend of decreasing frequency over time. It also shows that the variability decreases over time in both groups, suggesting more stability as time goes by. Group B (ET) shows a generally lower heart rate that group A (LM). The heart rate is higher at T1 in both groups, but in group B is the highest. The variability over time decreases for both groups.

Measurements of mean arterial pressure (MAP) are presented in Figure 4. Both groups show a decreasing trend over time, although both groups start off with a high MAP and significant variability. Group A has wider confidence intervals at all points in time meaning a greater variability in MAP levels.

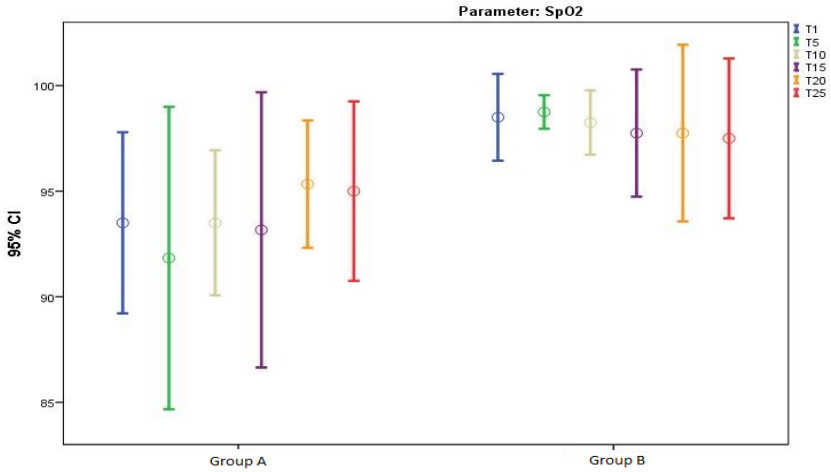

Fig. 2. Mean SpO_2 for the groups during the surgery at 95% CI

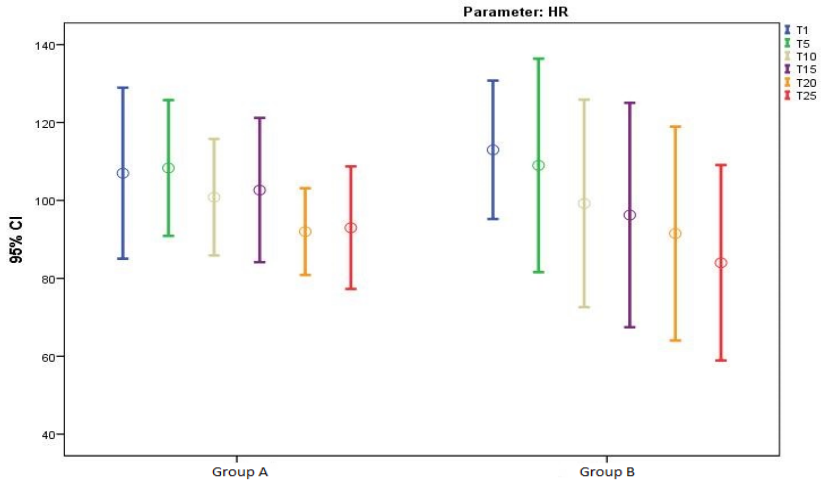

Fig. 3. Mean heart rate for both groups during the surgery at 95% CI

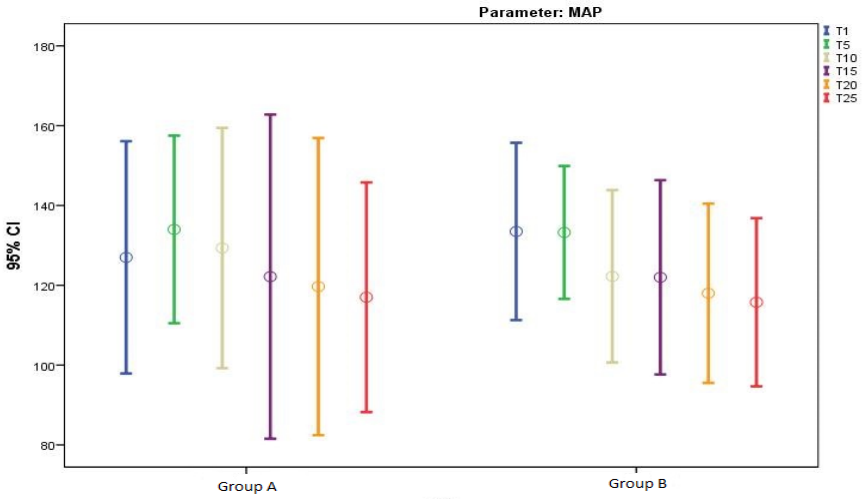

Fig. 4. Mean MAP for the groups during the surgery at 95% CI

Figure 5 shows the confidence intervals and mean $EtCO_2$ measured for each group. Initially, both groups start with a great variability. Over time, group A keeps on displaying great variability, unlike group B in which the measurements are by far more consistent for each data point. This emphasizes that ET is a more reliable way to measure the $EtCO_2$ unlike the LM that showed inconstant measurements.

After intubation, no patient presented signs of apnea. All patients breathed spontaneously during the interventions. Capnography was used to evaluate the patients EtCO2 and respiratory rate. In group A in one case the capnograph failed to register the EtCO2 for the first 7 minutes, but registered the breaths of the cat. After it started to register, it showed lower than normal readings. In another case, the capnograph stopped registering the EtCO2 after 20 minutes of surgery and it started to register again when the position of the laryngeal mask was readjusted.

Figure 6 shows the respiratory rate for both groups during the surgery. All cats start with a low respiratory rate, but by the end of the measurement in both groups confidence interval gets wider suggesting a greater variability in respiratory rate. Also, in both groups there is an increasing trend over time. This is to show that both groups start bradypneic and the respiratory rate increases over time.

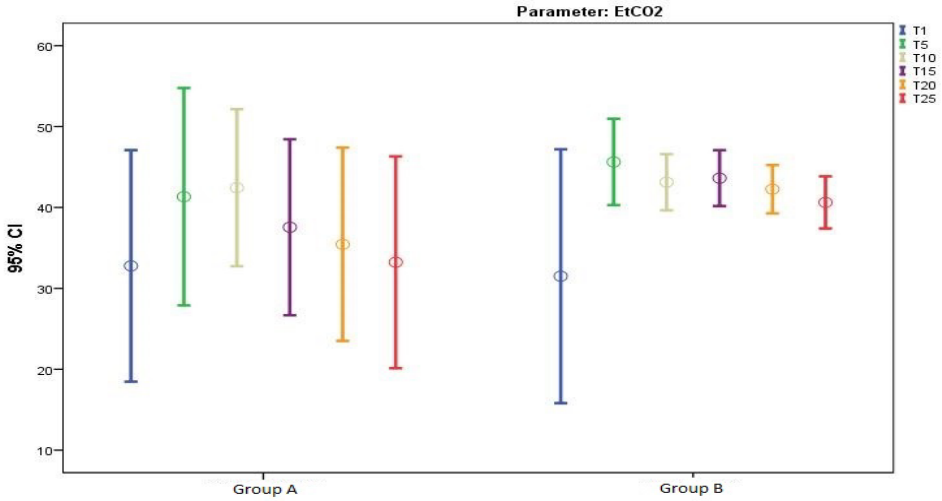

Fig. 5. Mean EtCO2 for the groups during the surgery at 95% CI

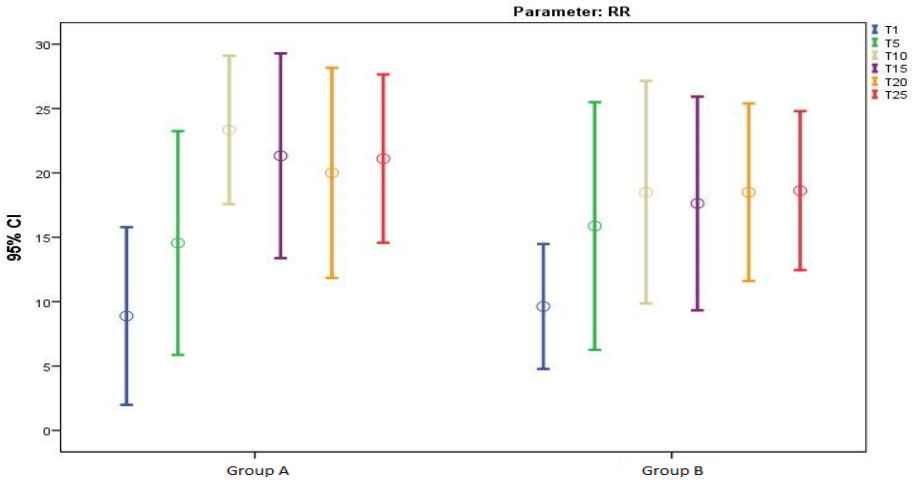

Fig. 6. Mean RR for the groups during the surgery surgery at 95% CI

Conclusions

Medetomidine buprenorfine and ketamine combinations had the same effect for all cats in the time span the measurement were registered.

Both groups start with a high heart rate that subsides over time. Mean arterial pressure in both groups starts high and throughout the period that was monitored, no clear sign of a decreasing trend was noticed.

Oxygen saturation fluctuated greater in group A (LM) that in group B (ET). Group A showed a higher variability in SpO_2 in most of the time points. In both groups respiratory rate was low at first, but in the other time points it started to show a great variety.

$EtCO_2$ shows a great variability in the first measurement. As time goes by, group A keeps displaying a great variability unlike group B in which the measurements are by far more consistent for each data point.

The results of this preliminary study is that unlike endotracheal intubation, which is a safe and verified way of securing the airways, laryngeal mask has both advantages and disadvantages.

The advantages of using the laryngeal masks are that they are easy to place and there is no risk of arytenoid cartilages and/or tracheal trauma. When properly fitted, they offer good sealing of the airways, giving the possibility of for positive pressure ventilation. In our tests, we evaluated pressures of 10 mmH_2O that are ideal for protecting the lung parenchima. Above 15 mmH_2O all cases in group A had leaks.

The disadvantages of using the laryngeal masks are that they can not be properly fitted in small cats, therefore they run the risk of moving and not sealing the airway. Another risk is misplacing the supraglottic by inserting it further into the esophagus. In brachycephalic cats is a greater risk for them not to seal the upper airways properly.

In our study, the laryngeal mask failed to properly secure the airways 44.44% of the time. This high percentage can be attributed to the fact that the device was not secured directly to the head and was locked in place by the rebrething circuit that was placed on stander.

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CASE STUDY: ANESTHESIA MONITORING AND PAIN EVALUATION FOR PYOMETRA SURGERY IN A BITCH USING BUTORPHANOL, MEDETOMIDINE AND LOCAL ANESTHESIA WITH LIDOCAINE

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Abstract

Ovariohysterectomy is an elective procedure in bitches. It can also be performed in uterine pathologies. For closed cervix pyometra surgery is required as quickly as possible, but most of the time the female already has some other pathologies making the anesthesia protocol a challenge. An eleven years old bitch was diagnosed with closed cervix pyometra. Clinical exam, blood analysis and sonography were corroborated to reach the diagnosis. For anesthesia, butorfanol was used in premedication combined with medetomidine in order to acquire heavy sedation. Local infiltration of linea alba, ovarian pedicles and cervix with lidocaine was performed in order to supplement analgesia. Anesthesia monitoring of the 11 years old bitch showed that this protocol maintained proper sedation and anlagesia. The heart rate registered between 42 and 71 beats per minute. Oxygen saturation registered between 93% and 97%. The dog was oxygenated by endotracheal tube. Mean arterial pressure registered 73 to 114 and the temperature was maintained above 36.3℃. Because of thrombocytopenia an electric scalpel and bipolar forceps were used to ensure a better hemostasis.

***Keywords**: Anesthesia, Local, Pyometra, Radioelectrosurgery, Ultrasound.*

Pyometra is a uterine pathology characterized by the presence of purulent content in the uterus. It can be with an open or closed cervix, the one with a closed cervix having the potential to put the animal's life in danger. It is a frequently encountered pathology, especially in elderly females (15).

Although there are studies that show that drug treatment is effective in treating pyometra (1), surgical treatment is the safest to treat this pathology, even in dogs that are suffering from other comorbidities. For this reason, the anesthesia must be adapted according to the patient's physiological state.

Anesthesia was achieved using medetomidine for muscle relaxation and butorphanol for analgesia (6). Meloxicam was administered preoperative (10). Lidocaine has been used locally to supplement analgesia (2). It was administered by injection on the incision line and then infiltrated inside the ovarian pedicles and intramural cervix (3).

Ketamine was avoided because the biochemical examination suggested that liver and kidney function were impaired and could have prolonged the excretion time of ketamine and increased the risk of possible complications. We also wanted to evaluate the depth of anesthesia using only medetomidine and butorphanol.

During the pyometra surgery, the dog was monitored by following heart rate (HR), pulse rate (PR), end tidal CO_2 ($EtCO_2$), respiratory rate (RR), blood pressure and temperature. The purpose of anesthesia is to provide muscle relaxation, analgesia and amnesia.

Materials and methods

An 11-year-old, 40 kg female was presented because she had hematuria, poor appetite, and apathy. Following the clinical examination, the only remark was abdominal tenderness and a temperature of 38.9℃. On the first day, blood samples were collected for hematology and biochemistry. The results are exhibited in Figures 1 and 2.

The hematological examination (Fig. 1) revealed neutrophilia, monocytopenia and thrombocytopenia. Following the biochemical examination of the blood (Fig. 2), a decrease in albumin and Alanine Aminostrasferase and an increase in creatinine and globulin were observed. These changes were suggestive of the presence of an acute infectious process, but also of liver and kidney failure.

The next day, following the evaluation of the results of the blood tests, the ultrasound examination was decided, where the uterus with anechoic content (Fig. 3) was identified and the uterine vessels showed an obvious Doppler signal (Fig. 4). Ovarian cysts, on both left and right ovaries have been noted during the ultrasound exam.

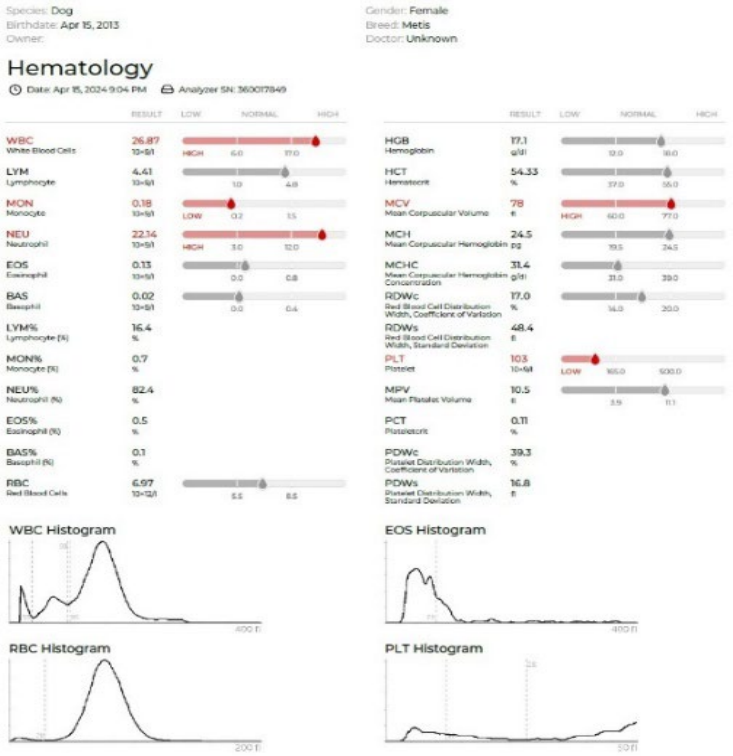

Fig. 1. Hematologic examination

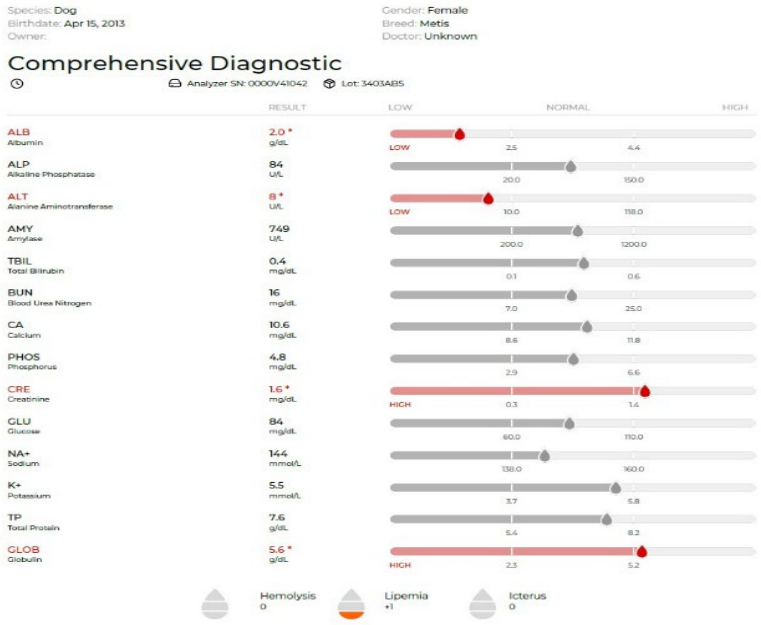

Fig. 2. Biochemical examination

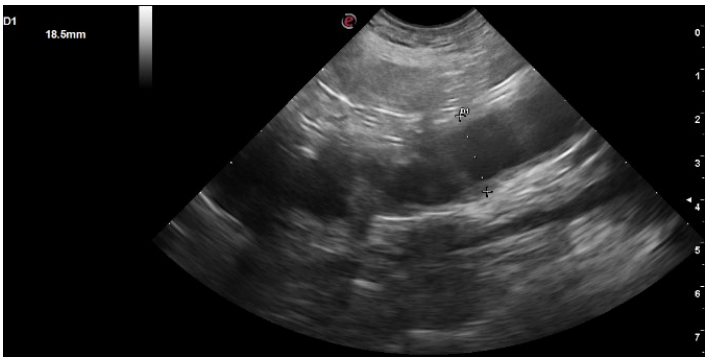

Fig. 3. Uterine horn distended by anecogenic content measuring 18.5 mm

Following ultrasound and blood tests, the diagnosis of closed cervix pyometra was established and surgical approach was decided.

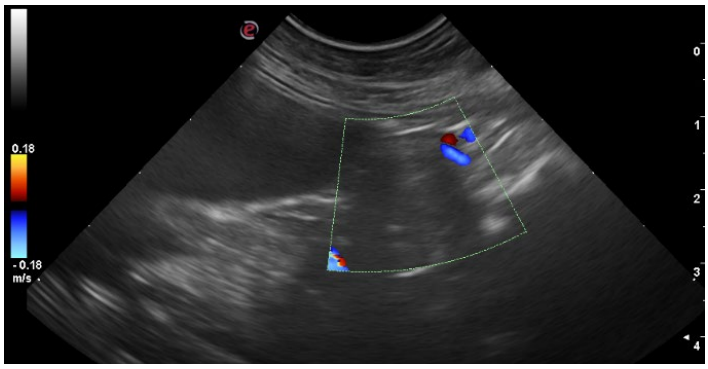

Fig. 4. Uterine body with Doppler signal

Premedication was initiated by administering 8 mg of meloxicam subcutaneous. Anesthesia was achieved by administering 40 mg of Butorphanol intramuscular followed 10 minutes later by the intravenous administration of 0.4 mg of medetomidine hydrochloride. Muscle relaxation set in within 4 minutes. The palpebral reflex was present and the mandibular tone very weak. It was decided to administer 10 mg of propofol i.v. to further enhance muscle relaxation and allow intubation. At the time of intubation, the dog coughed. It was positioned in a dorsal recumbent position, the sidestream capnograph and the rebreathing circuit from the anesthesia machine were connected to the patient.

The sensors from the anesthesia machine that were then connected are: EGK, pulse oximeter, thermometer and sphygmomanometer.

After preparing the surgical field for surgery with alcohol and betadine, the laparotomy incision line was infiltrated with a total volume of 1 ml of Lidocaine 20 mg/ml.

The surgical drapes were put in place and a 10 cm incision was made. Signs of discomfort during the incision were noted on the monitor, but no muscle contractions were noted, suggesting that the animal was in the IIIrd plane of anesthesia.

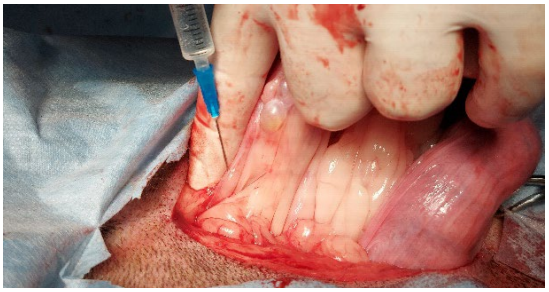

Fig. 5. Ovarian ligament injection of lidocaine

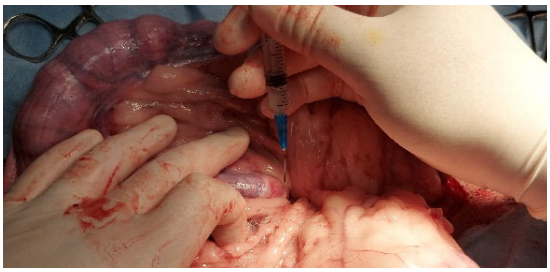

Fig. 6. Lidocaine injection of the dorsal aspect of the cervix

The uterine horns and ovaries with ovarian ligaments were brought out. An increase in heart rate was observed when the ovarian ligaments were pulled. 0.5 ml of lidocaine was administered in each ovarian pedicle (Fig. 5.) and then a total volume of 0.8 ml of lidocaine was administered on the dorsal side (Fig. 6.) and on the ventral side of the cervix.

We than waited for 2 minutes before proceeding to the ligation and vascular sealing of the ovarian pedicles (Fig. 7). It was decided to use the 2 methods of hemostasis because the blood vessels were surrounded by a thick layer of adipose tissue and were not isolated. The ligation was performed en bloc. The 2 methods have similar efficiency, but the use of bipolar forceps reduces surgical time (13).

A transfixing ligature was used for cervix using PGA USP 2 suture wire.

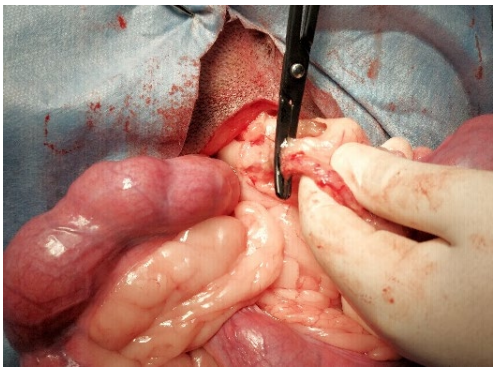

Fig. 7. Ovarian blood vessels sealing

Abdominal wall suture was performed in two steps: peritoneum-muscular layer using PGA USP 2 thread in simple continuous suture and skin with subcutaneous tissue in simple points with Nylon USP 1 practicing for each point 8-9 knots.

Results and discussions

The duration of the intervention was 78 minutes, from the time of administration of lidocaine until the installation of the last suture.

Considering that anesthesia is a deliberate intoxication, it must be adapted to each individual patient according to the physiological state.

This case study aimed to evaluate the possibility of performing a major intervention using a protocol that in a clinically healthy animal provides only sedation, without complete muscle relaxation.

The heart rate measured preoperative was 96 beats/min and the respiratory rate was 23 breaths/min. The temperature measured on the first day was 38.9°C, decreasing on the 2nd day to 37.8 ℃ preoperative.

Figure 8 shows the values extracted from the monitor every minute for the following parameters: SpO_2 in gray, $EtCO_2$ in blue, respiratory rate (RR) in orange. SpO_2 remained between 93 and 97% throughout the monitoring period. $EtCO_2$ had been recorded with values between 24 and 39 mm Hg. These low values were attributed to the flowmeter set at 3l/min. The respiratory rate was influenced by both the skin incision and the tugs on the ovary and uterine body when they were removed. During the skin incision, the heart rate increased to 16 breaths/minute, but during the muscle wall and skin incision it decreased to 6-7 breaths/minute. This suggested better local analgesia of the white line than on the skin. Tugs on the reproductive apparatus increased the respiratory rate from 7-8 breaths per minute to 10-12 breaths/min.

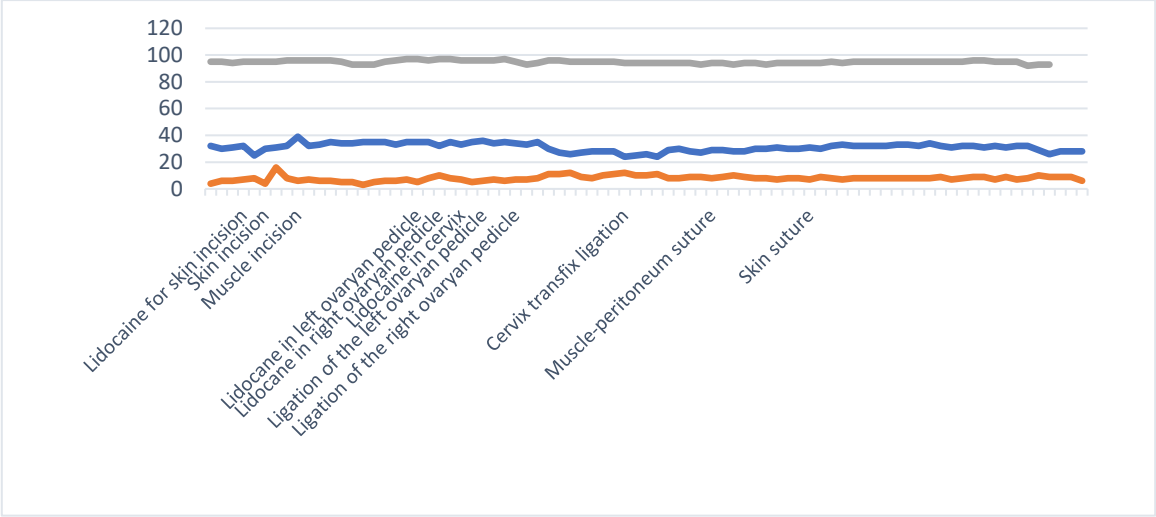

Fig. 8. SpO2 (gray), EtCO2 (blue) and RR (red) during surgery

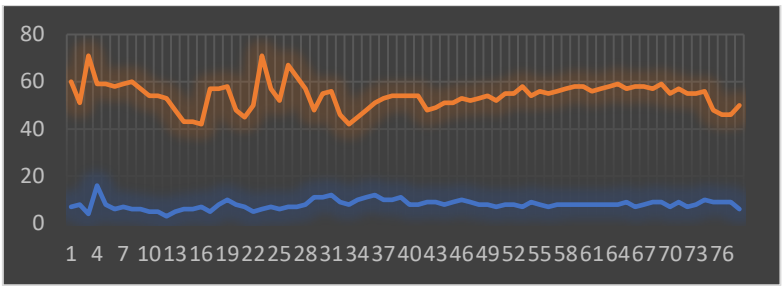

Fig. 9. Heart rate (HR) - blue vs pulsatility rate (PR) - red

Figure 9 shows the differences between heart rate (HR) highlighted in blue and pulsatility rate (PR) in orange. They are not of significant clinical importance in this case, being in correlation with the blood pressure variation shown in Figure 10.

In Figure 10, the chart for oscillometrically measured systolic (SAP), diastolic (DAP) and mean (MAP) blood pressure was superimposed on that heart rate to demonstrate that the difference between HR and PR is influenced by blood pressure variation. Blood pressure was noted to increase at the time of skin incision as well as at the time of traction on the reproductive apparatus.

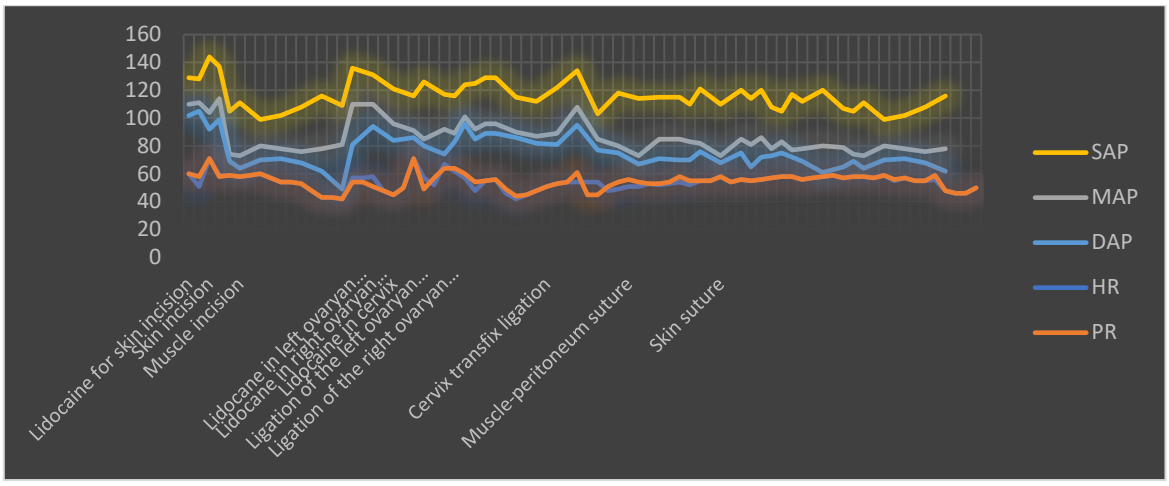

Fig. 10. Systolic arterial pressure (SAP), Medial arterial preassure (MAP), diastolic arterial pressure (DAP), HR and PR

Conclusions

The medetomidine butorphanol protocol was studied by Robinson et al. (12) and proved a 55% decrease in heart rate and a 62% decrease in respiratory rate.

The anesthetic protocol used induced deep muscle relaxation but insufficient analgesia for a major surgery. Although local anesthesia was used, the dog felt some discomfort that was noted by the increase of RR, HR, blood pressure (5). These increases were temporary and no muscle contractions were noted, thus considering that the animal was in stage III anesthesia (4). Also, the presence of pain can be attributed to insufficient local anesthesia associated with strong tugging on the reproductive system due to short ovarian ligaments.

Anesthesia monitoring is essential because it provides us with data on the functioning of the respiratory and cardiac systems. An anesthesia plan that is too shallow is not enough to perform safe surgical procedures, and an anesthesia plan that is too deep can lead to sequelae or even the death of the patient.

The anesthetic protocol used may suffice for older dogs with other comorbidities, but does not for a routine spay in a healthy dog.

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STURGEONS - AQUACULTURE, PRODUCTION AND DISEASES

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Abstract

Data on bacterial pathogens of sturgeon are quite limited, most likely because sturgeon aquaculture is more diverse than other species and not as well known, and because, as they grow to a certain size, sturgeons become more resistant to disease. Sturgeons, listed by the International Union for Conservation of Nature (IUCN) as being on the Red List, are considered one of the fish species threatened with extinction, with up to 85% of them at risk. The recovery of sturgeon species has been hampered by their specific traits, which have developed over many years. Several viral diseases triggered by fungal, parasitic and bacterial pathogens have been reported worldwide. These infectious diseases represent a major threat to aquaculture.

Keywords: *fish, sturgeon, diseases, aquaculture.*

Sturgeons belong to the Acipenseridae family, which includes 27 species, the best known of which are: the cod (*Huso huso*), the trout (*Acipenser stellatus*), the sturgeon (*Acipenser gueldenstaedtii* – Fig. 1), the chub (*Acipenser ruthenus*), the Siberian sturgeon (*Acipenser baeri*), and the white sturgeon (*Acipenser transmontanus* -Fig. 2) (10).

Fig. 1. Sturgeon Caviar (16)

Fig. 2. White Sturgeon (15)

The meat and caviar of sturgeons are highly valued for their nutritional value and commercial value, and their aquaculture has a major impact on the economy of several countries. Compared to other fish species, the development process of sturgeons takes longer and requires more special growing conditions (7, 11).

Due to water pollution, fish poaching, overfishing and habitat destruction, the number of sturgeon specimens is constantly decreasing, which has led to the development of aquaculture (10).

According to the International Union for Conservation of Nature, sturgeons are an endangered fish species. Consequently, most of the caviar in the market comes from sturgeons raised in ponds (11).

Several factors such as: species, season, maturity, sex, etc., can influence the elemental chemical composition of sturgeons.

Sturgeon meat is rich in essential amino acids, vitamins such as niacin, pyridoxine, vitamin B12 (Fig. 3), minerals such as potassium, magnesium and phosphorus, omega-3 fatty acids, docosahexaenoic acid (DHA) (3.8–11.1%) and eicosapentaenoic acid (EPA) (4.9–6.8%). Due to the presence of glutamic acid in a proportion of 18.1%, sturgeon meat has a special, distinctive taste. The consumption of sturgeon fish is essential for human health, taking into account the high content of unsaturated fatty acids. The benefits of consuming sturgeon are multiple and include, among others, skin regeneration, metabolism, and blood pressure (11).

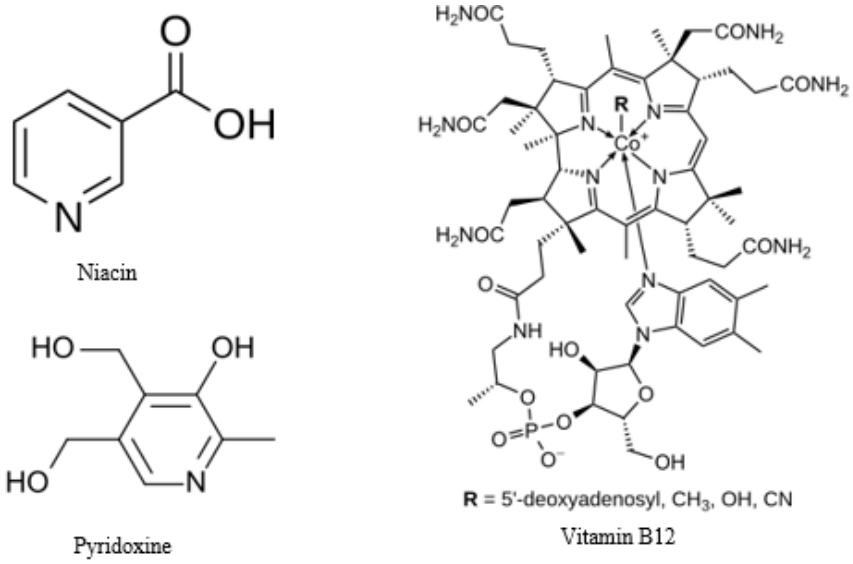

Fig. 3. Chemical structure of the main vitamins in sturgeon (17)

Background of Sturgeon Aquaculture

Although initially viewed with skepticism due to its environmental impact, aquaculture has undergone significant improvements through advances in technology, the study and understanding of best practices, and their implementation, leading to a sustainable source of fish for human consumption that reduces pressure on wild sturgeon populations.

Sturgeons, listed by the International Union for Conservation of Nature (IUCN) as being on the Red List, are considered one of the most threatened fish species, with up to 85% of them being threatened with extinction. The recovery of sturgeon species has been hampered by their specific characteristics, which have developed over many years. These unique traits include a lifespan of over a century, differences between males and females in terms of maturation stage, and intermittency in spawning, with females having a lower reproductive rate than males. While these traits have helped sturgeons adapt over the years, they have been subjected to certain anthropogenic factors with significant impacts over several generations for most species (1).

Most sturgeon species from the wild are fragile and require appropriate conservation measures. The multiple action of biological, ecological and economic factors has resulted in overfishing and poaching of these species. In this context, aquaculture has emerged as a life-saving solution for the sturgeon caviar industry, becoming a more sustainable alternative to the exploitation of wild specimens. In order to prevent further decline in the number of sturgeon fish, but also to maintain the species at its current level, a series of measures have been taken by implementing several programs for the conservation and restoration of aquaculture (1, 9).

Sturgeon production

To obtain 1000 kg of caviar, around 20,000 kg of sturgeon meat are needed. In the last two decades, sturgeon aquaculture has experienced significant growth, driven by the development of the industry in China. According to FAO, in 2002 world production was 4,100 tonnes, of which about half came from Russia and the other half from the European Union. In 2003, with the reporting of Chinese production exceeding 9,000 tonnes, world production tripled. Four years ago, China accounted for over 80% of world sturgeon production, followed by Russia with 4% (4,836 tonnes) and Armenia with 3% (4,200 tonnes).

The largest producer of sturgeon (Fig. 4) in the European Union is Italy. In 2020, Italy produced over a thousand tons of sturgeon, down 19% from 2019, but up 5% from 2016. The estimated value of sturgeon production in 2020 in Italy, according to the FAO, was around 7 million euros. In the last 10 years, Poland and Bulgaria have been second and third after Italy, respectively (14).

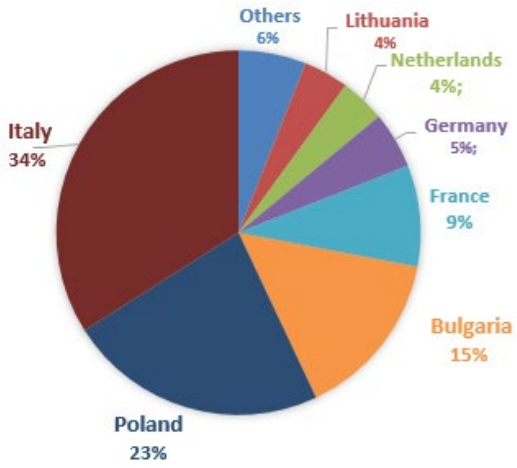

Fig. 4. Sturgeons main EU producers (13)

CITES export data also records sturgeon by-products and products traded worldwide (13, 14).

Common sturgeon diseases

In many countries in Asia and Europe, sturgeon farming is an important source of income (4). With the development of sturgeon aquaculture activities, outbreaks of infection have also occurred, generating multiple infectious diseases that can represent a serious problem in the cultivation of cultured sturgeon. Several viral diseases triggered by fungal, parasitic and bacterial pathogens have been reported worldwide. These infectious diseases represent a major threat to aquaculture, due to the fact that we have very little knowledge about the verification methods and epizootiology. In addition, neither the World Organization for Animal Health (OIE) nor the EU have regulated any of the diseases reported in sturgeon (10).

Although bacterial and parasitic diseases affect sturgeon, viruses seem to be the most serious problem (8), especially those of the *Iridoviridae* family, which has recently been unofficially classified as *Mimiviridae*. Currently, more than 10 types of specific viruses have been identified in sturgeons (12).

The most frequent mortality outbreaks in sturgeons have been caused by infection with viruses from the *Herpesviridae* and *Iridoviridae* families, which are major threats to sturgeon aquaculture. Iridoviruses can cause very serious diseases with a mortality rate of over 50%. *Acipenser iridovirus* European (AcIV-E) has recently been identified in sturgeons in Europe (10).

Bacterial diseases

According to B. Austin and D. A. Austin *Vibrio anguillarum* is the most common bacterial pathogen associated with saltwater sturgeons (*Huso huso* and *Acipenser gueldenstaedtii*) (3). It has also been reported that *V. alginolyticus* and *Pasteurella sp*. can cause mortality in *Acipenser baerii* sturgeons. In recent years, bacteria isolated from sturgeons include *Flavobacterium johnsoniae*, *A. hydrophila* (*Acipenser gueldenstaedtii*) and *Flavobacterium columnare* (*Acipenser oxyrinchus desotoi*). In the study by Kayiş, Ş. et al., the following were isolated from sturgeons: *A. hydrophila*, *Acinetobacter radioresistens*, *Aeromonas sobria*, *A. salmonicida*, *B. mycoides*, *Citrobacter freundi*, *Pseudomonas fluorescens*, *Pseudomonas putida* and *Serratia sp*. (7). *Streptococcus dysgalactiae*, a Gram-positive bacterium, has been identified in chinese sturgeon *Acipenser schrenckii*. These bacteria are known to cause serious diseases such as hemorrhage, exophthalmos, and ulcers, resulting in the death of aquaculture fish (7).

Parasitic diseases

Sturgeons can be hosts for a multitude of parasites, such as protozoa, trematodes, nematodes, monogeneans, helminths and argulids. These parasites are among the main factors that lead to weight loss, impotence, abnormal behavior, gill deformation and the appearance of epithelial lesions. Parasites and their communities represent essential bioindicators for establishing the quality of the environment because they are part of aquatic biodiversity, being directly or indirectly influenced by it through their hosts, although they are most often not taken into account when ecological assessment is made (5).

As a rule, young specimens from aquaculture are the most prone to parasitic diseases, which are caused by ciliates of the genus *Trichodina* and other genera of the family Ur-ceolariidae. Infections caused by *Ichthyophthirius multifilis* and *Chilodonella cyprini* are less common.

In fish farms, helminthic diseases of sturgeon are very rare, even though there are representatives of several species of

Monogenea, *Trematodes*, *Cestodes* and *Nematodes* in sturgeon from these fish farms (10).

Fungal diseases

During the incubation of sturgeon eggs, diseases caused by *Saprolegniaceae* frequently occur. During this period, egg mortality can reach approximately 70-90%. Thirteen species of pathogens have been identified, including *Saprolegnia* (7 species), *Achlia* (2 species), *Aphanomyces* (1 species), *Dactyunus* (2 species) and *Zeptolognia* (1 species). Jalilpoor et al. were able to isolate from sturgeon eggs, *Acipenser persicus*, *Penicillium spp*., *Fusarium spp*., *Mu-corspp*. and *Saprolegnia sp*. (6, 10).

Another infectious agent causing fungal diseases among white sturgeon (*Acipenser transmontanus*) from aquaculture includes *Saprolegnia sp*. It is found mainly in adults and spawning fry (4, 7).

To achieve higher fish production, a key element is maintaining the health of the biological material, which is influenced by the interaction between biotic and abiotic factors that affect the fish's immune system and thus decrease their resistance to disease (2).

Conclusions

Sturgeon meat and caviar are very valuable due to their nutritional value. Sturgeon meat is rich in essential amino acids, vitamins such as niacin, pyridoxine, vitamin B12, minerals such as potassium, magnesium and phosphorus, omega-3 fatty acids.

In many countries in Asia and Europe, sturgeon farming is an important source of income. The largest producer of sturgeon in the European Union is Italy. Worldwide, most viral diseases are caused by fungal, parasitic and bacterial pathogens. The most frequent mortality outbreaks in sturgeons have been caused by infection with viruses from the *Herpesviridae* and *Iridoviridae* families.

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QUALITY AND SAFETY OF HONEY IN CENTRAL ROMANIA

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Abstract

Honey production remains culturally and economically significant in Romania, particularly in Alba and Hunedoara counties, where the demand for high-quality honey has increased both domestically and internationally. However, the industry faces challenges such as competition from imported honey, health issues affecting bees, and climate change impacts. This study analyzed 32 honey samples from Alba and Hunedoara counties, classified into polyfloral, acacia, and rapeseed honey. The physicochemical parameters analyzed included moisture, diastase index, invert sugar, sucrose, hydroxymethylfurfural (HMF), acidity, and antibiotic residues. The moisture content ranged from 16% to 20%, with polyfloral honey showing the highest levels. Diastase activity ranged from 5.6 to 7.5 Gothe units, confirming adequate enzymatic activity. Invert sugar levels were highest in polyfloral (77%) and acacia honey (76%), while sucrose content remained below 3.9%. HMF levels were low, indicating freshness and authenticity. No antibiotic residues were detected, confirming safety for human consumption. These results align with national standards and European regulations, validating the authenticity, freshness, and safety of Romanian honey. Further studies on a larger sample size are recommended to reinforce the findings. The study highlights the importance of adherence to best practices in honey production and quality assurance to ensure consumer's safety.
Keywords: *honey, polyfloral, hydroxymethylfurfural, antibiotic residues, quality assurance.*

Honey, as defined by the Codex Alimentarius, is a sweet substance produced by honeybees, specifically *Apis mellifera* and *Apis cerana*, derived from the nectar of flowers or from sweet secretions found on living plants, which bees collect, process, and store in honeycombs (5, 20, 26). Over the past century, beekeeping has evolved into a global industry, with advancements in technology significantly enhancing the monitoring of bee health and honey production (7, 13, 21). Concurrently, growing awareness of honey's nutritional and medicinal benefits has elevated its status as a healthy and versatile food source (8, 22). In Romania, the average annual honey consumption per capita is estimated at 1–1.5 kg, which, while increasing, remains lower than that of Western European countries where per capita consumption can reach 2–3 kg (4, 24, 29). Romania is among the largest honey producers in the European Union, yielding between 20.000 and 30.000 tons annually, depending on climate conditions and bee health (13, 22). A significant portion of this production is exported, particularly to EU countries such as Germany, France, and Italy, while domestic consumption continues to grow due to promotional campaigns and a rising awareness of honey's health benefits.The Romanian honey market is characterized by a preference for polyfloral honey, followed by acacia and linden honey, alongside an increasing demand for value-added products such as honey with propolis, royal jelly, and pollen (1, 3, 10). However, the market faces challenges including competition from imported honey, often sold at lower prices, and issues related to bee health and climate change, which impact production (15, 18). Additionally, honey is highly susceptible to adulteration, particularly through substitution with sweet substances or addition of non-permitted preservatives aimed at preventing fermentation (2, 11, 14).

This study seeks to evaluate the physicochemical properties, integrity, and freshness of three distinct types of honey, alongside the detection of residues of antimicrobial compounds. By assessing these parameters, this research aims to verify compliance with national and international standards of quality and safety. Through detailed analysis of physicochemical parameters and contaminant presence, this work provides essential information for beekeepers, researchers, and consumers, thereby promoting best practices in honey production and trade.

Materials and methods

Between March 2023 and March 2024, a total of 32 honey samples were collected and analyzed. The samples were sourced from various authorized locations, each labeled appropriately to ensure full traceability. The collection area was centralized in Romania, with 18 samples (56.25%) gathered from Alba County and 14 samples (43.75%) from Hunedoara County. In terms of honey varieties from these two administrative regions, the samples were categorized as follows: 16 samples (50%) were polyfloral honey, 12 samples (37.5%) were acacia honey, and 4 samples (12.5%) were rapeseed honey (Fig. 1.).

Honey samples were collected in sterile glass containers (500 grams, single-use) for physicochemical analyses and residue detection. Analyses followed standardized protocols consistent with national and European legislation, ensuring accuracy and reliability.

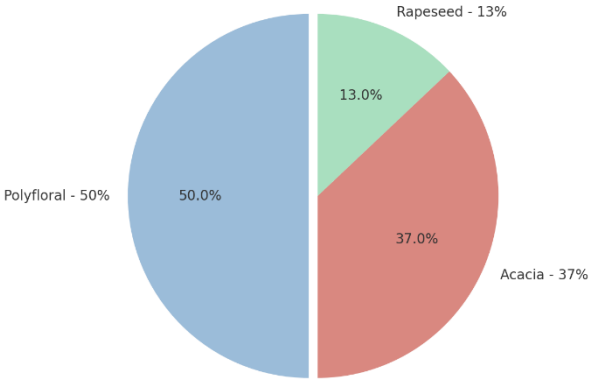

Fig. 1. Distribution of honey varieties by percentage

Organoleptic examination

The sensory analysis of honey samples was conducted to evaluate their appearance, consistency, color, aroma, and taste. Prior to analysis, the samples were homogenized and filtered to remove any visible impurities, ensuring that the observed characteristics were representative of the honey's natural properties. The sensory evaluation was carried out under controlled conditions to maintain consistency and reliability of the results. The samples were assessed for their overall visual appearance, including the clarity and color range, as well as the texture, which was evaluated for fluidity and viscosity. Aroma and taste were also carefully documented, with a focus on identifying specific floral notes, sweetness, and any other distinguishing sensory features. The results of this analysis provided valuable insights into the organoleptic qualities of the honey, contributing to a comprehensive understanding of its sensory profile.

Physicochemical Examinations

Moisture content

The moisture content of the honey samples was determined using the refractometric method, which was performed repeatedly to ensure precision and reliability of the measurements. In cases where discrepancies were observed or further confirmation was required, the drying oven method was employed. This method involved heating the samples at 103 ± 2°C until a constant mass was achieved, providing a more definitive assessment of the moisture content.

Diastase index

The diastase index, an important indicator of the enzymatic activity in honey, was measured to assess amylase activity per gram of honey. The determination was carried out at an optimal pH and temperature of 45°C. The diastase number was calculated based on the hydrolysis of starch in a controlled reaction, providing insights into the freshness and quality of the honey.

Invert sugar content

Invert sugar content in the honey samples was quantified using the Elser method, which involves titration with copper sulfate to reduce it to cuprous oxide in a heated reaction. This method allows for the precise measurement of invert sugars, which are a critical component of honey's sweetness and overall composition.

Sucrose content

The sucrose content of the honey was determined through acid hydrolysis of the invert sugar content, both before and after inversion. The resulting measurements were used to calculate the direct reducing sugar content, offering an indication of the sucrose levels present in the honey, which is an important factor in evaluating its overall quality and composition.

Hydroxymethylfurfural (HMF) levels

HMF is a chemical compound that serves as an indicator of honey's freshness and storage conditions. HMF levels were measured using a spectrophotometric method at a wavelength of 550 nm, as described in Winkler's method. This test provides valuable information on the potential aging or overheating of honey, as elevated HMF levels are typically associated with reduced quality.

Freshness evaluation

The freshness of the honey samples was assessed through titration to measure acidity levels. This analysis is a reliable indicator of the honey's overall quality, as increased acidity can signal degradation or poor storage conditions. By measuring the pH and titratable acidity, this evaluation helps in determining whether the honey meets established freshness criteria.

Residue Detection

To ensure the safety and quality of the honey, potential antibiotic residues were detected using the CHARM II system, a rapid and sensitive assay designed for food testing. Honey samples were incubated on test strips specific to various antibiotic classes. The presence of any antibiotic residues was analyzed to ensure that the honey met food safety standards and was free from harmful contaminants, providing assurance of its suitability for consumption.

Results and discussions

Sensory analysis results

The organoleptic analysis of polyfloral honey samples revealed a color spectrum ranging from light yellow to golden yellow, with a slight opalescent quality and an absence of visible impurities. The consistency of the samples varied from fluid to viscous, with a homogenous texture. All samples exhibited a well-defined aroma, characterized by a diverse array of floral notes of moderate yet pleasant intensity. The perceived taste was pronounced and well-balanced, predominantly sweet with subtle acidic nuances that enhanced the aromatic complexity attributed to the wildflower sources. This flavor profile offered a rich, layered sensory experience that reflected the varied botanical origins of the honey.

The acacia honey samples demonstrated an appearance from colorless to very light yellow, showcasing a transparent quality free from foam or visible impurities. The consistency ranged from fluid to slightly viscous. This variety was marked by a delicate aroma, specific to acacia flowers, with a discreet but pleasant intensity. The taste profile was distinctly sweet and well-pronounced, without secondary flavor notes, offering a refined and gentle sweetness that is highly palatable. This subtle yet distinct flavor, devoid of complex undertones, makes acacia honey particularly appealing and suitable for versatile consumption.

In the organoleptic assessment, rapeseed honey samples displayed a color range from light yellow to yellow-orange, with a slight opalescence and no visible impurities. The consistency was fluid to viscous, and homogenous throughout the samples. The aroma was specific to rapeseed flowers, with a distinct and well-pronounced intensity. The taste was balanced, with moderate sweetness complemented by subtle bitter undertones and a mildly astringent sensation. This particular flavor profile provided a unique and slightly complex tasting experience, with the bitterness contributing to an enhanced depth, characteristic of rapeseed honey.

Results of physicochemical examinations

As a result of the physicochemical analyses conducted on the 32 honey samples, obtained from different beekeepers, the findings indicated that the results did not exceed the limits established by the current legislation.

Moisture content

Moisture content, being an important parameter for evaluating the overall nutritional value of honey, which can influence its organoleptic properties, particularly appearance and consistency, was found to have an average value of 18%, with a minimum of 16% and a maximum of 20% (Table 1). Excess moisture beyond the maximum threshold can lead to the fermentation of honey.

Table 1

The average moisture content of the honey varieties analyzed

No.	Sample type	Number of samples	Moisture content (%)
1	Polyfloral honey	16	17-20%
2	Acacia honey	12	16-18%
3	Rapeseed honey	4	17-19%

Among the 32 samples analyzed, it was observed that polyfloral honey and rapeseed honey exhibited a higher average water content compared to acacia honey. Of the three honey varieties analyzed, polyfloral honey tends to have the highest moisture coefficient, followed by rapeseed honey, while acacia honey has the lowest moisture coefficient.

The water content in polyfloral honey showed significant variation, influenced by the diversity of floral sources. The moisture levels in the analyzed samples ranged between 17% and 20%.

Acacia honey was characterized by a relatively low water content, with results ranging from 16% to 18%. This characteristic contributes to the product's high stability, reducing the risk of fermentation, and enhances the reputation of acacia honey for its excellent preservability.

Rapeseed honey exhibited a moderate water content, with values ranging between 17% and 19%. This moisture level is sufficient to prevent fermentation, although careful attention during storage is necessary to maintain product quality.

The results of the analyses are consistent with the requirements of Directive No. 110/2001/EC for all 32 samples (30).

In a study conducted by Vranić et al. (23), which analyzed the water content in 372 honey samples, it was found that none of the samples exceeded the maximum permissible value of 20%, demonstrating compliance with the current regulations.

The findings of this study align with those reported in previous research on 201 honey samples collected from across Serbia in 2009, where the average moisture content ranged from 16.12% in acacia honey samples to 17.98% in sunflower honey samples (16). Similarly, another study evaluating 187 honey samples from north-western Spain reported an

average moisture content of 17.6% (9). Furthermore, an analysis of 39 honey samples from Greece showed water content ranging from 10.50% to 20.50% (12, 19).

Diastase index

The honey samples analyzed exhibited the following diastase index values: 6.5 Gothe units for polyfloral and rapeseed honey, and 6.8 Gothe units for acacia honey. The highest value, 7.5 Gothe units, was observed in 3 out of 32 samples (9.4%) from Alba County, consisting of two polyfloral honey samples and one acacia honey sample. Conversely, the lowest diastase index value, 5.6 Gothe units, was recorded in a polyfloral honey sample (3.2%) from Hunedoara County. These values indicate adequate enzymatic activity in the analyzed honey samples, falling within an acceptable range according to the quality standards of the beekeeping industry (Table 2.).

The compliance of the results with the values outlined in Directive No. 110/2001/EC (30), which specifies a range of 3-8 Gothe units, further demonstrates the conformity of the analyzed samples with the current European legislation.

Table 2

The average diastase index for the analyzed samples

No.	Sample type	Number of samples	Diastase index (cm^3/g)
1	Polyfloral honey	16	6.5
2	Acacia honey	12	6.8
3	Rapeseed honey	4	6.5

Invert sugar

The values obtained for invert sugar in the polyfloral (77%), acacia (76%), and rapeseed (70%) honey samples demonstrated that the analyzed honey is of high quality and authenticity (Table 3). These results are in compliance with quality standards, reflecting good beekeeping and processing practices. Therefore, the final product is reliable for consumers, providing a rapid source of energy and long-term stability, which highlights excellence in the management and processing of the honey product.

Among the three types of honey, as shown in Table 3.3, acacia and polyfloral honey exhibited high invert sugar content, ranging from 70% to 80%. Rapeseed honey displayed a moderate to high invert sugar content, ranging from 65% to 75%.

Table 3

The average invert sugar content for the analyzed samples

No.	Sample type	Number of samples	Invert sugar (%)
1	Polyfloral honey	16	77
2	Acacia honey	12	76
3	Rapessed honey	4	70

Sucrose

As a result of the physicochemical analyses conducted on the 32 honey samples (Table 4). For polyfloral honey, the average sucrose content was 2.4%, with a maximum value of 3.9%, accounting for 50% of the total samples analyzed. For acacia honey, the average sucrose content was 2.1%, with a minimum value of 1.5%, representing 31.25% of the total samples. Rapeseed honey had an average sucrose content of 2%, constituting 18.75% of the total samples. The analyses revealed a low sucrose content in all types of honey examined, indicating that the results are in compliance with the current legislation (Directive No. 110/2001/EC) (30).

Table 4

The average sucrose content for the analyzed samples

No.	Sample type	Number of samples	Sucrose (g/100g)
1	Polyfloral honey	16	2.4
2	Acacia honey	12	2.1
3	Rapessed honey	4	2

According to the findings of Vranić et al. (23), a total of 11 honey samples were found non-compliant due to low reducing sugar content and a sucrose content higher than the legally permitted limit, representing 52.38% of all the acacia honey samples examined. Additionally, 14 polyfloral honey samples (35.90%) were non-compliant based on reducing sugar content, and 5 polyfloral honey samples (12.82%) were non-compliant based on sucrose content. Moreover, 2 samples of honeydew honey (50%) were non-compliant due to reducing sugars. In 2015, 18 acacia honey samples (51.43%) were non-compliant for low reducing sugar content, and 20 samples (57.14%) for high sucrose content. For polyfloral honey, 33 samples (44.59%) were non-compliant due to reducing sugar content, and 24 samples (32.43%) due to sucrose content. Additionally, 8 honeydew honey samples (88.89%) were non-compliant for reducing sugars, and 6 samples (66.67%) for

sucrose. In 2016, 10 acacia honey samples (13.16%) were non-compliant for low reducing sugar content, and 5 samples (6.58%) for high sucrose content. Among polyfloral honey, 8 samples (7.41%) were non-compliant for reducing sugar content, and 5 samples (4.63%) for sucrose. The results obtained for reducing sugars and sucrose content in all honeydew samples examined in 2016 were in accordance with the compositional criteria defined by the current national regulations.

Hydroxymethylfurfura

The values for HMF in polyfloral, acacia, and rapeseed honey were as follows: 0.55 mg/kg, 0.35 mg/kg, and 0.65 mg/kg, respectively (Table 5). These results are in compliance with the maximum allowable limits for this compound in honey, as outlined in Directive No. 110/2001/EC (30). In terms of the maximum limits for HMF in honey, the recorded values fall within the legal parameters.

Table 5

The average hydroxymethylfurfural content for the analyzed samples

No.	Sample type	Number of samples	Hydroxymethylfurfural (mg/100g)
1	Polyfloral honey	16	0.55
2	Acacia honey	12	0.35
3	Rapeseed honey	4	0.65

Vranić et al. [38] observed that all HMF levels were below the maximum permissible limit of 40 mg/kg, indicating that the honey could be considered fresh. In their 2014 study, the HMF range was between 0.384 and 3.306 mg/100g, and in 2016, it ranged from 0.157 to 3.281 mg/100g. Generally, the HMF values obtained in this study were higher than those reported in the literature for honey from Turkey (0.252 mg/100g) (25), and Argentina (0.898 mg/100g) (6).

Freshness results

The acidity values determined for polyfloral, acacia, and rapeseed honey were as follows: 2.5 acidity degrees, 1.9 acidity degrees, and 2.2 acidity degrees, respectively. The lowest values were recorded for acacia honey, with acidity ranging from 1.5 to 2.7 acidity degrees (Table 6). The maximum allowable acidity for honey intended for consumption is 5 acidity degrees. The results are within the permissible limits established by the relevant regulations, in accordance with Directive No. 110/2001/EC (30).

Table 6

The average acidity for the analyzed samples

No.	Sample type	Number of samples	Acidity (ml NaOH 1N/100g)
1	Polyfloral honey	16	2.5
2	Acacia honey	12	1.9
3	Rapeseed honey	4	2.2

The average values for the free acidity of honey reported by Vranić et al. (23) varied from the lowest values of 10.82 meq/kg (2014), 10.87 meq/kg (2015), and 8.23 meq/kg (2016) for acacia honey, to 17.44 meq/kg (2014), 14.65 meq/kg (2015), and 16.46 meq/kg (2016) for polyfloral honey, with the highest values reaching 26.03 meq/kg (2014), 18.53 meq/kg (2015), and 23.59 meq/kg (2016) for honeydew honey. The free acidity values for all the honey samples analyzed were below the legal limit (less than 50 meq/kg). The results obtained in this study are consistent with the data reported in the literature.

Control of antibiotic residues

The analysis conducted demonstrated the absence of antibiotic residues in the honey samples, indicating compliance with food safety standards (Table 7). The absence of antibiotic residues in the analyzed honey samples confirms their conformity with the applicable European regulations, thus ensuring food safety for consumers.

In a study conducted in Italy, out of a total of 153 analyzed samples, 5.2% showed the presence of tetracyclines, while 12% of 74 samples tested positive for sulfonamide antibiotics. In another study carried out in Spain, 21% of a sample of 215 honey samples tested positive for antibiotics from the sulfonamide class, such as sulfathiazole and sulfadiazine (6, 16, 17, 25).

Table 7

Results regarding antibiotic residue detection in honey samples

No.	Sample type	Number of samples	Classes of residues analyzed	Result
1	Polyfloral honey	16	Aminoglycosides Macrolides Sulfonamides Tetracyclines	Negative
2	Acacia honey	12	Aminoglycosides Macrolides Sulfonamides Tetracyclines	Negative
3	Rapeseed honey	4	Aminoglycosides Macrolides Sulfonamides Tetracyclines	Negative

Overall, the results obtained from the honey samples in our study align with the regulations stipulated in both national and European legislation concerning apicultural products. Relevant legislative norms include Regulation (EC) No. 852/2004 on the hygiene of foodstuffs (27), Regulation (EC) No. 853/2004 on the hygiene of foodstuffs of animal origin (28), as well as national standards for apicultural products. This legislative framework ensures that honey produced and marketed meets the strict safety and quality requirements, thus protecting public health and promoting sustainable beekeeping practices.

Conclusions

The low levels of HMF in the analyzed honey samples indicate their freshness and authenticity. HMF is a reliable marker for honey quality, and its low concentration suggests minimal processing and proper storage. The significant variations in chemical composition reflect the diverse floral sources and bee habitats, highlighting the environmental impact on honey characteristics.

Additionally, the absence of antibiotic residues in the honey confirms its safety for human consumption, meeting established food safety standards. However, to increase the confidence in these results, further studies with a larger sample size, covering a broader range of beekeepers and production periods, are needed. This would provide more comprehensive data and enhance the reliability of the findings.

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30. *** DIRECTIVA 2001/110/CE A CONSILIULUI din 20 decembrie 2001 privind mierea

A COMPREHENSIVE STUDY ON THE IMPACT OF DIETARY IMPROVEMENT ON JENNY MILK PRODUCTION

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Abstract

Donkey milk is increasingly recognized for its nutritional benefits and potential role in sustainable farming. This research examines the effects of supplementing a pasture-only diet with complementary compound feeds to improve milk yield. Initially, the jennies were fed a natural diet consisting solely of pasture, grass, and various plants. To enhance nutritional intake, their diet was supplemented with specially formulated complementary feeds. A total of 15 jennies were monitored over a one-month period, with baseline milk production recorded prior to the introduction of a complementary feed. Before supplementation, the average daily milk yield ranged from 390.88 ml to 920.42 ml. Following the dietary intervention, the average daily milk production increased significantly, ranging from 515.49 ml to 1008.62 ml. The study demonstrates that the strategic inclusion of dietary supplements can lead to substantial improvements in milk production in jennies, highlighting the critical role of nutrition in optimizing lactation. These findings provide valuable insights into effective feeding strategies for improving productivity in equine management, suggesting further investigation into long-term dietary impacts on health and performance in lactating animals.

Keywords*: donkey, ration, balanced nutrition, jenny milk production.*

Donkey milk has been recognized for its high nutritional value, containing bioactive compounds and properties that make it suitable for human consumption, particularly for individuals with cow milk allergies (1, 7, 9). In recent years, there has been a growing interest in donkey milk production as part of sustainable agriculture, driven by its nutritional benefits and potential market expansion (2). However, one of the main challenges in donkey farming is optimizing milk yield during lactation, which is closely linked to the animal's diet and overall health (14).

In traditional farming systems, donkeys are typically fed a natural diet based on pasture, grass, and other forage plants (3, 10). While this diet is often sufficient to maintain basic health, it may not provide the optimal nutrients required for high milk production (4). The natural forage is often subject to seasonal variations in nutrient availability, which can lead to fluctuations in milk output. To address this issue, recent advancements in animal nutrition suggest that the introduction of compound feeds, designed to balance the diet with essential proteins, carbohydrates, fats, vitamins, and minerals, can significantly enhance both the quantity and quality of milk produced (6, 13).

This study aims to investigate the effects of dietary modification on milk production in jennies by comparing milk yield before and after the introduction of compound feeds into a previously pasture-only diet.

Materials and methods

This study aimed to underline the effects of dietary changes on the milk production of lactating jennies and it was conducted on a donkey farm in Romania. The farm was specifically chosen for its traditional feeding practices, which align closely with natural foraging behaviours. Donkeys were kept on a pasture, bordered by forests and with access to a natural water source. This setup closely mimics a natural habitat and allows donkeys to graze freely. The diet of the jennies consisted solely of pasture with no concentrates, highlighting the need for dietary improvement. During the winter, when pasture resources were limited, the diet was supplemented with mountain hay rich in cellulose.

A total of 15 lactating jennies were selected for this study. The age of the individuals ranged between 5 and 10 years, with similar sizes and weight. The lactation stage varied, including females from 4 to 8 months of lactation. This selection was made to optimize results and enhance the relevance of the data obtained. Regarding milking, it was conducted manually once a day in the morning, prior to allowing the animals to graze. During the research period, the females were maintained on pasture with no other modifications made to their daily management practices.

The dietary supplementation involved incorporating a complementary compound feed into the daily diet of the animals. This consisted of a pelleted mixture of grains, sunflower meal, and vitamins. Bentonite was added to the mixture to serve as a binding agent and to enhance storage stability (Table 1). The proportions were determined according to the established nutritional requirements (8, 11). Sunflower meal was selected to improve the protein intake while ensuring good digestibility,

as it has a higher fibre content (12). The pellets were administered daily, at a rate of 2 kg per individual over a one-month period, during which time the quantity of milk produced was closely monitored.

Prior to supplementing the diet, for a duration of one week, the quantity of milk produced by each individual was monitored daily, for a comprehensive assessment of the herd's overall productivity.

Table 1

Jenny feed ration

Feed Type	%	D.M. (kg)		Crude Fiber (%)		Feed units		D.C.P. (g)		Ca (g)		P (g)	
Corn	48.50	0.88	42.68	2.00	97.00	1.05	50.93	71.00	3443.50	0.20	9.70	2.70	130.95
Wheat	40.00	0.88	35.20	2.00	80.00	1.02	40.80	95.00	3800.00	0.40	22.00	3.90	156.00
Sunflower meal	8.00	0.91	7.28	20.00	160.00	0.90	7.20	320.00	2560.00	3.00	21.75	7.00	56.00
Bentonite	2.50		0.00		0.00		0.00		0.00		0.00		0.00
Zoofort	1												
Total Concentrate	100	85.16		300.00		98.93		9803.50		49.70		342.95	
For 1 kg	1	0.85		3.00		0.98		98.03		0.49		3.42	

Results and discussions

At the beginning of the research period, the average daily milk yield per individual jenny ranged between **390.88** and **920.42 ml/day** (Table 2). This initial measurement established a baseline that reflected the existing dietary conditions, which primarily consisted of pasture without any supplementary feeds. The variability in milk yield within this range could be attributed to factors such as age, and individual differences among the animals.

By the end of the study, the average daily milk yield had increased significantly, ranging from **515.49** ml to **1008.62 ml/day** (Table 2). This improvement can be directly linked to the introduction of a complementary diet, which included a balanced mixture of grains, sunflower meal, and essential vitamins. The increase in milk production indicated not only a positive response to dietary supplementation but also demonstrated the importance of providing adequate nutrition to enhance lactation performance in jennies.

Table 2

Average milk production ml/day

Individuals	*Before supplementation*	*After supplementation*
1	870.12	1000.5
2	750.45	860.32
3	880.67	990.75
4	500.3	600.2
5	920.42	1008.62
6	450.5	650.45
7	700.21	830.11
8	580.8	680.9
9	780.55	890.67
10	610.32	820.15
11	540.11	750.85
12	430.29	580.81
13	600.25	800.78
14	500.67	625.6
15	390.88	515.49

The introduction of a balanced ratio of energy and protein in the diet of the jennies was a crucial factor in enhancing lactation performance. Specifically, by administering a daily intake of **6% crude fiber** and **196.06 g of crude protein** /individual, we ensured that the nutritional needs of the lactating animals were adequately met. (Table 1).

Crude protein is essential for milk production and overall health, as it provides the necessary amino acids required for the synthesis of milk and supports bodily functions. The inclusion of crude fiber plays a significant role in maintaining digestive health and ensuring optimal nutrient absorption.

Elevating phosphorus levels in the diet, to

3,42 g relative to calcium was essential for achieving a proper mineral balance. This adjustment considered the higher calcium concentrations commonly found in pasture grass. Maintaining an optimal calcium-to-phosphorus ratio, typically around 2:1, is vital for ensuring efficient absorption and utilization of these minerals within the animal's body. Increasing phosphorus intake helps to mitigate the risk of potential deficiencies that could negatively impact skeletal development, energy metabolism, and overall health. This approach supports the maintenance of mineral homeostasis and promotes optimal physiological functions during critical periods, such as growth and lactation.

A comprehensive understanding of the nutritive requirements of donkeys during the lactation period is crucial for optimizing milk production. Such an understanding not only facilitates improved milk yields but also significantly contributes to the welfare of both the jennies and their foals. By addressing these specific needs, we can enhance the overall health of both jennies and their foals.

This study's findings align with previous research in the field. Yue et al. (15) investigated the effects of varying dietary concentrate-to-forage ratios on milk production, milk amino acid composition, and milk protein synthesis in lactating donkeys. Increasing the proportion of forage in the diet (specifically alfalfa replacing low-quality roughage) while maintaining equal energy and protein levels significantly improved milk production, enhanced milk protein synthesis, optimized amino acid composition, and promoted better nutrient utilization in lactating donkeys (15).

Another study conducted by Zhang et al. (16) in 2015, investigated the effects of varying energy density in the close-up dry period diet and postpartum supplementation with extruded full-fat soybeans on the metabolic and hormonal status and lactation performance of dairy cows. It was reported that feeding a lower energy density diet in the close-up period, combined with supplemental extruded full-fat soybeans post-partum, can be beneficial for optimizing metabolic status and improving milk production in early lactation, reinforcing the effectiveness of dietary improvements (16).

Furthermore, Liang et al. (5) examined the impact of different dietary crude protein (CP) levels on milk production, nutrient digestibility, and serum metabolites in lactating donkeys. The researchers measured various parameters, including milk yield, milk composition, nutrient digestibility and serum metabolites. The diets containing 14.2% and 15.3% CP resulted in significantly higher milk yield and yields of several milk components compared to the low CP diet (13.1%). The study concluded that a dietary CP level of 14.2% is sufficient to optimize milk production and nutrient utilization in lactating donkeys. Higher levels provide no further benefit. The results highlight the potential of dietary manipulation to improve donkey milk production (5).

Overall, the results of this study contribute to a growing body of literature that underscores the importance of nutritional interventions in managing lactation in jennies. By comparing these findings to existing studies, it becomes evident that effective dietary strategies can lead to improved productivity and well-being in lactating animals.

Conclusions

The study revealed significant insights into the impact of dietary supplementation on milk production among the jennies. Over the one-month period, a clear increase in the quantity of milk produced was observed in the supplemented group compared to the baseline measurements recorded prior to the introduction of the pelleted feed. The incorporation of the complementary feed, which included grains, sunflower meal, and vitamins, played a crucial role in enhancing nutritional levels.

The observed increases in milk yield highlight the potential benefits of implementing balanced dietary practices in the management of lactating animals. This underscores the significance of nutrition in supporting milk production and the overall health of the animals, paving the way for further research into optimal feeding strategies for equine species.

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A MINI-REVIEW OF THE ZOONOTIC SIGNIFICANCE OF ENTEROCYTOZOON BIENEUSI

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Abstract

Enterocytozoon bieneusi is a microsporidian parasite that has gained increasing public health relevance due to its zoonotic potential. This parasite primarily targets the intestinal epithelial cells of humans and various animal hosts, including livestock and wildlife. The zoonotic transmission of *E. bieneusi* poses a significant concern for both human and animal health, making it crucial to understand its epidemiology and genetic diversity. This review synthesizes current knowledge on the parasite, with a focus on its zoonotic implications, transmission patterns, and genetic variation across different host species. A systematic search of the Google Scholar database was conducted, and 20 relevant articles were included in the final analysis, providing a global perspective on *E. bieneusi*. The findings emphasize the widespread nature of this parasite and its potential to cross species barriers, underscoring the importance of surveillance and research to better understand its role in public health.

Keywords: *Enterocytozoon, zoonotic importance, epidemiology.*

Microsporidia is a group of obligate intracellular parasites, causing microsporidiosis in both humans and animals. Among the roughly 1600 designated species of microsporidia, 17 have been identified as capable of infecting humans, including relatively common species, such as *Encephalitozoon cuniculi*, *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, and *Encephalitozoon hellem* (10, 26).

Enterocytozoon bieneusi is the most important zoonotic microsporidian species worldwide and is transmitted predominantly through the fecal-oral route because it is capable of infecting a broad range of hosts, including humans, domestic animals, poultry, companion animals, birds, and wildlife. Moreover, *E. bieneusi* is known to be responsible for gastroenteric episodes clinically characterized by chronic diarrhea, malabsorption, and abdominal pain, predominantly observed in immunocompromised individuals (26, 27).

*Enterocytozoon bieneus*i was first identified in human immunodeficiency virus (HIV) patients in 1985, and with the expansion of the HIV epidemic, the number of cases of *E. bieneusi* infections in humans has increased (12, 27).

Microsporidia have the capacity to affect individuals with compromised immune systems, encompassing those afflicted by the human immunodeficiency virus (HIV) and individuals undergoing cancer treatment. There is a growing body of evidence reporting instances of *E. bieneus*i infections in individuals with immune disorders or weakened immunity, such as children and the elderly (12).

Various modes of contracting *E. bieneusi* exist, encompassing transmission through food and water. Additionally, the identification of numerous genotypes capable of infecting both humans and domestic animals underscores the zoonotic nature of microsporidiosis (10).

While infections in individuals with competent immune systems typically manifest as asymptomatic, the shedding of spores during such instances holds the potential to contaminate the environment, posing an epidemiological concern (17).

The epidemiology of *E. bieneusi* remains ambiguous, and understanding its transmission routes remains inadequate. Nevertheless, through comparative analysis of ITS genotypes across various hosts, potential sources for both humans and a diverse array of animals are gradually being recognized (21).

Materials and methods

For the purpose of conducting this review, the integrated articles were obtained through a systematic search of the Google Scholar database.

The searching methodology employed specific keywords including: *Enterocytozoon bieneusi* zoonotic potential, epidemiology of *Enterocytozoon bieneusi,* prevalence of *E. Bieneusi*. A total of 300 publications were identified, and after the titles and abstracts reviewed, 35 papers were selected for full-text reading. According to the inclusion and exclusion criteria, 20 studies were finally considered to summarize the relevant data and to assess the zoonotic potential of *E. bieneusi*.

Results and discussions

Enterocytozoon bieneusi has been found in a wide range of hosts, including wild and domestic mammals, birds and reptiles. Actually,

various genotypes infecting animals can infect humans, supporting that zoonotic transmission may be common (15).

Different modes of transmission of *E. bieneusi* are probable, but it is thought to occur mainly by ingestion of spores from the environment. Contaminated food or water are, therefore, considered to be the main vectors of contamination, along with zoonotic, and human-to-human transmission (12, 15).

Analyzing the 20 relevant publications on the zoonotic impact of *Enterocytozoon bieneusi*, the data on the prevalence of genotypes in animals and humans have been summarized in the following two tables. (Table 1, Table 2).

Table 1

Prevalence of *E. bieneusi* genotypes in animals

Location	Host	Sample type	Method	No. Examinated	No. Positive (%)	Genotype	References
Germany	Cat	feces	PCR	60	3 (5)	K, L	(5)
	cattle	feces	PCR	60	7(12)	F, I, J (3), M, N	(5)
	Pig	feces	PCR	50	5(10)	F(3), G, H, O	(5)
	Llama	feces	PCR	1	1 (100)	P	(5)
China	Pig	feces	PCR	94	88 (94)	EbpA, EbpC, EbpD, Henan-I	(11)
USA	beaver	feces	PCR	85	13 (16)	D, EbpC, WL15	(25)
	Fox	feces	PCR	67	9(14)	D, EbpC, WL11, WL15	(25)
	Muskrat	feces	PCR	239	20(9)	D, EbpC, WL15	(25)
	Raccoon	feces	PCR	55	15 (28)	D, EbpC, WL15	(25)
	otter	feces	PCR	19	2 (11)	EbpC	(25)
Peru	guinea pig	feces	PCR	8	7(88)	Peru16	(4)
Japan	pig	feces	PCR	30	10 (33)	D, EbpC	(1)
	cat	feces	PCR	7	1(15)	Type IV	(2)
Czech Republic	pig	feces	PCR	79	74 (94)	D	(18)
Korea	cattle	feces	PCR	538	60 (15)	D	(8)
	pig	feces	PCR	472	67 (14)	CAF1	(7)
Abu Dhabi	falcon	feces	PCR	137	6 (5)	D	(14)
Colombia	horse	feces	PCR	195	21 (11)	D	(20)
	cat	feces	PCR	46	8 (17)	D, Type IV, Peru10 WL11	(22)

In a study conducted in China, Li et al. (10, 11) used the PCR method to identify the EbpA, EbpC, EbpD, and Henan I genotypes in 94% of fecal samples collected from pigs. In contrast, other studies carried out in the Czech Republic by Sak et al. (18) and in Korea by Lee et al. (8) identified a different genotype, D, also from pig fecal samples.

In the USA, Sulaiman et al. (25) analyzed 465 fecal samples from different fur-bearing wild mammalian hosts (85 beavers, 67 foxes, 239 muskrats, 19 otters, and 55 raccoons). Fifty-nine specimens were *E. Bieneusi*-positive (9 isolates from foxes, 13 isolates from beavers, 20 isolates from muskrats, 15 isolates from raccoons, and 2 isolates from otters). Genotype analysis revealed: D, EbpC, WL15, WL11 (25).

Additionally, genotype D has been identified in other animals, such as falcons in a study conducted by Müller et al. in Abu Dhabi, as well as in horses and cats in a study by Santín et al. in Colombia. In the same study, cats were also found to carry genotypes Type IV, Peru10, and WL11 (14, 22).

In 2010, Abe and Kimata (1) examined in Japan, 30 fecal samples from pigs to investigate *E. bieneusi* infection. Ten pigs (33%) were found to be positive when assessed by polymerase chain reaction. Among the genotypes with zoonotic potential, D and EbpC were identified. Also in Japan, Abe et al. (2) isolated the Type IV genotype from a cat after analyzing 7 fecal samples using the PCR method.

In Germany, Dengjel et al. (5) examined fecal samples from 26 humans and 350 animals across 37 species, identifying *E. bieneusi* in 18 samples from humans, cats, pigs, cattle, and a llama. Molecular epidemiological analysis of these data offers convincing evidence for a zoonotic potential of *E. bieneusi*. *E. bieneusi* DNA was detected in two humans (genotypes C

and Q), seven cattle (genotypes F, I, J, M, and N), five pigs (genotypes F, G, H, and O), three cats (genotypes K and L), and one llama (genotype P) (5).

Finally, the most frequently isolated genotype in animals was D, followed by EbpC, WL15, Type IV, F, WL11, CAF1, J, G, H, O, P, and so on.

Zoonotic transmission has been historically supported by the observation of identical genotypes in both animals and humans. In line with this, Cama et al. reported on a child infected with an unusual genotype (Peru 16), who had close contact with infected household guinea pigs. (Table 2). In Peru, other genotypes isolated from human stool samples include D, EbpC, Type IV, WL11, Peru6, and WL15 (4, 15, 24).

Table 2

Prevalence of *E. bieneusi* genotypes in humans

Location	Sample type	Method	No. examinated	No. Positive (%)	Genotype	References
Germany	feces	PCR	26	2 (8)	C, Q	(5)
Cameroon	feces	PCR	758	22 (3)	D, Type IV CAF1	(3)
	feces	PCR	181	8(5)	Type IV	(23)
Gabon	feces	PCR	822	25(3)	D, CAF1, TypeIV	(3)
Thailand	feces	PCR	180	6 (3,3)	D, EbpC	(13)
	feces	PCR	90	5 (6)	D	(19)
	feces	PCR	33	24(73)	D, EbpC, O, PigEBITS7	(9)
Peru	feces	PCR	13937	212 (2)	D, EbpC, Peru10, Type IV, WL11, Peru6	(24)
	feces	PCR	388	31(8)	D, EbpC, Peru16, Type IV, WL11, Peru6, WL15	(4)
Vietnam	feces	PCR	270	27(10)	D, CAF1,EbpC, Type IV,	(6)
England	feces	PCR	25	13(52)	D, Type IV,	(16)

In Cameroon and Gabon, Breton et al. (3) analyzed stool samples by an immunofluorescence antibody test and PCR. Twenty-five out of 822 HIV-positive patients from Gabon and 22 out of 758 villagers from Cameroon were found to be positive for *E. bieneusi*. Genotype analysis revealed D, CAF1, TypeIV (3).

Similarly, in Thailand, multiple studies have reported a high prevalence of genotype D in patients positive for *E. bieneusi*. In addition to this genotype, other genotypes such as EbpC and O were also detected, which have been identified by other researchers in animals as well, further highlighting the zoonotic impact of *E. bieneusi* (9, 13, 19).

Espern et al. (6) after examining 270 stool samples from individuals in Vietnam, found that 27 (10%) were positive for *E. bieneusi* by real-time PCR, with most cases occurring in HIV-positive individuals. The identified genotypes included D, CAF1, EbpC, and Type IV (6).

Another study conducted in England by Sadler et al. (16) highlighted the presence of genotypes D and Type IV in fecal samples positive for *E. bieneusi*.

The most frequently identified genotype in humans across the reviewed studies, as in animals, was D, followed by Type IV, EbpC, CAF1, O, WL11, Peru 6, and others.

Conclusions

The present review provide compelling evidence for the zoonotic potential of *Enterocytozoon bieneusi.* The presence of identical genotypes in both human and animal hosts suggests frequent transmission between species. The most commonly identified genotype in both humans and animals, especially in regions with high prevalence, is genotype D. Additionally, genotypes such as EbpC, Type IV, and WL11 have been detected across diverse host species, further supporting the zoonotic nature of the infection.

The prevalence of *E. bieneusi* varies significantly across different regions, with higher infection rates observed in immunocompromised populations, particularly those living with HIV/AIDS. In countries like Thailand, Cameroon, Gabon, and Peru, genotype D is the most frequently identified in both human and animal samples. These findings emphasize the need for region-specific epidemiological surveillance and control strategies in order to minimize the risk of infection in both humans and animals.

The zoonotic nature of *E. bieneusi* presents significant challenges for public health, particularly in regions with poor sanitation and where humans and animals live in close proximity. Given the potential for widespread

environmental contamination through asymptomatic human carriers, there is a need for improved hygiene practices, water sanitation, and food safety measures.

The data reviewed strongly supports the zoonotic transmission of *Enterocytozoon bieneusi* and underscores the importance of cross-species surveillance to reduce its impact on both human and animal health. Further research into the transmission routes and genotypic diversity of *E. bieneusi* is essential to enhance prevention efforts and control strategies.

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STAPHYLOCOCCUS SPP. IDENTIFIED FROM DOGS WITH OTITIS EXTERNA AND THEIR ANTIMICROBIAL RESISTANCE

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Abstract

This study investigated the antimicrobial resistance of *Staphylococcus species* isolated from dogs with otitis externa. Out of the 71 samples collected, *Staphylococcus spp.* was isolated in 65% of cases.. Isolation, identification of the species and antimicrobial susceptibility testing was carried out first using cultural microbiological methods and with the Vitek 2 system. The most common species identified were *S. pseudintermedius* (41.3%), followed by *S. aureus* (19.6%), *S. intermedius* (17.4%), and *S. schleiferi* (17.4%). Penicillin resistance was significant in *S. intermedius* (50%), *S. aureus* (44.4%), and *S. pseudintermedius* (31.6%). Clindamycin resistance was observed across all species. Multiple drug resistance (MDR) was observed in 13% of the strains, while no oxacillin resistance was noted. The study emphasizes the need for regular antimicrobial susceptibility testing to avoid ineffective treatments and control MDR strains.

Keywords**:** *Staphylococcus species,* antimicrobial resistance, dog, otitis externa.

Infections with multidrug-resistant bacteria are becoming an increasingly serious challenge, in veterinary medicine as well as in human medicine (11), having a significant impact on animal health and public health (2).

Inflammation of the external auditory canal, including the ear pinna, is known as otitis externa. It can be either acute or chronic (recurrent or persistent for at least 3 months). In the case of chronic inflammation, changes that may occur in the external auditory canal include dilation and glandular hyperplasia, hyperkeratosis, and epithelial hyperplasia (3).

Increased production of cerumen in the external auditory canal is usually the result of these changes, leading to increased humidity and local pH, making the ear more susceptible to secondary infections (3).

Inflammation, pain, ear discharge, head shaking and scratching of the ear are the most frequently observed clinical signs of otitis externa, and in prolonged cases, symptoms such as erythema, edema, pruritus, and an unpleasant odor may appear (20).

Otitis, a common infection in dogs, is caused by multiple factors and is also one of the most common conditions encountered in dogs. Among the main factors observed are the anatomical conformations of the ears and ear canal, immune suppression, and changes in the microbiota, which act as predisposing factors (19). Acute and simple inflammation of the external ear canal can be effectively treated in many cases, but chronic or recurrent inflammation of the external ear is much more difficult to manage (3).

The pathogens most commonly involved in the etiology of otitis include species from the genus *Staphylococcus*, predominantly *S. pseudintermedius*, which is usually found in small quantities in healthy ears. Regarding gram-negative microorganisms, although they are not normally isolated from healthy ear canals, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis* also play a role in the development of otitis (6, 14).

Given that species from the *Staphylococcus* genus exhibit a high rate of conjugation, constantly acquiring plasmids that carry genes associated with antimicrobial resistance, it is essential to periodically update knowledge regarding resistance to antimicrobial drugs. The emergence of multidrug-resistant strains and therapeutic failure may occur due to empirical treatment being administered without performing antimicrobial susceptibility tests (14).

Due to the danger of the increasing emergence of antimicrobial-resistant bacterial strains and the difficulty in treating external otitis caused by these bacterial strains, the aim of this study was to update knowledge regarding the antimicrobial resistance of *Staphylococcus* species isolated from dogs with external otitis.

Materials and methods

Secretions and wax samples from the ears were collected from 71 dogs with otitis externa using sterile swabs.

For the primary bacteriological examination, the samples were inoculated into nutrient broth and incubated at 37°C for 24 hours. From each test tube containing the mixed bacterial culture, inoculations were then performed on selective media to isolate *Staphylococcus spp.* The inoculations were done using a sterile loop and streak plate technique on Chapman and Baird-Parker

media, which had been previously poured into Petri dishes. The plates were maintained at 37°C for 24 hours under aerobic conditions. From each plate with these selective media, a characteristic colony was picked and examined by the Gram staining method, after which it was transferred onto blood agar plates for 24 hours at 37°C under aerobic conditions. *Staphylococcus species* identification was performed using the Vitek 2 Compact system, employing identification cards for Gram-positive bacteria. From each culture obtained on blood agar, a bacterial suspension was prepared in 3 ml of physiological saline in a 12 x 75 mm plastic tube.

Each bacterial suspension was adjusted to a density of 0.5 – 0.63 McFarland. Specific identification cards were used for bacterial strain identification, with each card containing 64 wells. Due to the various carbohydrates and enzymes present in these wells, the bacterial strains could be identified based on their biochemical characteristics. Due to the storage conditions of the testing cards, specifically refrigeration at 4°C, the cards were allowed to equilibrate to room temperature for 10 minutes before use. Afterwards, they were placed in the vacuum pressurization compartment alongside the bacterial suspension, with each tube of bacterial suspension positioned near the corresponding card, enabling transfer of the bacterial suspension to each individual card. The cards, along with the bacterial suspension, are maintained within the system at 33.5°C, with each well being periodically checked by the device's spectrophotometer. Each metabolic reaction generated by the enzymes on the card is recorded at 15-minute intervals, and upon completion of the process, which lasts between 8 and 24 hours, the results are displayed and interpreted via the AES system, with data stored on the computer attached to the device.

Antimicrobial susceptibility test was also performed using the Vitek 2 Compact system, following the manufacturer's instructions.

Results and discussions

Regarding the identification of species isolated from otic secretion samples collected from dogs with otitis, performed using the Vitek 2 system, the results are presented in Figure 1. Thus, *Staphylococcus spp.* was identified in 65% (n=46) of the collected samples. Specifically, the following *Staphylococcus species* were identified: *S. pseudintermedius* (n=19, 41.3%), *S. aureus* (n=9, 19.6%), *S. intermedius* (n=8, 17.4%), *S. schleiferi* (n=8, 17.4%), and *S. lentus* (n=2, 4.3%) (Fig. 1).

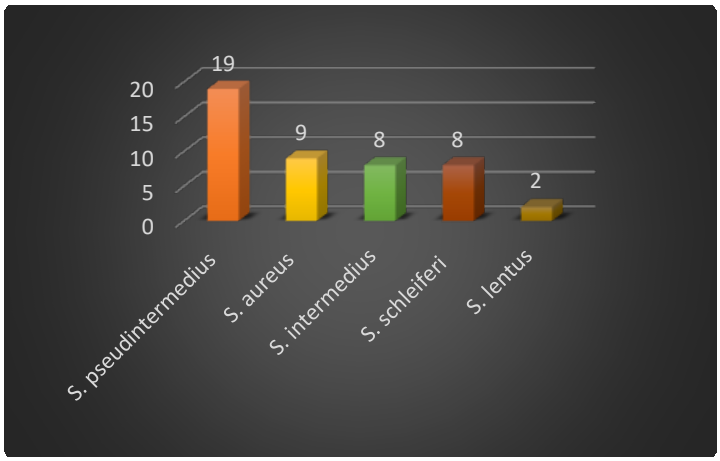

Fig. 1. The identified *Staphylococcus spp* isolated from otic secretion samples collected from dogs with otitis

In this study, the most frequently isolated species was *S. pseudintermedius* (41.3%), and our results are similar to those obtained in the study conducted by Hassan et al. (7), where *S. pseudintermedius* was isolated in 41.6%. Furthermore, our findings are comparable to those reported by Tesin et al. (20), where *S. pseudintermedius* was isolated in 54.7%. Additionally, our results are similar to those of the study by Popa et al. (15), where *S. pseudintermedius* was also the most frequently isolated species, at a rate of 30%. *S. pseudintermedius* is an opportunistic pathogen commonly associated with skin infections (13, 16), otitis externa (4, 13, 16, 18), abscesses, wound infections (4), and urinary tract infections in dogs (4, 18). It is a coagulase-positive staphylococcus, identified as a separate species from *Staphylococcus intermedius* in 2005 (18), and is included in the *Staphylococcus intermedius* group

(SIG) along with four other species: *S. intermedius*, *S. delphini*, *S. ursi*, and *S. cornubiensis* (1, 12).

Staphylococcus aureus is present in both animals and humans and is a commonly encountered bacterium (8). In this study, *S. aureus* was isolated at a rate of 19.6%, with our results aligning closely with those obtained by Hassan et al. (7), where *S. aureus* was isolated at 22.2%. *S. intermedius* was isolated in this study at a rate of 17.4%, which is similar to findings by Diren Sığırcı et al. (5), where *S. intermedius* was isolated at 18.7%, being the most frequently isolated species in that study. In contrast, *S. intermedius* was isolated at a higher rate of 58.8% in the study by Lyskova et al. (10), where it was also the most frequently isolated species. Regarding *S. schleiferi*, it was identified in our study at a rate of 17.4%, differing from the findings of Lee et al. (9), where *S. schleiferi* was isolated at 39%.

The results of bacterial susceptibility testing to antibiotics are shown in Table 1.

Table 1

Antimicrobial Susceptibility of Identified Bacterial Strains

Isolated Species	Antibiotic	Susceptible		Resistant	
		n	%	n	%
***S.pseudintermedius* (*n*=19)**	Penicillin	13	68.4	6	31.6
	Oxacillin	19	100	0	0
	Gentamicin	17	89.5	2	10.5
	Tetracycline	14	73.7	5	26.3
	Ciprofloxacin	19	100	0	0
	Moxifloxacin	19	100	0	0
	Erythromycin	19	100	0	0
	Clindamycin	15	78.9	4	21
	Linezolid	19	100	0	0
	Teicoplanin	19	100	0	0
	Vancomycin	19	100	0	0
	Fusidic acid	19	100	0	0
	Tigecycline	19	100	0	0
	Rifampicin	19	100	0	0
	Trimethoprim + Sulfamethoxazole	19	100	0	0
***S. intermedius* (*n*=8)**	Penicillin	4	50	4	50
	Oxacillin	8	100	0	0
	Gentamicin	8	100	0	0
	Tetracycline	8	100	0	0
	Ciprofloxacin	8	100	0	0
	Moxifloxacin	8	100	0	0
	Erythromycin	6	75	2	25
	Clindamycin	5	62.5	3	37.5
	Linezolid	8	100	0	0
	Teicoplanin	8	100	0	0
	Vancomycin	8	100	0	0
	Fusidic acid	8	100	0	0
	Tigecycline	8	100	0	0
	Rifampicin	8	100	0	0
	Trimethoprim + Sulfamethoxazole	8	100	0	0
***S. schleiferi* (*n*=8)**	Penicillin	8	100	0	0
	Oxacillin	8	100	0	0
	Gentamicin	8	100	0	0
	Tetracycline	8	100	0	0
	Ciprofloxacin	8	100	0	0
	Moxifloxacin	8	100	0	0
	Erythromycin	8	100	0	0
	Clindamycin	6	75	2	25
	Linezolid	8	100	0	0
	Teicoplanin	8	100	0	0
	Vancomycin	8	100	0	0
	Fusidic acid	8	100	0	0
	Tigecycline	8	100	0	0
	Rifampicin	8	100	0	0
	Trimethoprim + Sulfamethoxazole	8	100	0	0

***S. aureus* (*n*=9)**	Penicillin	5	55.6	4	44.4
	Oxacillin	9	100	0	0
	Gentamicin	6	66.7	3	33.3
	Tetracycline	7	77.8	2	22.2
	Ciprofloxacin	9	100	0	0
	Moxifloxacin	9	100	0	0
	Erythromycin	8	88.9	1	11.1
	Clindamycin	5	55.6	4	44.4
	Linezolid	9	100	0	0
	Teicoplanin	9	100	0	0
	Vancomycin	9	100	0	0
	Fusidic acid	9	100	0	0
	Tigecycline	9	100	0	0
	Rifampicin	9	100	0	0
	Trimethoprim + Sulfamethoxazole	9	100	0	0
***S. lentus* (*n*=2)**	Penicillin	2	100	0	0
	Oxacillin	2	100	0	0
	Gentamicin	2	100	0	0
	Tetracycline	2	100	0	0
	Ciprofloxacin	2	100	0	0
	Moxifloxacin	2	100	0	0
	Erythromycin	2	100	0	0
	Clindamycin	1	50	1	50
	Linezolid	2	100	0	0
	Teicoplanin	2	100	0	0
	Vancomycin	2	100	0	0
	Fusidic acid	2	100	0	0
	Tigecycline	2	100	0	0
	Rifampicin	2	100	0	0
	Trimethoprim + Sulfamethoxazole	2	100	0	0

Regarding multidrug resistance (MDR), it was present in 6 strains (13%). The MDR strains belong to the species *S. pseudintermedius*, *S. aureus*, and *Staphylococcus intermedius*. Resistance to oxacillin was not identified in any of the strains.

In the present study, the *S. pseudintermedius* strains isolated from the otic secretions of dogs with otitis exhibited the highest resistance to penicillin and tetracycline. Our results are similar to those obtained in the study by Rosales et al. (17), where *S. pseudintermedius* also showed the highest resistance to penicillin and tetracycline.

In this study, *S. schleiferi* showed 25% resistance to clindamycin, which is somewhat different from the results obtained in the study by Rosales et al. (17), where *S. schleiferi* exhibited 6.3% resistance to clindamycin. Regarding *S. intermedius*, our study found high resistance to penicillin (50%), which is similar to the findings of Zamankhan Malayeri et al. (21), where *S. intermedius* also showed high resistance to penicillin (61.7%). In the present study, *S. lentus* exhibited 50% resistance to clindamycin. In the study by Zamankhan Malayeri et al. (21) *S. lentus* showed 100% resistance to ampicillin, ceftriaxone, oxytetracycline, and penicillin G.

Conclusions

The study reveals a high prevalence of *Staphylococcus* bacteria in dogs with otitis externa, particularly the species *S. pseudintermedius*, which was isolated most frequently (41.3%). This is an opportunistic species, often involved in skin and ear infections in dogs.

Antimicrobial susceptibility testing showed that the bacterial strains exhibited variable resistance to different antibiotics, with high resistance to penicillin and clindamycin.

The emergence of MDR strains complicates clinical treatments. In this study, 13% of the strains were classified as multidrug-resistant, highlighting the need for careful management and continuous monitoring of bacterial susceptibility to prevent therapeutic failures.

The results suggest that antimicrobial susceptibility testing is essential before starting treatment in order to choose the appropriate antibiotic and reduce the risk of resistance development.

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RESEARCH ON THE QUALITY OF TRADITIONALLY CHEESE COMMERCIALIZED IN THE AGRI-FOOD MARKETS LOCATED IN BEIUȘ AND ȘTEI MUNICIPALITIES, BIHOR COUNTY

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Abstract

Cheeses are products made by processing milk obtained from different species, which are highly appreciated by consumers, not only in Romania but also worldwide. The production of traditional cheeses in different regions of our country has a considerable tradition, especially in the south-eastern part of Romania, but also in mountainous areas like Carpathian Mountains. A large part of the cheeses are produced in small-scale units, following traditional recipes, which has made it possible to obtain a diverse number of varieties that are commercialized mainly on the local market. Traditional Romanian cheeses are typically produced using artisanal methods that have been passed down through generations. The quality of these cheeses is influenced by various factors such as milk type (cow, sheep, goat, or buffalo), natural fermentation processes, and specific ripening conditions, often in wooden barrels or caves. Sensory attributes, including texture, flavor, and aroma, are largely shaped by traditional production methods and the use of native microbial cultures. In addition to their distinctive organoleptic properties, these cheeses exhibit notable nutritional benefits, being rich in proteins, fats, and minerals. The present study focuses on analyzing the physicochemical properties and nutritional composition of cheeses, with a particular emphasis on the preservation of traditional methods while meeting modern quality and safety standards. Last but not least, the research also investigates methods for detecting the falsification of cheeses, such as counterfeiting with non-traditional milk types or other non-compliant ingredients. The findings highlight the importance of preserving Romania's cheese-making traditions while ensuring product authenticity, quality, and safety in response to contemporary consumer demands.

Keywords**:** *Romania, food-safety, traditional cheese, dairy product analysis.*

A traditional product is defined as a food item produced within the national territory using local raw materials, free of food additives, and prepared according to a traditional recipe, production method, and/or technological process. Such products stand out from similar items in the same category due to their distinctive traditional elements. Traditionality refers to the unique characteristics or set of features that differentiate a product from other similar items. It is not limited by composition, quality, quantity, or specific production methods established by community or national regulations or voluntary standards, unless these are explicitly set to define traditionality. Furthermore, a traditional product is also considered an ecological product, requiring compliance with environmental protection standards (7, 15).

Traditionally, cheese has always been an important food source, of animal protein, which has sustained human evolution. At the same time, cheese represented a way of preserving and valorizing the milk. The process of making cheese is easy to understand and can be carried out by the uninitiated people, but creating varieties and specialties requires some experience. It all consists in separating the milk protein (casein), which precipitates at high temperatures, under the action of acidifying agents or when the isoelectric pH is reached. Casein is highly digestible and well absorbed in the digestive tract (6, 10).

In the beginning, cheese was obtained by simply coagulating the curds without much intervention or processing. As the diet became more varied, people had to create new types or varieties. Another aspect that determined the diversity of cheeses was the degree of civilization and cultural education of the people (1, 2).

Maturated hard cheese - the beginnings of the cheese-making process are not well known, but it is known that by 300 BC, cheese had already become a more elaborate product. Cheese has different names depending on the country of origin, such as in Albania *Kaçkavall*, in Bulgaria *Кашкавал* (*Kashkaval*), in Serbia *Kačkavalj* and in Sicily *Cascavaddu*. Cheese is made especially from cow's or sheep's milk. Originally produced in Sicily, it is now found all over the world, but mainly in the Netherlands, France, England, Germany, Austria, the Czech Republic, Spain, and through the Balkans to Romania, Bulgaria, Serbia, Hungary, Serbia, Turkey, Greece, Slovenia, Croatia, Greece and, as a result of mass emigration around the 18th century, it can be found in the United States and Canada (1, 4).

Kneaded cheese - depending on the containers in which it is ripened, it has been given several names, such as burduf cheese (stored in sheep skin), cheese stored in the sheeps stomach and bladder, basket or bark

cheese and cheese stored in wooden containers. It is a type of cheese made from curd, cut and mixed with salt, kneaded and left to maturate in the aforementioned containers. The milk used is unpasteurized sheep's milk. After storage in the various containers and maturing, it acquires a soft consistency, a specific aroma and a slightly spicy, sour taste. Recipes differ from country to country. In our country it is specific to the south-eastern part of Transylvania. It has a creamy consistency and is recognized among Nordic peoples and countries such as Germany, Slovakia, Poland and Ukraine (9).

Whey cheese is a milk product, more specifically whey, obtained from sheep's milk but also from cow's milk. It is produced by boiling and precipitating whey proteins (alpha lactalbumin and beta lacto-globulin). Curd is the dairy product with the highest nutritional and biological value, usually made from whey from sheep's milk (which also gives the highest yield), but also from cow's or goat's milk. It is also produced in other European countries such as Macedonia, Bulgaria, Serbia and Ukraine. It can be found under other names like “Orda” or “Zsendice”. In terms of appearance, it is a soft, crumbly, slightly granular dairy product (8, 9).

Telemea cheese is a brine-ripened cheese made from sheep's milk as well as cow's milk. The production process is simple and differs from curd production in that the curds are cut several times to facilitate the draining of the whey After production, telemea cheese is stored to mature in brine, where it can be kept for several months. Of all the types of cheese produced in our country, telemea cheese is the most widespread and appreciated by consumers. This cheese is also known as white cheese or “cheese of Brăila”. According to some authors, the ancestor of cheese in brine is considered to be an Egyptian cheese known since ancient times. Depending on how the milk is processed, different types of “telemea” are produced in different countries: Romanian, Bulgarian, Serbian, Grece. At the beginning, “telemea” was made only in the lowlands from sheep's milk and was a craft product. With the transition to industrial cheese production, the production of cottage cheeses from cow's milk was also introduced (3, 8).

Cottage cheese is made from curdled milk. It is a type of semi-soft, unsalted (sometimes lightly salted) fresh white cheese made from sheep's or cow's milk. After maturing in brine, this curd turns into cottage cheese after 2-3 weeks, and after another maturing process, it can be made into curd cheese or burduf cheese.

Small-scale producers must comply with all hygiene requirements for primary animal-origin products intended for human consumption, as outlined in Annex 1 of Regulation (EC) No. 852/2004 on food hygiene. Direct sales of such products are permitted in markets, fairs, exhibitions, and during events organized for religious or public holidays by local or regional authorities nationwide (14).

Products offered by small-scale producers must meet specific criteria: they must originate from healthy animals, be handled by healthy individuals, remain uncontaminated, not be adulterated, and be transported or presented in clean, suitable packaging. Products must be identifiable with proper labeling, comply with hygiene standards for milking and milk storage, meet raw milk criteria for germ count and somatic cell count, and ensure product traceability. Producers participating in holiday markets or fairs must maintain proper hygiene and wear clean protective gear, including coats, aprons, caps, and gloves (13). Direct and retail sales are permitted only for products originating from facilities registered or authorized by veterinary and sanitary authorities. Such sales may be restricted to specific locations, including the point of production, other registered retail units, or during markets and public events organized by local authorities (11, 12).

Materials and methods

Between May and June 2021, a total number of 15 samples representing different assortments of traditionally produced cheeses from two agri-food markets located in Bihor County were collected. The cheeses were gathered in the form of whole pieces, in a quantity of approximately 250 grams. The varieties monitored belonged to the following categories: sweet cow's cheese, cottage cheese, soft cheese, sweet curd, salted curd, cheese curd, ripened cottage cheese and sweet udders (Fig. 1). Samples were collected in sterile polyethylene bags and individually labeled. The date on which the sample was collected and the assortment were noted on the label. the samples were transported to the laboratory under refrigerated conditions. The samples were transported to the laboratory under refrigerated conditions as soon as possible.

In order to determine the quality, hygiene and authenticity of the assortments, physicochemical and microbiological analyses were carried out. The concentration of sodium chloride, water and the percentage of fat and dry matter were determined together with the detection of counterfeit cheeses made from sheep's milk by the addition of cow's milk. For this determination, the “I.C. Bovino” test was used. The principle of the method is based on

the formation of the antigen-antibody complex, with the production of a colored compound on the test strip. The test is used to detect immunoglobulin G, specific for cow's milk, in cheese declared as made from sheep's or buffalo milk.

The investigations were carried out in several research laboratories of the Faculty of Veterinary Medicine from Timisoara including: the Laboratory of Food Hygiene and Technology, the Laboratory of Interdisciplinary Research Platform and the Laboratory of Microbiological Risk Surveillance in Food located in "Horia Cernescu" Research Complex.

Detection of counterfeit sheep's milk cheeses by the addition of cow's milk. The principle of the method was based on the antigen-antibody complex formation, with the appearance of a colored compound on the test strip. The test was used to detect cow's milk specific immunoglobulin G in cheese declared as made from sheep or buffalo milk. The procedure included the following successive steps (*i*) 5 g of cheese were weighed and 10 ml of distilled water were added and mixed until a homogeneous solution was obtained, (*ii*) the product obtained was centrifuged at 3000 - 3500 rpm for 10 minutes. (As an alternative method the samples can be filtered using filter paper), (*iii*) the resulting filtrate was used for testing. Next, 3 drops of diluted solution were mixed with 1 drop of the resulting supernatant extract from the cheese. When the mixture was complete, a strip was immersed with the arrow pointing downwards into the resulting substance for 5-10 minutes.

Interpretation of the results was made according to the number and color of the bands that appear:

- negative: if only one blue line appeared on the immersed strip;
- positive: if a blue and a red line appeared on the immersed strip.

Determination of water percentage by oven drying technique. The principle of the method is based on exposing the product to a heat source until constant weight. The weight loss, calculated as a percentage, represents the water content. It is recommended that the determination should be carried out on 2 samples in parallel for each sample taken in work. Weigh the two empty vials, numbered in advance. If sea sand is used, the weight includes the sand as well as the short glass rod. From the minced and homogenized sample, place in each vial, approx. 5 g of the product is spread in an uniform layer. Weigh the vial with the product and subtract the weight from this quantity to obtain the exact quantity taken in the sample. It is necessary that the product be put into the vial, spread out in a uniform layer and weighed as quickly as possible in order to avoid loss of water by evaporation during these operations. After weighing, it is no longer necessary to place the vials in the exicator.

After weighing, the vials were placed in an oven, previously heated to 103˚C. The exposure time averaged between 16 to 18 hours. At a certain time interval the samples were removed from the oven, weighed and noted, after which they were reintroduced and maintained (depending on the nature of the product), ½-1 hour. This process was repeated until constant weight was reached. Constant weight is represented by the weight when there the difference between two successive weighings was not more than 0.005 g. The water content of the sample was calculated according to the following formula (5):

$$\% H_2O = \frac{m - m1}{m2} \; x \; 100$$

m = vial weight + the product before drying;
m1 = vial weight + the product after drying;
m2 = the quantity of the product + the product before drying minus the vial weight;

Determination of fat by the acid-butyrometric method. The principle of the method was represented by partial hydrolysis of protein substances using sulfuric acid and the separation of fat with the help of amilic alcohol by heating and centrifugation. The first step was represented by exact weighing of 3 g of cheese, finely crumbled placed into the the butyrometer. The sulfuric acid was introduced through the lower part of the butyrometer and then, the butyrometer was placed on the water bath until the cheese was completely dissolved. After that, one milliliter of amilic alcohol and sulfuric acid were added up to the 35 division from the butyrometer. The butyrometer was then inserted into the water bath. After centrifugation, the amount of fat was read.

The quantity of fat related to the total dry substance (T.D.S) was calculated using to the following formula (5):

$$\% Fat/T.D.S = \frac{G}{(100 - A)} \; x \; 100$$

G = the quantity of fat from the butyrometer;
A = the quantity of water in percentage;

Determination of sodium chloride (NaCl) content. The principle of the method was represented by the direct titration of chlorine ions using silver nitrate solution in the presence of potassium chromate as the indicator (5).

The results were obtained using the

following formula:

$$NaCl\ (g/100) = 0.00585\ x\ \frac{V}{G}\ x\ \frac{V1}{V2}\ x\ 100$$

V = the volume in milliliters of the 0.1 N silver nitrate solution used for the titration;
V1 = total volume of water extract;
V2 = the volume of analyzed extract;
G = sample weight in grams;

Results and discussions

The results of the tests for the presence of cow's milk in cheeses declared as made only from sheep's or buffalo milk, based on the identification of immunoglobulin G, are schematized in Table 1. Following the interpretation of the results, out of the 15 cheese samples analyzed, 7 (46.6%) contained cow's milk. Of these samples found to be counterfeit, 13 (86.6%) were declared as sheep and 2 (13.4%) as buffalo cheese.

Figure 1 shows the I.C. Bovino test for the detection of cheese counterfeiting from sheep's milk by the addition of milk from other species.

Table 1

I.C. Bovino Test Results

No.	Cheese variety	Origin of Raw Milk	I.C. Bovino Test Result
1.	Cottage Cheese	Sheep	-
2.	Salted Cottage Cheese	Sheep	-
3.	Telemea cheese	Buffalo	+
4.	Telemea cheese	Sheep	-
5.	Maturated Hard Cheese	Sheep	+
6.	Telemea cheese	Sheep	-
7.	Telemea cheese	Sheep	+
8.	Telemea cheese	Sheep	-
9.	Telemea cheese	Sheep	+
10.	Telemea cheese	Sheep	+
11.	Telemea cheese	Sheep	+
12.	Telemea cheese	Sheep	-
13.	Telemea cheese	Sheep	+
14.	Telemea cheese	Buffalo	-
15.	Telemea cheese	Sheep	-

Legend: - negative; + positive;

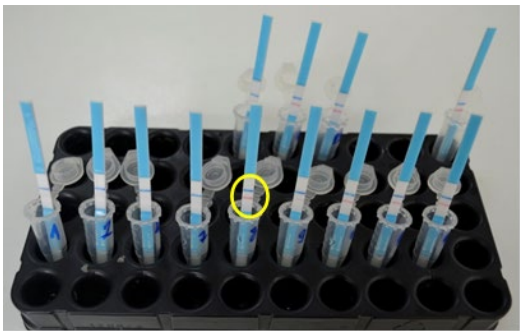

Fig. 1. Positive result of I.C. Bovino test

The obtained results regarding the main physicochemical characteristics of the products are centralized in Table 2. Of the nine types of cheese processed for the determination of sodium chloride, four (44.4%) recorded values above the maximum permissible limit regulated by the standard, and the types that did not meet the standard were: salted cottage cheese and buffalo and sheep's milk cheeses.

In the four samples of sweet cottage cheese, the average water content was 52%, which is within the limits set by the standards. However, only two samples (50%) did not exceed the maximum permissible limit laid down in the standard. Regarding the fat content (percentage) in total dry substances (%F/T.D.S), the average obtained (54.8%) was 10% above the standard value (16).

Tabel 2

Results of the physicochemical composition of cheese varieties

No.	Cheese variety	T.D.S.%	T.D.S.% Minimum Standard Values	Water %	Fat/ T.D.S.%	Fat/T.D.S% Standard Values	NaCl %	NaCl % Standard Values
1.	Sweet Cottage cheese	50.90	48	49.10	64.14	45	-	-
2.	Salted Cottage cheese	40.28	52	59.72	49.65	45	3.73	3.5
3.	Telemea (buffalo) cheese	43.48	48	56.51	56.33	50	4.58	2.5-4
4.	Telemea cheese	49.49	45	51.51	51.55	47	3.55	2.5-4
5.	Sweet cheese	26.01	20	73.99	-	-	-	-
6.	Maturated hard cheese	69.66	52	30.34	30.14	40	1.68	2.5
7.	Telemea cheese	54.86	45	45.13	41.91	47	8.74	2.5-4
8.	Burduf cheese	38.86	52	61.13	69.46	45	2.47	3
9.	Telemea cheese	45.05	43	54.95	49.83	47	2.95	2.5-3.5
10.	Dry cottage cheese	39.43	48	60.57	39.31	45	2.59	3.5
11.	Whey cheese	38.01	32	61.98	39.45	25	-	-
12.	Sweet cottage cheese	38.82	48	61.18	45.07	45	-	-
13.	Sweet cottage cheese	48.06	48	51.94	65.54	45	-	-
14.	Telemea cheese	45.19	43	54.81	45.14	47	4.34	2.5-3.5
15.	Sweet cottage cheese	54.18	48	45.82	44.29	45	0	0

The average water content of the salted cottage cheese samples was 60% compared to the standard value of 48%, significantly exceeding the percentage of water content characteristic for this variety. Regarded the values of %F/T.D.S, for dry cottage cheese was less than 39% and salted curd more than 49%, compared with the minimum value of 45% (17).

In the telemea cheese samples, the average value of water content was 52.6%, compared to the average standard value of 66% for this variety. The %F/T.D.S. content averaged 49% compared to the standard value of 47%. The highest value (56.3%) was recorded for telemea cheese (from buffalo milk) among all the assortments studied (16, 17).

The water content of the sweet cheese was 74%, which is within the maximum permissible limit (80%) in the standard for this variety. The acidic-butyrometric method did not reveal any detectable % F/T.D.S. content, which meant that the cheese could be classified as a low-skimmed sweet cheese (16).

The maturated hard cheese showed a lower water (30.4%) and fat (30.1%) content compared to the standard values (48% and 40% respectively) (16, 17).

Compared with the standard values, the

water content (61.3%) and the %F/T.D.S. (69.5%) of the burduf cheese were higher (16, 17).

The water content of whey cheese showed a lower value (62%) compared to the standard value (68%) and a higher percentage in %F/T.D.S. (39.5%) compared to the standard value of 25% (16, 17).

Conclusions

The present research conducted on traditionally produced cheeses sold in the agri-food markets of Beiuș and Ștei municipalities underscores the rich diversity and cultural significance of Romanian cheese-making traditions. However, findings reveal notable challenges in maintaining product authenticity and quality. Instances of adulteration, such as the addition of cow's milk to cheeses labeled as sheep or buffalo milk, compromise the integrity and nutritional value of these products. Physicochemical analyses further highlight deviations from standard values, particularly in water content, fat percentages, and sodium chloride levels across various cheese types. These discrepancies suggest a need for enhanced quality control measures to preserve the artisanal heritage while ensuring consumer safety and satisfaction. Future initiatives should focus on stricter regulatory compliance, improved testing methodologies, and educational efforts to safeguard the legacy and market viability of traditional Romanian cheeses.

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CASE REPORT: HYPOSPADIAS IN TWO GOAT KIDS

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Abstract

Congenital malformations in animals are a major concern for farmers and breeders due to the increased economic loss that these pathologies produced. Hypospadias, disorder of sex development, is a rare congenital anomaly that occurs when the male urethra opens on the ventral side of the penis, or on the perineum, and can be associated with failure to develop other male genital parts, such as penile aplasia, penile hypoplasia, incomplete formation of the prepuce or even cryptorchidism. In this report, we described two cases of hypospadias seen in goat kids of two and a half months old. The cases were documented in April 2021, in a herd of 300 goats, located in Alba County, Romania. On the andrological examination, the goat kid from case no 1 presented with perineal hypospadias and the goat kid from case no 2 presented with scrotal hypospadias. In both cases, hypospadias was associated with abnormal growth length of the body of the penis, hypoplasia of the penis, and incomplete development of the prepuce.

Keywords: *hypospadias, disorder of sex development, congenital malformation, goat kid.*

Congenital malformations are rare and unfortunate cases, that can be caused by genetic factors, environmental factors, or a combination of them (5). One of the most common congenital defects of the male reproductive and urinary system, both in humans and animals, is called hypospadias (4). Placing second after cryptorchidism, hypospadias (OMIA 001187-9913) is a genital development abnormality, which results from the failed fusion of the urogenital folds, causing the male urethra to open on the ventral side of the penis or on the perineum. It can be associated with failure to develop other male genital parts, such as penile aplasia, penile hypoplasia and incomplete formation of the prepuce (7, 11). Depending on where the urethra opens, hypospadias can be classified as glandular/balantic, penile, on the proximal, middle or distal part of the penis, scrotal, perineal and even near the anus. The most severe cases are the posterior positions, precisely scrotal, perineal and anal locations (6, 7, 8, 16). The schematic representation of the different types of hypospadias are seen in Figure 1.

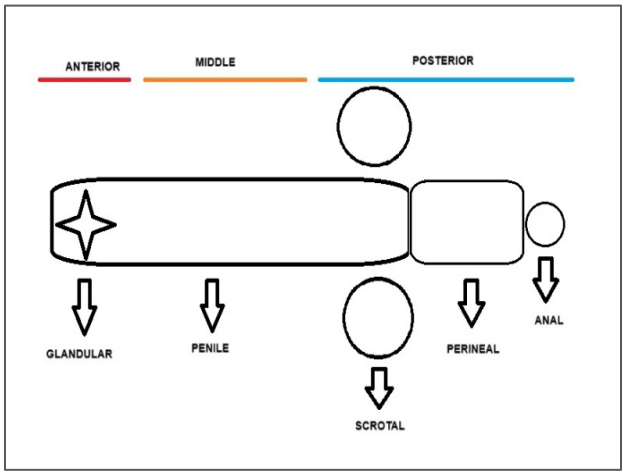

Fig. 1. A schematic representation of the classification of hypospadias (orig.). The degree of severity of hypospadias is given by the position of the urethra opening, from mild hypospadias in the anterior and middle region to severe hypospadias in the scrotal, perineal or anal regions.

Among animals, hypospadias is rarely found. For farmers and breeders is a major concern when a case is found, due to the increased economic loss that these pathologies produce. Species that were diagnosed were small and large ruminants, most frequently goats, sheep's and cattle's, dogs, cats, horses, rams and pigs (7, 13). It was also reported to be found among wild animals like polar bears and rhesus monkeys (13).

In human medicine, this congenital anomaly is a serious topic, since it affects approximately 1 in 150 - 200 newborn males

(3, 8). Also, patients with hypospadias, confront psychological problems, like mental retard, and physical difficulties with urination and sexual functions (3, 6). Hypospadias International Society (HIS), together with other organizations, help and successfully treat patients with this anomaly, thanks to the dedication and implication of doctors and researchers, that annually meet up and find new alternatives to cure this congenital disease (17).

Understanding congenital deviations in the genitalia region requires understanding the embryogenesis of the genital tract. Thus, during the gonadal development, there are primordial germ cells (PGCs) that migrate into the gonadal primordium from the yolk sac. PGCs become a small cluster of cells in the extraembryonic mesoderm, near the allantois, and after that, they shift to a site in the mesoderm. Migrating in the wall of the hindgut and through the dorsal mesentery, the cells will reach a newly formed genital ridge (day 22 in sheep and goats). Developing normal male genitalia, includes the existence of specific and important hormones, like Müllerian inhibiting substance (MIS) (15).

Etiologically speaking, hypospadias seems to be multifactorial, including genetic, environmental and endocrinological factors (10). Studies showed that between 10–30% of human hypospadias cases are attributed to specific gene mutations, for instance, *AR* gene mutation and *SRD5A* type II deficiency, while 70% of cases possibly have a combination of genetical and environmental factors. (1, 14). Many genes (*SRD5A2, ATF3, MAMLD1*, *MID1, BNC2, HSD17B3, WT1, SF1, BMP4, BMP7, HOXA4, HOXB6, FGF8, FGFR2, AR*, etc.) are involved in the development of male external genitalia, that is why different mutations and changes in them could cause hypospadias (1, 2, 14, 16). Moreover, environmental factors were noted in the etiology of hypospadias, that affect the mesenchymal-to-epithelial process and disrupt the hormonal cascades during urethral development. Some endocrine disrupting compounds are organic solvents, atrazine, bisphenol A, pesticides and phenol. Also, studies show that maternal exposure to different hazardous air pollutions can be a hypospadias risk for the fetus (8, 11). Endocrinologically speaking, hypospadias can be caused by disfunction of the androgen synthesis, characterized by a lack of Müllerian structures regression. Enzymes involved in the production of testosterone and dihydrotestosterone starting from cholesterol, are identified in 46, XY disorders of sex differentiation. One of the enzymatic steps involves the steroidogenic acute regulatory (STAR) protein and p450 oxidoreductase (POR), causing the cleavage of cholesterol to pregnenolone. Pathogenic mutations on the gene or enzyme can produce congenital adrenal hyperplasia, which furthermore can be associated with hypospadias (2, 12).

This study is designed to present the clinical findings of hypospadias in two documented goat cases in Romania, in Alba County.

Materials and methods

The reported cases were discovered in April 2021, in a herd of 300 Carpathian goats, located in Alba County, Romania. The subjects were perceived by the caretakers as females at birth, even though over time the persistence of urine around the ventral perineum was noticed. No additional observations were made at the time. The diagnosis of hypospadias was made when they were around 3.5 months during slaughter. In both cases, the testicles were located just outside the abdomen, and there was an absence of female reproductive organs, which ruled out the possibility of hermaphroditism.

From an andrological standpoint we can observe an incomplete and improper development of the penile and the prepuce as well as an open perineal urethra. The examination of the scrotum revealed the presence of two normally developed testes for the age of the animal, with the location slightly altered and the absence of scrotal sacs. The urethra opened close to the anus with urethral folds deepened over the site of the opening alongside the penial gland developed forming structures that superficially resemble a reverted vulva. The Case no 1 (brown and white male goat) is presented in Figure 2.

Fig. 2. Clinical phenotype of the Case no 1. The brown and white male goat kid showed a perineal hypospadias. The urethra (urethra opening marked with an arrow) opened about 5-8 cm ventrally the anus (marked with a circle). The testes (marked with stars) were in the inguinal region

The case no. 2 (white male goat) is presented in the Figure 3. In this second case, similar as the one prior had the same improper development of the penile and urethra, but in this case the opening of the urethra was located closer to the scrotal area. Upon closer examination of the scrotal area, it was observed that the testes were present and positioned as expected.

Fig. 3. Clinical phenotype of the Case no 2. The white coated male goat kid with scrotal hypospadias (urethra opening marked with an arrow and testes marked with stars)

Aside from the absence of the scrotal sacs, which were not developed, the testes located subcutaneous appeared to have developed normally, showing no signs of abnormalities. The development of the penial gland, located at the opening of the urethra, displayed characteristics that made it resemble a vulva on the surface, despite being anatomically part of the male reproductive system.

Results and discussions

The cases of the two goat kids are rarely found in the animal world. Both cases are particularly interesting because they have severe types of hypospadias and other genitalia malformations. Due to not having any severe complications with urination, the two goat kits were able to live a decent life for 3.5 months.

Looking at the first case, the brown and white coated male goat kid had the most severe form of hypospadias. First of all, the urethra opening was located on the perineal part of the genital area, 5 to 8 cm close to the anus. The penis was undeveloped and mislocated. Normally, the penis together with the urethra should be located anterior to the testes, close to the umbilical area. Moreover,

the testes were normally developed, but the scrotum was not formed. That way the testes were only covered by an epithelium, that possibly couldn't offer the right conditions to have normal functional testes. The prepuce was also improperly and incompletely developed.

On the other hand, the second case, with the white coated goat kid, was considered severe as well, due to the posterior location of the urethra opening, specifically on the scrotal area. The testes were normally developed but without the formation of the typical scrotum. The penile gland was also underdeveloped but located between the testes. The difference between the two goats was mainly regarding the location of the opening of the urethra, one being on the perineal part and one on scrotal area.

Both hypospadias cases are severe and rare, which makes the treatment and the management of the goat kids unattainable. Unfortunately, these cases ended with the scarification of the two goat kids.

However, many types of instances of hypospadias have been documented, not only among animals, but humas as well. As a matter of fact, this disorder has raised the curiosity of many researchers, who successfully managed to come up with a detailed study of hypospadias.

Hypospadias (OMIA 001187-9913) is a male congenital disorder, a genital development abnormality, which results from the failed fusion of the urogenital folds, causing the male urethra to open ectopically on the ventral side of the penis and can be associated with failure to develop other male genital parts, such as penile aplasia, penile hypoplasia and incomplete formation of the prepuce. The etiology of the disorder is multifactorial, hence it includes genetical, endocrinological and environmental factors.

Depending on where the urethra opens, hypospadias can be classified as glandular/balantic, penile (on the proximal, middle or distal part), scrotal, perineal and even near the anus. Another commonly used classification as follows: anterior (sub coronal and glandular), middle (distal, proximal penile and midshaft), posterior (scrotal, perineal and anal). 50% of the cases are located on the anterior part of the penis and are the minor and easy cases; 20% have a middle location and the rest are posterior, which are rare and the most severe cases (18).

The primary sign of hypospadias is the abnormal placement of the urethral opening. It may be positioned anywhere along the ventral surface of the penis, from near the base to closer to the scrotum, depending on the severity of the condition. In some cases, the goat may experience difficulty urinating normally, especially if the urethral opening is located near the scrotal area. Urination may be weak, redirected, or splashed in different directions due to the abnormal positioning. Additionally, the penis may appear shortened, curved, or malformed, and in more severe instances, it may be underdeveloped or incomplete (13).

The two goats, from the presentation, had the urethral opening located on the penile gland, with the opening positioned in the perineal area, in one case, and in the scrotal region in the other. The penis appeared shortened and showed signs of hypoplasia, presenting traces of urine on the fur in the area. In some cases, the penile gland appeared to function like a sphincter, causing pressure to build up inside the urethra during urination. Or in other cases, openings on the urethra which not communicating with the exterior can also be present. This resulted in urine leaking into the subcutaneous tissue of the ventral abdominal wall and prepuce, leading to skin damage, compromising tissue integrity and causing persistent discomfort to the affected animals. In this cases a surgical intervention is necessary to assure drainage and reconstruct the urethra, preventing further damage and discomfort to the animal. The males are also withdrawn from breeding and castrated (9, 10).

The treatment in animals with hypospadias is generally surgical. Many species like dogs, cats, goats, sheep, cattle, horses and even wildlife animals were treated with surgical intervention regarding the opening of the male urethra. It is essential to know that mostly anterior and midshaft cases of hypospadias are easily treated, due to the fact that they are manageable and minor cases. Posterior location is the most sever type of hypospadias, thus it can't be treated surgically. In farm animals, most cases end with the animal being culled.

More elaborated surgical technics and reconstructions take place in cases affecting humans, trying to ensure as close to normal sexual development as possible. That is why many organizations and the Hypospadias International Society (HIS) have come up with successful chirurgical treatments and support for those who suffer from this congenital disorder (17).

Conclusions

To conclude, hypospadias is a rare congenital disorder of male sex development, that occurs when the urethra opens on the ventral side of the penis, scrotum or even perineum, and can be associated with failure to develop other male genital parts, such as penile aplasia, penile hypoplasia, incomplete formation of the prepuce or even cryptorchidism. It has multifactorial etiology, which includes genetical, endocrinological and environmental factors.

The two cases documented and presented in this article describe severe types of hypospadias. The brown and white coated goat kid (Case no 1.) presented perineal hypospadias, with the opening of the urethra at few centimeters away from the anus, and the white coated goat (Case no.2) had scrotal hypospadias with the testes located subcutaneous. Both came with abnormal development of other genital parts, like abnormal growth length of the body of the penis, hypoplasia of the penis, and incomplete development of the prepuce.

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RESEARCH ON CANINE POPULATION MANAGEMENT IN GORJ COUNTY, IN THE PERIOD 2013-2023

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Abstract

In Romania, the ever-growing number of stray dogs has become a well-known and current problem of the modern population, although its roots date back for at least three centuries. Even if in our times, the majority of people tend to live in large urban agglomerations, we seem to not be able to escape the attention of our "best friend", the dog. The present study proposes to deepen in the the problem of stray dogs in Romania, to review the current legislative framework related to the welfare of pets, with or without an owner, but also to observe the application and effectiveness of methods aimed at combating the problem of the number of stray dogs in Gorj county, by the local public forces, in parallel with the animal protection associations that exercise their activity on the territory of the county and the Municipality of Târgu-Jiu. Data regarding the numbers of stray dogs, in the 2013-2016 period, in the county was collected during in-person visits at the Târgu-Jiu townhall and from the local and national press in order to asses the situation, the last known number being 2000 stray dogs in the year 2016. A guide of animal welfare, based on specific indicators was redacted and applied in order to evaluate the living condition of dogs accomodated in a private *“Open-Shelter*” type animal shelter, located in Gorj County. The guide was applied three times during the study, each time a number of 80 dogs were being evaluated, for a total of 240 diffrent dogs. In coclusion, the study highlights the diffrence between the management of the stray dog population between the local administrative forces and private animal protection associations.
Keywords: *stray dog, legistaltive framework, animal protection, welfare*.

The first definition of welfare was proposed by the Universal Declaration on Animal Welfare, drafted in Boston, USA by the World Organization for Animal Health (WOAH). The first part of the definition refers to the lexical, explanatory type and defines welfare as "The degree to which the animal's physical, behavioral and psychological requirements are met". Currently, this first part of the definition has acquired a new form, found within the general considerations of Terrestrial Animal Health Code’s chapter 7 drafted by WOAH: "Animal welfare means the physical and mental state of an animal in relation to the conditions in which it lives and dies". The second part of the definition introduces the normative concept of the "five freedoms", recognized by the World Veterinary Association in the 44th General Assembly in Paris in 1992, which must be ensured simultaneously:

• freedom from hunger, malnutrition and thirst (by ensuring access to fresh water and specific food);

• freedom from heat stress or physical discomfort (by providing an adequate environment, including comfortable shelter and rest);

• freedom from pain, injury and disease (through prevention of pain, injury, rapid diagnosis and treatment of disease);

• freedom from fear and distress (by eliminating fear and mental suffering);

• freedom to express normal behavior (by providing the space, facilities and company necessary for the expression of normal behavior) (17).

Romania’s stray dog problem is well known and cited since before its War of Independence, the first known written record being in 26 November 1876. Since then, the legislative framework has undergone many changes, along with the ruling regime of the country. The backbone of the legislative framework was first drafted in 2001, through the 155 Emergency Ordinance of The Government of Romania, being adapted into law later, in 2002. The effects of this law will shape the future of stray dogs in the country for the next 23 years.

The Emergency Ordinance of 2001 places the responsibility of stray dog population management on local councils, who were obliged to set up services to deal with the growing problem of stray dogs. In the shelters belonging to the associations and foundations for the protection of animals, stray dogs can only be accommodated after the sterilization, anti-rabies vaccination and tattooing procedures were done by veterinary medical services. Stray dogs will be captured and transported to shelters, where they will be housed for a period of up to 14 days, except for aggressive, chronically ill and incurable dogs (22). These last categories will be euthanized as soon as they were brought to the shelter. Euthanasia was defined and performed exclusively by the veterinarian and only by administering a lethal injection of barbiturates after anesthesia. The obligation of euthanasia rested with the local councils (19).

In May 2004, the Parliament adopts the Framework Law 205 on the protection of animals. With the purpose of ensuring the living conditions and well-being of animals with or without owners, the normative act regulated the situation of all animals, establishing a series of rights and obligations both for their owners and for the state authorities. Analysis of the content of Law no. 205/2004 highlights a series of obligations for animal owners. Compliance with the sanitary and veterinary rules regarding housing, feeding, care, reproduction, exploitation, protection and welfare of animals was required, abandonment was prohibited and the owner's obligation to properly care for and treat a sick or injured animal was established. They have the obligation to behave without brutality towards them, to provide them with appropriate shelter according to their ethological needs, breed, sex and age. It is forbidden for a dog owner to apply treatments such as: beating, strangulation and other such cruelties. Ill treatment and cruelty to animals was defined and criminalized, introducing fines and in specific cases prison terms from 3 months to a year (20).

In 2013, "Ionuț's Law" was adopted, after the death of a four-year-old child, the latest law concerning the stray dog problem of Romania. The public services for the management of stray dogs at the local level, as well as the shelters belonging to the organizations and foundations for the protection of animals, have the obligation to communicate monthly to the sanitary-veterinary and food safety departments of the county and the municipality of Bucharest the number of registered dogs and the number of microchips or the alternative means of identification, established by the National Veterinary Sanitary and Food Safety Authority (ANSVSA). All data are centralized at national level by the National Veterinary Sanitary and Food Safety Authority (21).

During the years 1990-2020, several authors (3, 4, 5) published studies carried out in Europe and the USA that demonstrate the predominance of dogs that have owners when it comes to cases of aggression. However, the risk associated with the bites and scratches of stray dogs must also be taken into account, especially the risk of spreading the rabies virus to people, in public spaces in cities or villages. According to the WHO, more than 29 million people are vaccinated annually against rabies worldwide, with children between the ages of 5-14 being the most affected. The annual cost of global rabies prophylaxis amounts to 8.6 billion dollars per year (15, 16).

In Romania the situation is just as worrying. According to the statistics made in 2023 in Bucharest, over 3,500 people bitten by dogs arrived at the "Matei Balș" anti-rabies center, of which over 1,000 were attacked by stray dogs (8). At the beginning of 2024, over a period of three weeks, more than 150 people were bitten by dogs, most of them being owned dogs. It is estimated that every day, between 10 and 15 people bitten by dogs arrive at the Anti-Rabies Center (13). In 2012, the Matei Balș Institute reported over 16,000 people bitten by dogs, of which 3,330 were children (9). In 2013, between January and August, over 10,000 cases of bite wounds were recorded (Table 1). At that time, a dose of anti-rabies vaccine cost 68 lei, a dose of anti-rabies serum 200 RON, and a dose of tetanus vaccine, 49 RON. The costs of the treatments amounted to 2.4 million RON, according to *Ziarului Financiar* (18). Equivaling in 2024, this amount would be equal to 4.1 million Romanian RON (824,086 EUR).

Regarding the severity of dog attacks in our country, it appears that, in the vast majority of cases, the bites were superficial and would have remained without consequences if the fear of rabies and tetanus had not necessitated specific anti-rabies and anti-tetanus prophylaxis. Approximately 1% of the attacks were more severe, and some even fatal, typically caused by dogs specifically trained to attack unfamiliar individuals who enter a particular area or approach certain persons or objectives (2).

Table 1

Number of people bitten by dogs in 2013, Bucharest

Month	Adults	Children	Total
January	912	141	1053
February	855	129	984
March	975	188	1163
April	1038	311	1349
May	1117	339	1456
June	1010	304	1314
July	1010	281	1291
August	868	281	1150

Materials and methods

In order to carry out this study, two diffrent time periods were created: the 2013-2023 period or the "observation period" where information and data was compiled from official government websites, visits at Târgu-Jiu's townhall, local and national articles regarding stray dogs and the 2019-2023 period regarding analyzation of canine population management in Gorj county. The following steps were undertaken in the second time period: collection of information regarding the management of the canine population in the municipality of Târgu-

Jiu, drafting of welfare assessment guidelines for stray dogs accommodated in shelters, the study of a private "open-shelter" and application of the welfare assessment guidelines in the same „open-shelter".

To achieve this goal, the official website of the Târgu-Jiu City Hall was accessed, where several Decisions of the City Council were obtained regarding the control of the stray dog population, the management of pets and the city's accession contract (12) to the Association for Intracommunity Development (A.D.I.), "*County service for the protection of stray animals*".

Following the practice carried out at a private *„Open-Shelter"* located in Gorj county, in 2019-2022 and the various subsequent visits in the period 2023-2024, enough information was collected in order to be able to understand the contribution that the staff employed in carrying out the canine population management program, as well as assessing the well-being of stray dogs housed in the shelter. Assessment of dog welfare housed in the shelter was made based on a guide containing specific indicators.

Data was also collected from social networks, from the official website of the shelter and from local and national press, in order to be able to appreciate the effectiveness of the canine management programs implemented in Gorj County and Târgu-Jiu Municipality by local authorities as well as their collaborations with the Animal Protection Associations in the county and outside it.

When talking about farm animals (dairy cows, beef cows, pigs, laying hens, etc.), complex and complete guides have been drafted that are internationally recognized (Ex: Welfare Quality Assessment Protocol) (14), which organize the important indicators in the evaluation animal welfare. Even if there is no official guide, there are studies that describe the behavior of stray dogs but also the risk factors that can appear in dog shelters (1).

The evaluation guide is made up of several indicators:

1. Evaluation indicators at shelter level (Table 2);
2. Evaluation indicators at pen level (Table 3);
3. Evaluation indicators at individual level.

Table 2

Evaluation indicators at shelter level

SHELTER LEVEL	
Evaluator name:	Date:
Shelter name:	No. dogs hospitalized:
No. housed dogs:	No. housed dogs (last year)
No. adopted dogs (this year)	No. adopted dogs (last year)
No. resheltered dogs (this year)	Temperature, humidity (T°, UR):

Table 3

Evaluation indicators at pen level

PEN LEVEL	
No. pens single capacity:	No. pens pair capacity:
No. pens <5 dogs capacity:	No pens >5 dogs capacity:
Total number of pens:	
EXERCISE	
Dogs get exercise:	□Daily (> 3 h)□ Daily (< 3h) □ Weekly □No
Are dogs walked on leash by shelter personnel or by volunteers	□Daily □ Weekly □ No
TRAINING AND REHABILITATION	
Presence of personnel trained for training with dogs	□ Yes □ No
Presence of personnel specialized in the rehabilitation of problematic dogs	□ Yes □ No
SURGERIES / PAIN CONTROL	
Presence of hospital pens	□ Yes □ No
Presence of operating procedures for post-surgical monitoring	□ Yes □ No
Presence of protocol of analgesia	□ Yes □ No
MORTALITY	

No. eutahasia caused by health problems (last year)	No. deaths (other than euthanasia):
No. euthanasia caused by behaviour problems:	No. aggressive dogs:
FEEDING	
Diet type: □dry pellets □ cooked □ wet/canned	Feeding regime: □once/daily □ twice/daily □ ad libitum
Special diets for puppies □ Yes □ No Special diets for geriatrics □ Yes □ No	Special diets for hospitalised dogs □Yes □ No
NOTES	
Time of assesment start____h	**Time of assesment end_____h**

The evaluation of **indicators at the individual level** refer to the general condition of the housed animals, the dogs' reaction to humans, body mass index (BMI) and the animal's body hygiene (the presence of certain dermatitis and skin lesions) as well as the presence of lameness.

Results and discussions

With the accession to the European Union, the obligation to comply with the principles of the European Convention for the Protection of Pets and Leisure Animals, signed in Strasbourg in 1988, was established in Romania. Since then, the country has transposed into the national legislation several laws, normative acts and government decisions that are concerning the welfare of all animals, with or without an owner. However, when it comes to compliance with the law, there are important differences between the country's cities, and especially regarding the effectiveness of local stray dog management programs. The canine population management program in the municipality of Târgu-Jiu was carried out taking into account the guidelines (17) developed in Geneva by the World Health Organization (WHO) and the World Organization for Animal Health (OIE).

According to the statement given by the director of the Gorj Animal Protection Directorate, the number of stray dogs in the county is currently unknown, a problem that first appeared in 2016. Using the information available from the local press, the municipality of Târgu-Jiu was identified as the most affected city in the county regarding the problem of stray dogs. Using the public data provided by the various associations dealing with the management of stray dogs and official press statements of local administration representatives, we can make an estimate of this number (Fig. 1).

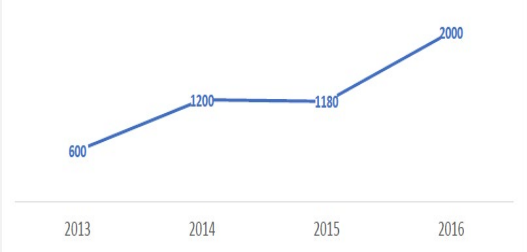

Fig. 1. Stray dogs in the Municipality of Târgu-Jiu, 2013-2016 period

In Gorj county there are five public dog shelters that collaborate with each other, with the aim of streamlining the management of the number of dogs. The municipality of Târgu-Jiu hosts the largest shelter in the region, with a maximum accommodation capacity of 400 dogs. Rovinari city hosts the second largest public shelter with a capacity of 200 dogs, Motru city's shelter capacity is of 70 dogs and the smallest ones are in the cities of Matăsari and Turceni, with a capacity of 40 and 50 dogs, respectively. In addition to public dog shelters, Gorj county enjoys the attention of several animal protection associations that house dogs in private shelters, organize free sterilization campaigns, offer dogs for adoption and inform the public on matters of interest.

The management of stray dogs in Târgu-Jiu was carried out in the past entirely by the *Societatea Edilitara Public* (10), which in addition to many other activities in the public domain, also had the role of overseeing the public dog shelter (Fig. 2), to look after the housed dogs, to capture and sterilize stray dogs and to give them up for adoption. Due to the very diversified activities carried out by the *Societatea Edilitara Public*, the segment of managing stray dogs has lagged behind, the public shelter being permanently at maximum accommodation capacity.

Fig. 2. Targu-Jiu's public shelter managed by SC. Edilitara

The situation of stray dogs could not be completely managed by the local authorities, thus, in 2023, by a decision of the local council and contract *No. 203210/19.06.23*, Târgu-Jiu Municipality joins *Asociația de Dezvoltare Intracomunitară (A.D.I.) "County service for the protection of stray animals"* (7, 12*)*, the service for managing stray dogs in Hunedoara county, based in Deva city. According to the contract, the service is obliged to carry out the catchings of stray dogs and the transport to shelters where they will register, consulted, sorted and housed. Also, they will be microchipped, dewormed, vaccinated, sterilized and registered in a unique record.

The association is contractually obliged to feed and care for the housed dogs, and at the request of local communities or groups, which assume responsibility in writing for the protection of the dogs, to release them, but not to let them loose on the public domain. The services will be kept in place as long as the Municipality of Târgu-Jiu is a member of A.D.I. The Association assumes the right to euthanize aggressive dogs or the ones affected by incurable diseases but also the unclaimed or unadopted dogs after 14 days, an activity preceded by a full veterinary consultation. The activity has to be carried out by a veterinarian using the substances permitted by law, provided that they do not cause animal suffering.

The Association started its activity of capturing stray dogs in September of 2023. On February 4, 2024, the deputy mayor of Târgu-Jiu municipality announced that over 700 dogs have already been captured of which, 190 were placed in the local paddock. In the January-August period of 2024, the Association has captured over 1400 dogs.

The "Pro Animals Romania" association, established in 1999, began its operations in 2000 and is unique to Gorj County. Among capturing and caring for stray dogs, the association also manages its own shelter and part of the city's public shelter. In 2023, it reported a number of over 1,500 dogs housed in the managed shelters (11). One of the most important activities done is the organization of free sterilization campaigns for dogs and cats. In 2016, through funds received from the donations of Germany, Finland and Romania's citizens, the first mobile veterinary clinic in the country was purchased (Fig. 3), equipped with apparatus necessary for surgical interventions, its purpose being to reduce the number of stray animals (6).

Fig. 3. Pro Animals Association's mobile clinic

Help from outside the country continued in 2019, when an animal protection association, established in Germany, began its activity near Târgu-Jiu city. Its purpose is to capture stray dogs from Gorj county cities, villages and countryside, to treat them in the association's „open shelter" type private shelter and finally, to give them all for adoption, in Romania or in Germany, no matter how long the process may be, euthanasia being done only by necessity. From its establishment until now, according to the data published on the association's official website, 1138 dogs have been adopted. The association includes a number of 37 members, of which 10 work in Romania and 27 in Germany.

When talking about the number of adoptions, data recorded from the winter of 2023 to the summer of 2024 reveal a slight increase in the number of adoptions carried out. In the January-June period of 2023, 41 adoptions were carried out (out of a total of 168), compared to the same period of 2024, where up to 43 adoptions have been currently made. According to the data from the shelter's registers, in 2022, 268 adoptions were made, in 2021, 408 of the housed dogs were given for adoption and in 2020, 342 were adopted. In the year of the association's debut (2019), it managed to give 61 dogs for adoption (Fig. 4).

The start of the project coincided with the onset of the pandemic caused by the new

SARS-CoV-2 coronavirus, the moment being favorable for a shelter that relies on funds coming from the donations of adopted dogs owners. One can see the rapid increase in the number of adopted dogs from 2019 until 2022, the end of the state of alert in Romania, when the number decreased drastically, more than halving. In the future of the shelter, these values are expected to be capped, as indicated by the similar number of adoptions in 2024, equal to that of 2023.

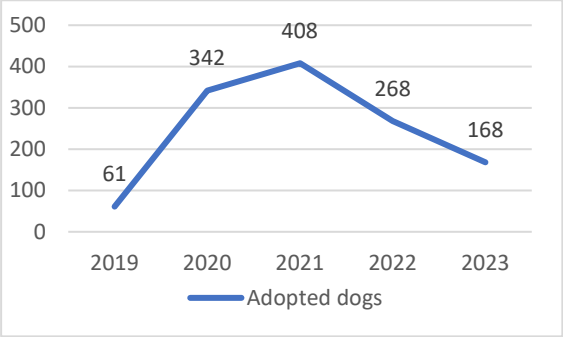

Fig. 4. Adopted dogs in a private shelter located in Gorj county, 2019-2023 period

In order to evaluate the well-being of the housed animals, visits were made between December 2023 and June 2024 and following the drafted guide, data of interest was collected and photo-video recordings were taken in order to capture the different behaviors of the housed dogs. The study was carried out on a total sample of 240 dogs, with 80 dogs from the shelter being observed at each trip. Using the newly drafted guide, following the 2023-2024 visits, regarding the shelter evaluation indicators, and the evaluation indices at the level of the pens and at the individual level, the following results can be found in Figure 5 and 6.

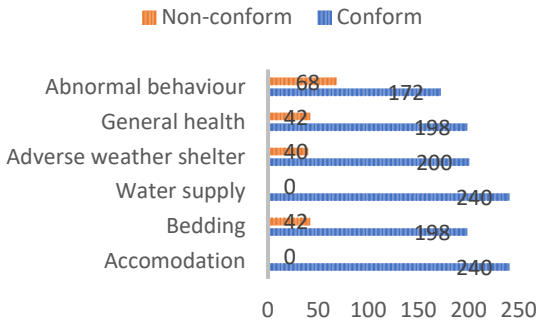

Fig. 5. Indicators at pen level

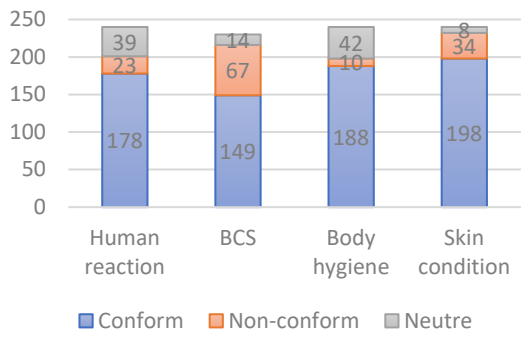

Fig. 6. Indicators at individual level

Conclusions

In Gorj county, the stray dog population management program is extensive, various animal protection associations being present, such as the Association for Intracommunity Development, the “Pro Animals” Association, but also international associations that manage the Open Shelter present in this study. Despite all these efforts, the problem of stray dogs is still present.

When it comes to animal welfare in shelters, staff interest, available funds and public opinion are key elements. In the private open shelter, the special care for animals can easily be noticed. At shelter level, hygienic conditions and well-maintained infrastructure ensure a healthy and safe environment for all dogs. The pens are spacious, clean and equipped with facilities that allow movement and play, contributing to the physical and mental health of the animals. On an individual level, each dog receives personalized attention, quality veterinary care and socialization activities, increasing their chances of adoption. Thanks to these measures, the shelter not only offers a temporary refuge, but also a real chance at a better life for the stray dogs housed.

Public shelters, whose activity is communicated extremely rarely and only through negative articles in the local press, have been shown to almost completely neglect the condition of the animals housed. On the contrary, in our study’s private shelter, everyone has access to information of interest, published on social networks, official websites or positive articles, the housed animals enjoy a high level of welfare.

The concept of welfare and protection of all animals must become common in people's consciousness.

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RESEARCHERS ON THE ISOLATION AND PRELIMINARY IDENTIFICATION OF SOME STRAINS OF *MYCOBACTERIUM SPP*. FROM CATTLE

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Abstract

Tuberculosis infection is found in all vertebrate species, but it causes economic losses especially in cattle, due to the necessary cuts, the uneconomical exploitation of animal productions, as well as expenses for combating the disease. Besides the economic importance of tuberculosis in cattle, the disease is also important for public health, being a zoonosis. Bovine lymph node samples from slaughterhouses where tuberculin-positive cattle were slaughtered were studied and analyzed. The bacteriological examination followed the isolation and preliminary identification of *Mycobacterium spp*. A number of 13 strains of *Mycobacterium spp*. were isolated. The strains of *Mycobacterium spp*., isolated were sent for confirmation and phenotypic and genetic identification (PCR) to IDSA. 12 strains of *M.bovis* and one strain of *M.caprae* were identified.

Keywords: *Mycobacterium spp., cattle, bacteriological examination, isolation and preliminary identification.*

Bovine tuberculosis is a chronic infectious disease caused by pathogenic members of the *Mycobacterium tuberculosis* complex (11, 16, 18, 21). Recent genomic analyses suggest that all *Mycobacterium tuberculosis* complex members belong to a single species – *M. tuberculosis*, with *M. africanum, M. bovis, M. caprae, M. microti* and *M. pinnipedii* considered heterotypic synonyms (variants) of *M. tuberculosis*; *and M. canettii, M. mungi,* and *M. orygis* recognised as strains of *M. Tuberculosis* (18, 20, 22). Tuberculosis also affects birds, predisposing them to other infections (11, 12, 17, 18). Bovine tuberculosis requires permanent surveillance, given the economic importance of this disease, but also the zoonotic risk that the disease presents (7, 20, 22).

Epidemiological surveillance in bovine tuberculosis consists of performing the allergic test, and among the reacted cattle they are sent to the slaughterhouse for control slaughter, in order to establish the presence of the specific infection, produced by *Mycobacterium spp.* (1, 6, 8, 9, 14).

From the macroscopic lesions, from the cows slaughtered for the control of tuberculosis, two *Mycobacterium spp. (Mycobacterium bovis* and *Mycobacterium caprae*), capable of producing morphopathological changes, were isolated (1, 2, 5, 6, 20, 22).

Bovine tuberculosis can coexist with other infections or diseases affecting cattle farms (2, 3, 4, 10, 13, 15, 18).

Materials and methods

The investigations that are the object of the present research were carried out over a period of one year, during which lymph node samples from 17 control cattle slaughtered in slaughterhouses were analyzed, in order to clarify the diagnosis of tuberculosis (6, 19, 21).

The lymph node samples came from cattle from a household and from cattle belonging to a commercial company located in the west of the country, as a result of the positive reaction to the tuberculin allergy test (TCS), carried out in order to establish the diagnosis of bovine tuberculosis (11, 14, 16, 18, 22).

The following groups of lymph nodes were sent individually for the laboratory examination: retropharyngeal, mandibular, parotid, tracheobronchial, mediastinal and retromammary lymph nodes (Table 1).

The samples of lymph nodes were examined anatomically-pathologically and bacteriologically, by seeding on special culture media and then they were incubated in appropriate conditions, necessary for the isolation and cultivation of the bacterium *Mycobacterium spp*. (2, 9, 12, 14, 19, 20, 21), in the Timiș Veterinary Sanitary and Food Safety Laboratory (LSVSA).

The strains of *Mycobacterium spp*. isolated and preliminarily identified in LSVSA were sent for confirmation and phenotypic and genetic identification (2, 5, 6, 7, 16, 21) to the Animal Health and Diagnostic Institute in Bucharest (IDSA).

Table 1

Synopsis of the samples examined for the isolation and cultivation of the bacterium *Mycobacterium spp*.

No. Crt.	Cattle breed	Age (months)	Trial details	Strain code
1.	MT	63	Caseo-calcified nodules in the mediastinal lymph nodes.	11594-1
2.	Holl	43	Caseo-calcified nodules in the retropharyngeal, tracheobronchial and mediastinal lymph nodes.	13405-2
3.	Holl	45	Caseo-calcified nodules in the tracheobronchial lymph nodes.	13405-3
4.	BR	20	Caseo-calcified nodules in the tracheobronchial and mediastinal lymph nodes.	13405-4
5.	MT	51	Caseo-calcified nodules in the tracheobronchial, mediastinal and retromammary lymph nodes.	13405-5
6.	Holl	86	Caseo-calcified nodules in the tracheobronchial lymph nodes.	13405-6
7.	BR	57	Caseo-calcified nodules in the tracheobronchial lymph nodes.	13405-7
8.	BR	68	Caseo-calcified nodules in the retropharyngeal, tracheobronchial and mediastinal lymph nodes.	13405-8
9.	Holl	41	Caseo-calcified nodules in the retropharyngeal and tracheobronchial lymph nodes.	13405-9
10.	Holl	37	Caseo-calcified nodules in the retropharyngeal lymph nodes.	13405-10
11.	BR	118	Caseo-calcified nodules in the retropharyngeal lymph nodes.	13405-11
12.	BR	84	Caseo-calcified nodules in the tracheobronchial and mediastinal lymph nodes.	13405-12
13.	Holl	55	Caseo-calcified nodules in the retropharyngeal lymph nodes.	13405-13
14.	Holl	55	Lymph nodes - without lesions;	13405-14
15.	Holl	42	Lymph nodes - without lesions;	13405-15
16.	BR	72	Lymph nodes - without lesions;	13405-16
17.	BR	55	Lymph nodes - without lesions;	13405-17

Legend: MT=metis; Holl=Holstein; BR=Bălţătă Romanian

For the isolation and preliminary identification of *Mycobacterium spp*. from bovine lymph nodes (Figure 1) the working protocol recommended by the European Reference Laboratory (21) was used (Culture protocol for mycobacteria isolation. European Union Reference Laboratory for Bovine - Tuberculosis. VISAVET Health Surveillance Centre, Complutense University of Madrid, Spain, 2017), having as a reference document *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*, WOAH, 8th Edition (20, 22), Chap. Mammalian tuberculosis (Infection with *Mycobacterium tuberculosis* complex).

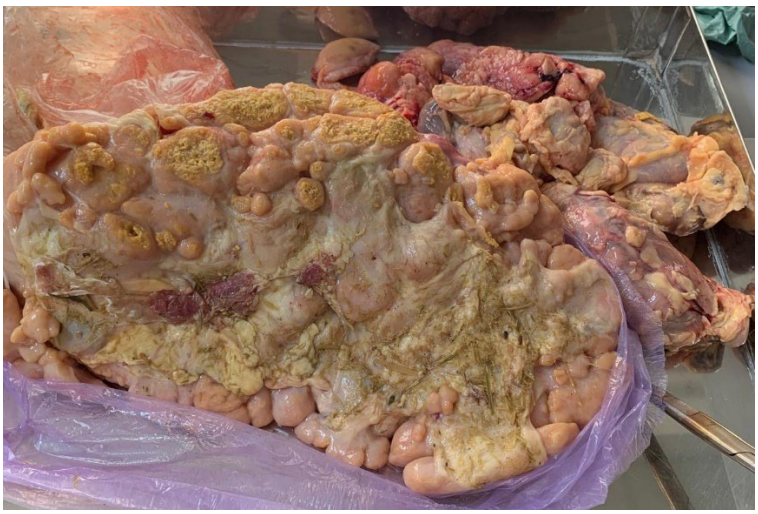

Fig. 1. Bovine lymph nodes with caseo-calcified nodules

For isolation and identification of *Mycobacterium spp*.fragments of lymph nodes were collected (11, 18, 21), 2.5 g/sample each, which were triturated and mixed with sterile distilled water 12 ml/sample. From the resulting suspension, 2 ml were taken for the counter sample and stored at -20ºC. The remaining suspension was transferred into a tube over which 10 ml of HPC (1-Hexadecylpyridinium Chloride) 0.75% (decontaminant) was added, followed by homogenization of the suspension for 30 min. Then the sample was centrifuged at 1300-1500 rpm for 30 min. After centrifugation, the supernatant was removed, and from the surface of the sediment, with the help of sterile swabs, inoculations were carried out on culture media (Coletsos). 4 tubes of Coletsos medium (23) were used for each sample. The seeded tubes were incubated at 37ºC, for a maximum period of 3 months. Periodically, the inoculated culture media were checked to observe the visible growth of *Mycobacterium spp*.

Morpho-tinctorial examination

Smears were taken from all the tubes in which colonies had developed. The smears were stained by the Ziehl-Neelsen method and were examined under a microscope with the immersion objective (4, 5, 11, 16, 18).

Results and discussions

The bacteriological examination carried out allowed the isolation and cultivation of the bacterium *Mycobacterium spp*., based on the work protocol recommended by the European Reference Laboratory, *Mycobacterium spp*., was isolated and identified in pure culture, in all the samples from the lymph nodes with lesions, according to the data from Table 2 (21). A total of 13 cattle, out of the 17 examined, were classified as being infected with the tuberculosis bacillus, based on the bacteriological examination performed. The age of the cattle from which *Mycobacterium spp*. were isolated and preliminarily identified was between 20 and 118 months.

Table 2

Distribution of samples with *Mycobacterium spp*., in correlation with the macroscopic appearance of the lymph nodes

No. Crt.	Age (months)	Strain code	Trial details	LSVSA Timiș result
1.	63	11594-1	Lymph nodes – with lesions;	Positive - *Mycobacterium spp*.
2.	43	13405-2	Lymph nodes – with lesions;	Positive - *Mycobacterium spp*.
3.	45	13405-3	Lymph nodes – with lesions;	Positive - *Mycobacterium spp*.
4.	20	13405-4	Lymph nodes – with lesions;	Positive - *Mycobacterium spp*.
5.	51	13405-5	Lymph nodes – with lesions;	Positive - *Mycobacterium spp*.
6.	86	13405-6	Lymph nodes – with lesions;	Positive - *Mycobacterium spp*.
7.	57	13405-7	Lymph nodes – with lesions;	Positive - *Mycobacterium spp*.
8.	68	13405-8	Lymph nodes – with lesions;	Positive - *Mycobacterium spp*.
9.	41	13405-9	Lymph nodes – with lesions;	Positive - *Mycobacterium spp*.
10.	37	13405-10	Lymph nodes – with lesions;	Positive - *Mycobacterium spp*.
11.	118	13405-11	Lymph nodes – with lesions;	Positive - *Mycobacterium spp*.
12.	84	13405-12	Lymph nodes – with lesions;	Positive - *Mycobacterium spp*.
13.	55	13405-13	Lymph nodes – with lesions;	Positive - *Mycobacterium spp*.
14.	55	13405-14	Lymph nodes - without lesions;	Negative - *Mycobacterium spp*.
15.	42	13405-15	Lymph nodes - without lesions;	Negative - *Mycobacterium spp*.
16.	72	13405-16	Lymph nodes - without lesions;	Negative - *Mycobacterium spp*.
17.	55	13405-17	Lymph nodes - without lesions;	Negative - *Mycobacterium spp*.

After incubating the samples at 37ºC in aerobic conditions, a slow and visible growth of the colonies was observed after 15 days of incubation, with the extension of the incubation up to 3 months. At the end of the incubation period, the inoculated culture media were examined culturally and morpho-tinctorially.

On the Coletsos culture medium (23), small and medium-sized, round, pale yellow colonies developed in all lymph node samples that showed macroscopic lesions (Figure 2). In 4 cattle whose lymph nodes did not show lesions, the culture media remained sterile (no colonies developed).

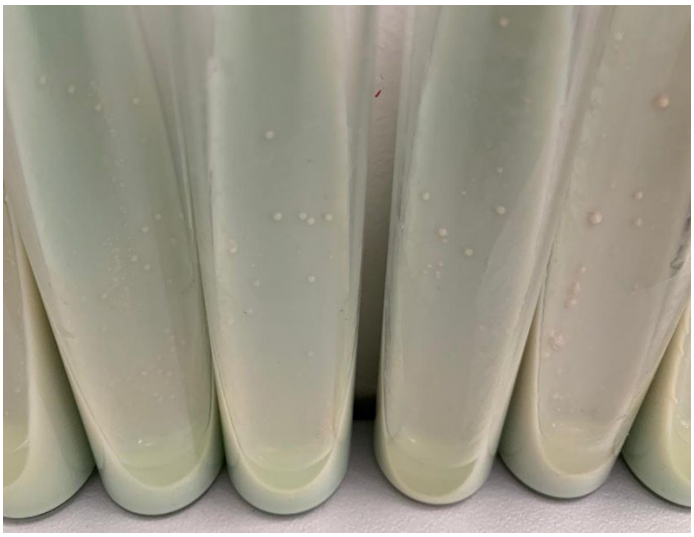

Fig. 2. *Mycobacterium spp*., in pure culture, on Coletsos medium

The results of the morpho-tinctorial examination

In smears stained by the Ziehl-Neelsen method, acid-alcohol-resistant, straight and/or slightly curved bacilli, colored red, characteristic of bacteria belonging to the genus *Mycobacterium spp*., were identified (Fig. 3) (4, 5, 11, 16, 18).

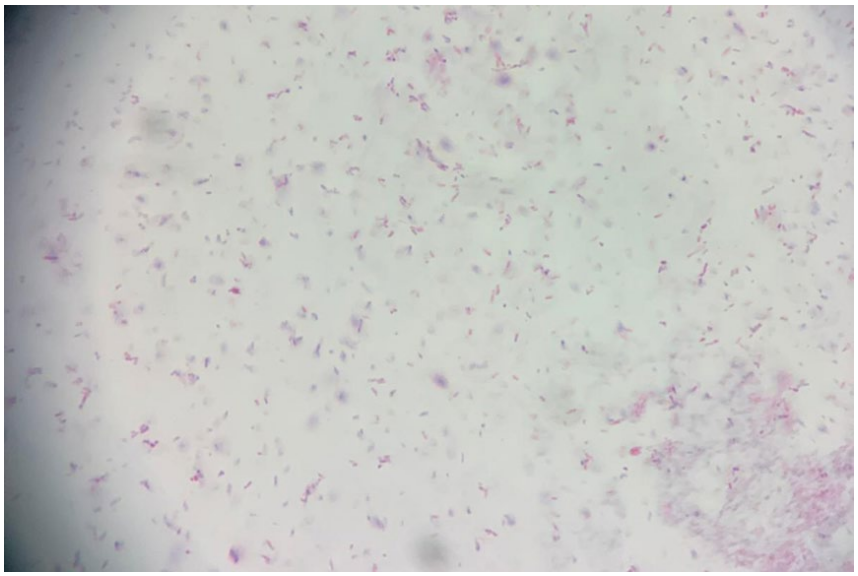

Fig. 3. *Mycobacterium spp*., in smear stained by the Ziehl-Neelsen method

The presence of characteristic caseo-calcified nodules in the lymph nodes, the cultural appearance, the slow growth of the colonies and the morphotinctorial characters of the isolated germs correspond to bacteria belonging to the genus *Mycobacterium*. Bacterial cultures were isolated only from triturated bovine lymph nodes that showed macroscopic lesions.

All 13 isolated bacterial strains were sent for phenotypic and genetic identification to IDSA. Following the investigations carried out within the IDSA, 2 species of Mycobacterium were identified: Mycobacterium caprae was isolated from the lymph nodes collected from cattle from a farm of a commercial company and *Mycobacterium bovis* was isolated from the lymph nodes collected from cattle from the household.

The appearance of several outbreaks of bovine tuberculosis both in the households of the population and in cattle breeding farms within a commercial company, represents an alarm signal for animal health and implicitly for public health and requires increased attention and more rigorous monitoring of livestock of cattle as well as the periodic medical control of

animal caretakers, especially those of cattle (18, 22).

The prevention of the spread of bovine tuberculosis outbreaks must continue to be carried out by carrying out annual tuberculin examinations for all cattle (starting with the age of 6 weeks in order to detect the disease early (11, 18, 22). The elimination of disease outbreaks will be carried out by eliminating, as quickly as possible, the reacted cattle from the herd positive in the allergy test, through slaughter and the application of restrictive measures and rigorous disinfection in disease outbreaks (11, 16). The importance of bovine tuberculosis is also given by the increased risk of transmission of the disease from animals to humans, and vice versa, which is why cattle caretakers should be examined periodically for tuberculosis (18, 20, 21).

Conclusions

The results obtained following the isolation and preliminary identification of *Mycobacterium spp*., from cattle slaughtered for the purpose of diagnosing tuberculosis, show the following:

- A total of 13 cattle, out of the 17 examined, were classified as being infected with the tuberculosis bacillus, based on the positive result of the bacteriological examination performed on samples from lymph nodes with lesions, on the Coletsos medium
- Two species of *Mycobacterium* were isolated: *Mycobacterium caprae* and *Mycobacterium bovis*;
- The age of the cattle from which *Mycobacterium spp*. were isolated and identified was between 20 months and 118 months;
- Bovine tuberculosis was diagnosed both in cattle that came from the population's household and in cattle that came from a commercial company.

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PREVALENCE OF GASTROINTESTINAL PARASITES IN HORSES IN EQUESTRIAN CENTERS IN WESTERN ROMANIA

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Abstract

Parasitic diseases are the main impediment to the growth and development of animal health worldwide. Horses, among domestic animals have been reported to be susceptible to infestation with various parasitic diseases. The aim of the present study was to determine the prevalence of gastrointestinal parasites of naturally infested horses in different equestrian centers in Western Romania. A total of 164 equine fecal samples (105 females, 59 males) were collected between September 2023 and February 2024. The horses belonged to the Friesian, Semigree and Gypsy Vanner (Tinker) breeds and were divided into three age categories: 6 months - 5 years, > 5 years - 10 years, > 10 years - 15 years. All fecal samples were examined by various methods (NaCl supersaturated solution flotation method, sedimentation method and Baermann method). The parasites identified by the flotation method belong to two classes of parasites: *Cestoda* (*Anoplocephala* spp.) and *Nematoda* (*Strongyles* spp. and *Parascaris equorum*), with a prevalence of 28%, 83.5% and 14%, respectively; by sedimentation and Baermann method no parasitic elements were revealed. Statistically significant differences were observed by sex and age ($p < 0.0001$). However, there were no statistically significant differences in the prevalence of gastrointestinal parasites by race. In conclusion, the present study revealed an increased prevalence of gastrointestinal parasites in naturally infested equidae.

Keywords: *horses, gastrointestinal parasites, Cestoda class, Nematoda class, Western Romania.*

The global equine population is approximately 122.4 million, consisting of 35% horses, 33% donkeys, 20% zebras and camels, and 12% mules (1). Globally, 98% of donkeys, 97% of mules, and 60% of horses are located in developing countries (20).

Parasitic diseases represent the primary obstacle to the growth and development of animal health worldwide (11, 15). Like other animals, equines are vulnerable to a variety of diseases, nutritional disorders, and other causes. Among the most common factors leading to poor health, suffering, and ultimately death are infectious and parasitic diseases. These significantly reduce the animals' working capacity, reproductive performance, and longevity (21, 22).

An apparently healthy horse can host over one and a half million gastrointestinal parasites, such as protozoa, trematodes, cestodes, and nematodes (12, 19). The most frequent intestinal parasites are migratory and non-migratory strongyles, ascarids (*Parascaris equorum*), pinworms (*Oxyuris equi*), and cestodes (*Anoplocephala* spp). This is because the gastrointestinal tract provides a favorable environment for the survival and proliferation of many of these parasites (7). Climatic variations, grazing and stable management, anthelmintic treatments, and the nutritional status of horses are the main epidemiological factors recognized in nematode infections (23).

Intestinal parasites, such as helminths, cause severe diseases in animals. Infected horses may show signs of weakness, emaciation, restlessness, agitation, diarrhea, anemia, and occasionally intestinal obstruction or perforation (19).

The objective of this study is to determine the prevalence of gastrointestinal parasites in horses from stud farms in Western Romania.

Materials and methods

The study was conducted between September 2023 and February 2024 in Timiș and Arad counties (Fig. 1).

The equines (males, n = 59; females, n = 105) included in the study belonged to several breeds: Friesian (n = 90), Semigree (n = 53), and Gypsy Vanner (Tinker) (n = 21). They were divided into three age categories: 6 months – 5 years, >5 years – 10 years, and >10 years – 15 years. The age of the animals was determined based on dentition. Equines under five years of age were classified as young, those between five and ten years as adults, and those over ten years as senior.

A total of 164 fecal samples were collected (Fig. 2). Immediately after defecation, the samples were placed in sterile coprocultures and properly labeled. All samples were kept at a temperature of 4°C and transported in isothermal boxes to the Department of Parasitology and Parasitic Diseases at the Faculty of Veterinary Medicine in Timișoara for examination.

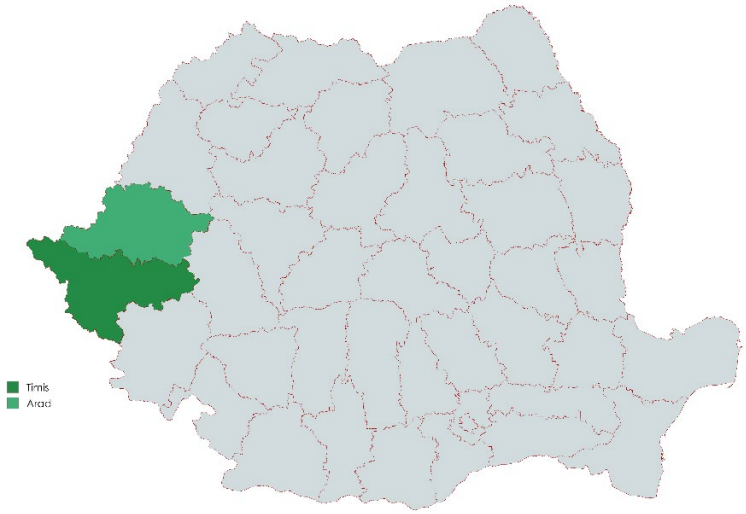

Fig. 1. Map of the counties surveyed (24)

Fig. 2. Samples collected

To determine parasite positivity, the standard screening method, namely the Willis flotation method, was used. Additionally, sedimentation and Baermann methods were also used. The presence of parasitic elements was identified using an optical microscope with 10x and 40x magnifications.

To evaluate potential statistically significant differences between the collected data and infestation prevalence, Fisher's Exact Test was applied using GraphPad QuickCalcs (2024) (25). Differences were considered statistically significant when the p-value was less than 0.05.

Results and discussions

Out of the 164 fecal samples examined, 137 (83.5%) tested positive for gastrointestinal parasitic infections. The prevalence of strongyles proved to be the most predominant at 137/164 (83.5%), followed by *Anoplocephala* spp. (46/164, 28%) and *Parascaris equorum* (23/164, 14%) (Fig. 3 – 6.).

The prevalence of gastrointestinal parasites based on breed, sex, and age categories is presented in Table 1 and 2.

Horses in the age group 6 months to 5 years and those > 5 years to 10 years were most affected by gastrointestinal parasites (Table 3).

No statistically significant differences were observed in infestation rates based on breed.

However, extremely significant differences were observed in infestation rates between sexes ($p = 0.0001$), as well as between age categories of >5 years – 10 years and >10 years – 15 years ($p = 0.0001$) and between 6 months – 5 years and >10 years – 15 years ($p = 0.0001$).

No statistical differences were found between the age categories of 6 months – 5 years and > 5 years – 10 years.

Coproparasitological examination in this study, using flotation, sedimentation, and Baermann methods, revealed a general prevalence of 83.5% for gastrointestinal parasites, which is higher compared to reports by Singh et al. (18), Goraya et al. (8), Matto et al. (13), and Ali et al. (1), who reported prevalence rates of 20.63%, 32.2%, 72%, and 74.06%, respectively.

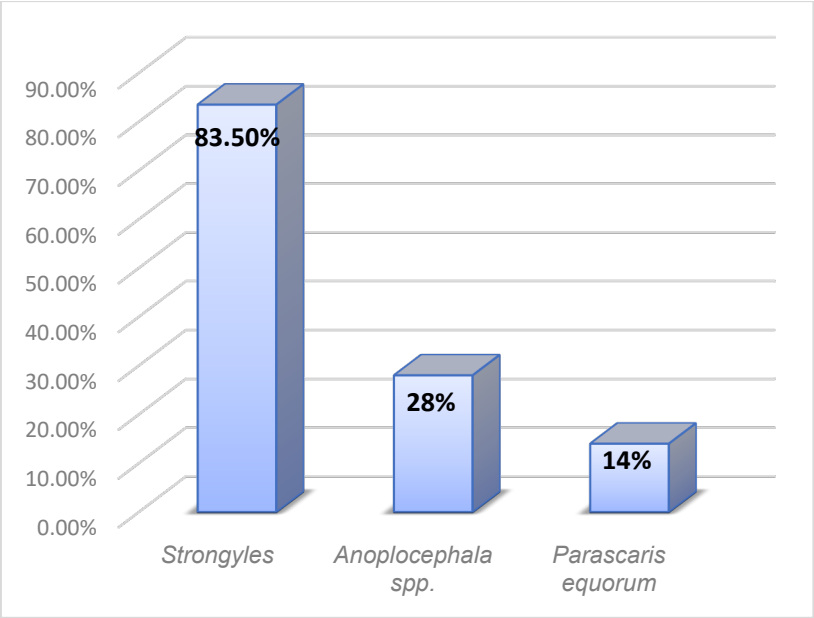

Fig. 3. Percentage distribution of gastrointestinal parasite species identified in the horses under study

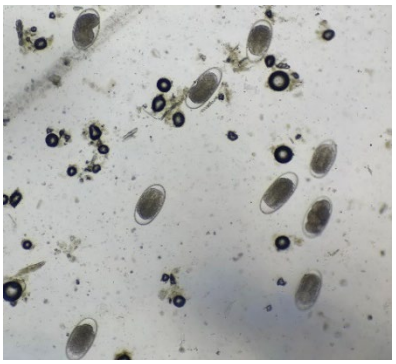

Fig. 4. Strongyles eggs

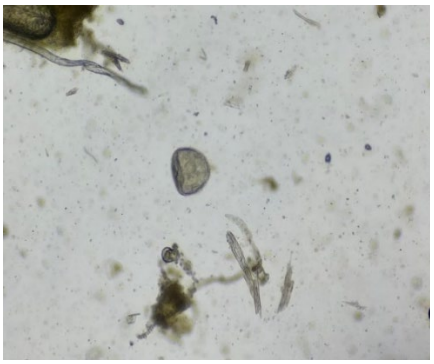

Fig. 5. *Anoplocephala* spp. Oncosphere

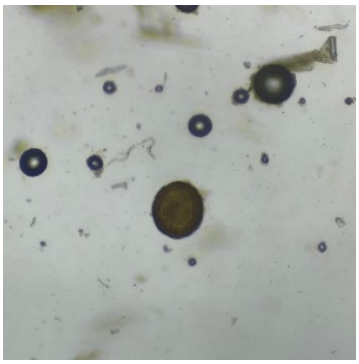

Fig. 6. *Parascaris equorum egg*

In Romania, a study conducted by Dreghiciu et al. (6) determined the prevalence of gastrointestinal parasites in horses. Fecal samples were examined using the flotation method, sedimentation method, and the Baermann technique. The overall prevalence was 43.5% for strongyles and 21.17% for *Parascaris* spp. Concurrent presence of at least two parasite species was observed in 17.64% of the examined horses (6).

Table 1

Prevalence of gastrointestinal parasites by horse breed

No. crt.	Breed	Number of animals examined	Positive animals	Prevalence
1.	Friesian	90	77	85.6%
2.	Semigree	53	44	83%
3.	Gypsy Vanner	21	16	76.2%

In 2012, Morariu et al. (14) examined 56 horses. Fecal samples were analyzed using the McMaster method. All fecal samples were found to be parasitized at a rate of 100%, with five species identified: gastrointestinal strongyles (73.21%), *Parascaris equorum* (28.57%), Strongyloides westeri (8.92%), *Oxyuris equi* (12.5%), and *Anoplocephala* spp. (19.64%) (14).

Bulgaru et al. (4) conducted a study on 156

horses. Of these, 28.57% tested positive for parasitism. Among the helminths identified, *Parascaris* spp. had a prevalence of 3.89%, while strongyles had a prevalence of 27.92% (5). In 2021, a study on 115 horses used flotation and sedimentation methods, revealing that 69 (60%) were positive. The prevalence of gastrointestinal strongyles was 35.65%, *Parascaris equorum* 19.13%, and *Eimeria leuckarti* 23.47% (4).

Table 2

Prevalence of gastrointestinal parasites by horse gender

No. crt.	Gender of animals	Number of animals examined	Positive animals	Prevalence
1.	Males	59	38	64.4%
2.	Females	105	99	94.3%

Table 3

Prevalența paraziților gastrointestinali în funcție de vârsta cabalinelor

No. crt.	Age of the animals	Number of animals examined	Positive animals	Prevalence
1.	6 months– 5 years	83	80	96.4%
2.	> 5 years – 10 years	54	52	96.3%
3.	> 10 years – 15 years	27	5	18.5%

A study by Aromaa (2) in Finland tested young horses for *Parascaris* spp. and strongyles. In this study, 112 horses aged between one and three years, housed in training stables, were evaluated. The prevalence of *Parascaris* spp. was 21%, while that of strongyles was 48% (2).

Avcıoğlu H. et al. (3) conducted a study in Turkey on 76 horses of varying ages, sexes, and breeds. Individual fecal samples were collected and examined using flotation and sedimentation methods, identifying the following species: strongyles (57.89%), Parascaris equorum (10.52%), *Dicrocoelium dendriticum* (2.63%), *Fasciola* spp. (2.63%), and *Eimeria* spp. (5.26%) (3).

In Germany, a study aimed at determining the prevalence of helminths in horses reported the following prevalences: *Cyathostominae* (98.4%), *Parascaris equorum* (16.7%), *Anoplocephala* spp. (14.3%), pinworms (8.7%), and *Strongyloides* (4.0%) (9).

A review of the literature on equine parasites in Iran revealed a cumulative prevalence of parasitic infestations of 28.8%, with helminths showing the highest prevalence (47.6%) (10). Parasitological investigations conducted on horses living in a tropical climate in Cuba determined a prevalence of 97% for strongyles and 10% for *Parascaris* spp. (16).

Parasite control is a global concern among researchers and horse breeders. In Italy, a study on strongyle infestations in horses found a prevalence of 39.5% (17).

Conclusions

The most widespread gastrointestinal parasites were *Strongyle*s (83.5%), followed by *Anoplocephala* spp. (28%) and *Parascaris equorum* (14%).

The highest prevalence rates were observed in young horses aged 6 months – 5 years, as well as those aged >5 years – 10 years, indicating that age represents a risk factor.

Statistically significant differences were observed between females and males, as well as between horses aged >5 years – 10 years and >10 years – 15 years, and between 6 months – 5 years and >10 years – 15 years.

No significant statistical differences were found in gastrointestinal parasite infestations based on breed.

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THE PREVALENCE OF ANTI-*LEISHMANIA INFANTUM* ANTIBODIES IN DOGS FROM WESTERN ROMANIA

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Abstract

Canine leishmaniasis (CanL) is caused by the parasite *Leishmania infantum*, which is transmitted by sand flies, with dogs serving as the primary reservoir for the disease. Romania has traditionally been considered non-endemic for CanL; however, climate changes over the past decade have significantly contributed to the increased presence of vectors in the environment, leading to a rise in disease incidence among animals and humans. Due to the zoonotic nature of the disease and the widespread prevalence of vectors, attention to this pathology is growing. The aim of this study was to determine the seroprevalence of CanL in dogs from three counties in Romania. Conducted between February and May 2024 in western Romania, the study involved serum sample collection from 100 asymptomatic dogs from four different shelters and one veterinary clinic, analyzed using indirect immunofluorescence assay techniques. Of all the samples analyzed, 1% tested strongly positive, while 8% showed weakly positive results. Considering these results as well as the increasing reports of the disease in endemic countries among resident animals, we recognize the importance of careful and rigorous monitoring of CanL in Romania.

Keywords: *CanL, Leishmania infantum, Romania, climate changes, dogs, indirect immunofluorescence.*

Canine leishmaniasis (CanL) is a zoonosis caused by protozoa belonging to the genus *Leishmania,* targeting the mammalian phagocytic immune system. Transmission occurs primarily through the blood-feeding activity of sand fly females from the *Lutzomyia* and *Phlebotomus* genera. This disease has a broad distribution, affecting both humans and animals across five continents. Although the disease has been identified in various animal species, dogs are considered the most important reservoirs and hosts for leishmaniasis worldwide (13, 3, 18).

Leishmaniasis has been identified primarily in China, Brazil, and the Mediterranean Basin. Its geographical distribution depends on the presence of vectors, which has increased in recent years due to climate changes that favor their development (4).

Romania has traditionally been considered a non-endemic region for Leishmania infections, with only isolated cases reported in humans. However, climate change over recent decades has created conditions favorable to *Phlebotomus* spp., the primary vectors for this infection, which have recently been identified in multiple areas across the country (11).

The disease can present in three forms: cutaneous, mucocutaneous, and visceral. The latter is the most common and severe form. Typical clinical symptoms include skin alterations, ulcers, localized or generalized lymphadenomegaly, weight loss, glomerulopathy, liver and spleen enlargement, ocular lesions, epistaxis, lameness, and onychogryphosis (2, 18). Atypical symptoms include monoclonal gammopathy, chronic colitis, hemostatic disorders, and disturbances in the cardiovascular, respiratory, and musculoskeletal systems (7). Thus, Leishmania infection can resemble many clinical and pathological characteristics of other canine diseases, emphasizing the importance of precise diagnostic methods (6).

Diagnosis is challenging to establish based on history and symptoms due to the disease's varied clinical manifestations. The most conclusive methods are serological tests targeting humoral and cellular responses and identifying the parasite through microscopic examinations (16, 12).

Serological surveys are useful tools for quantifying the spread of pathogens in specific areas, serving as the foundation for developing proper, risk-based surveillance approaches. The indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) are the most commonly used serological methods. Specifically, in-house IFAT prepared with whole-body parasites as antigens is considered the "gold standard" for serological diagnosis (14, 19).

Materials and methods

The study area included five localities (Deva, Răcășdia, Herculane, Tapia, and Timișoara), located in three counties (Hunedoara, Caraș-Severin, and Timiș) in western Romania (Fig. 1). The region is characterized by a temperate-continental transition climate, marked by some sub-Mediterranean influences, with mild winters and increased rainfall, especially in autumn. The

altitude ranges between 90 and 187 meters above sea level, with plains and meadow areas predominating (22).

Fig. 1. Number of samples collected from each county

The study was conducted between February and May 2024 and included the collection of 100 peripheral blood samples from dogs in shelters, collection centers, and veterinary clinics (Fig. 2). The origin of the samples by locality is presented in Table 1.

Fig. 2. Snapshot from a shelter in Timiș County (original)

The animals included in the study ranged in age from 3 months to 13 years, comprising 55 females and 45 males. At the time of sample collection, none of the dogs exhibited typical clinical signs of CanL.

Table 1

Number of samples collected from each county and locality

County	Locality	No. of Samples
Hunedoara	Deva	11
Caraș-Severin	Herulane	32
	Răcășdia	30
Timiș	Tapia	18
	Timișoara	9

Each blood sample was collected in 5 ml tubes without anticoagulants and transported to the laboratory for further study (Fig. 3). After individualizing the samples, they were centrifuged at 3000 rpm for 5 minutes to facilitate the separation of serum from the blood cells. The serum extracted from each sample was transferred into Eppendorf-type tubes using an automatic pipette. Serum samples were stored at −20 °C until analysis.

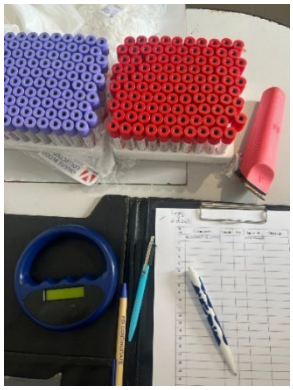


Fig. 3. Snapshot from the blood sample collection process (original)

The samples were examined using the Anti-Leishmania Antibodies (Leishmaniosis) Kit®, produced by ByoSistems, following the manufacturer's suggested usage guidelines. The results were interpreted with an imID® fluorescence microscope (BioSystems).

Indirect immunofluorescence (IFA) is performed by applying animal serum onto a slide coated with Leishmania promastigotes. Antibodies present in the serum bind to the promastigotes, with positivity evidenced by fluorescent secondary antibodies. Samples displaying homogeneous fluorescence under the microscope are considered positive (21).

Results and discussions

Positivity was noted by intense fluorescence and high homogeneity in a single sample out of the 100 examined. Microscopic analysis revealed that 8 dogs exhibited weaker fluorescence but maintained high homogeneity. Results for 24 animals were inconclusive, showing fluorescent fields alongside areas with reduced fluorescence, lacking homogeneity. The remaining 67 dogs were negative (Fig. 5).

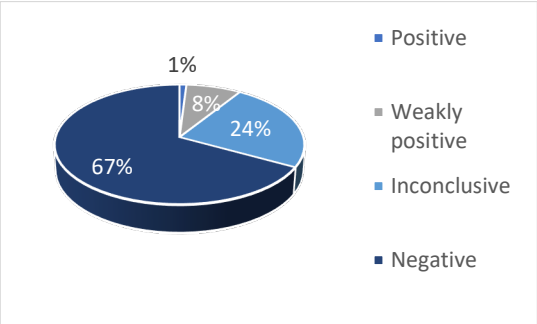
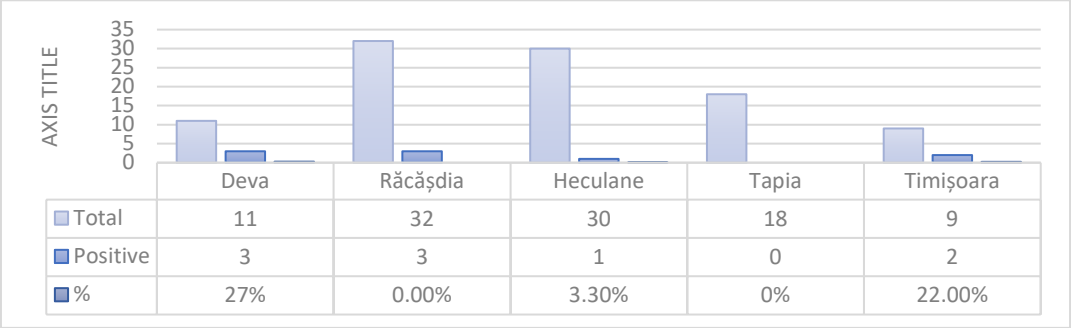


Fig. 5. IFAT results

The positive and weakly positive samples for each locality are presented in Figure 6.

Maximum fluorescence was observed in a 3.6-year-old female (Fig. 7). This serum sample was collected from a shelter in Herculane. Positivity could be associated with an infection or possible prior contact with this protozoan. The shelter is located in a southern point of Caraș-Severin County, near a forest with rocky terrain, relatively close to the Danube River. These characteristics are specific to the presence of vectors that prefer such habitats (1).



	Deva	Răcășdia	Heculane	Tapia	Timișoara
Total	11	32	30	18	9
Positive	3	3	1	0	2
%	27%	0.00%	3.30%	0%	22.00%

Fig. 6. Positive samples by locality

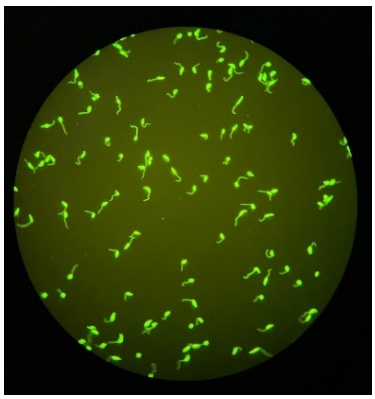


Fig. 7. Microscopic aspect of the positive sample showing fluorescence intensity and homogeneity (original)

In 2008, Miranda et al. (15) evaluated a group of 390 dogs confirmed with CanL, noting a significantly higher proportion of males compared to females, higher proportions among breeds like German Shepherds, Rottweilers, and Boxers, and a bimodal age distribution for disease onset, with a primary peak among adults aged 2–4 years and a secondary peak in older animals (over 7 years). In our study, the mean age of positive animals was 3.6 years, with 5 females and 4 males, all mixed breeds. As for sex, no studies have compared breed percentages in an affected population with those in a reference population for the same geographic area and time period (15).

Over time, epidemiological studies on CanL have highlighted a large number of positive but asymptomatic animals. This has led to the use of these methods for disease control in endemic and non-endemic countries (20).

Chargui et al. analyzed the efficiency of several diagnostic methods for CanL and observed a 93.3% accuracy for IFAT (10). Similarly, Camargo et al. studied the efficiency of three diagnostic methods (IFAT, ELISA, and PCR) in Brazil in 2010, noting that IFAT and PCR had 100% specificity, while the sensitivity of immunofluorescence was 97.77%, the highest among the three methods (8).

In a 2014 study, Proverbio et al. (17) observed that animals with the highest recorded IFAT titers had higher clinical scores, indicating a positive relationship between anti-Leishmania IgG antibodies and clinical manifestations, particularly in severe clinical forms of canine leishmaniasis (17).

In recent years, the distribution area of sand flies has expanded in latitude and altitude due to global warming, increasing the prevalence of leishmaniasis. The geographic expansion of sand flies has been noted in the Pyrenees, northern Italy, and altitudes previously unsuitable for their development. Global warming is increasingly evident in southeastern Europe (5). Researchers noted

that March 2024 was the second warmest March on record since 1901, with an average temperature of 8.4 °C, also recorded in western Romania (22).

A 2019 study in Romania over five years revealed changes in the distribution and diversity of sand fly species in various areas of the country compared to studies conducted at the beginning of the 20th century. Due to the emergence of these vectors in previously unpopulated areas, surveillance of this disease should be extended nationwide (9).

Conclusions

The study confirms the increasing incidence and geographic spread of canine leishmaniasis. Monitoring dogs, the natural reservoir of the disease, is essential for timely and geographically targeted prevention to prevent the spread of canine leishmaniasis in Europe.

Using the *Leishmania infantum* immunofluorescence test to detect anti-*Leishmania* antibodies is a reference method due to its high sensitivity and specificity, particularly in symptomatic animals. For asymptomatic animals, sensitivity is lower, but the test has shown good results even for these individuals.

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THE THERAPEUTIC IMPACT OF QUERCITINE ON CISPLATIN-INDUCED HEPATIC LESIONS IN RATS

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Abstract

Cisplatin is a substance with anti-tumour properties, which is why it is used in chemotherapy, but it has serious side effects such as peripheral neurotoxicity, nephrotoxicity and ototoxicity. In an attempt to reduce the liver toxicity of cisplatin, quercetin was administered to rats for 6 weeks. Histological sections of the liver were used to show structural changes in hepatocytes. Cisplatin administration for 6 weeks also resulted in degenerative phenomena in the liver. Hepatocytes became hypertrophied due to the accumulation of vacuoles in the cytoplasm, and the nuclei of some cells underwent pyknosis and karyolysis. Hepatocyte necrosis was observed in some areas. Structural changes also extended to the blood vessels, with dilation and vascular congestion. In the group given cisplatin with quercetin, the liver architecture is almost normal. The lesions described in the hepatocytes are reduced, almost absent, blood vessels of normal appearance and diameter. Taking into account these considerations and the fact that quercetin is a flavonoid with proven antioxidant activity, it can be concluded that the negative effects of cisplatin administration on the liver, which can occur during anti-tumour therapies, can be significantly reduced by the combined use of quercetin.

Keywords: *cisplatin, quercitine, hepatotoxicity, rats*.

Cisplatin has been a chemotherapeutic agent since 1978 when the United States Food and Drug Administration (FDA) approved its use for a number of malignancies. Since then, it has been on the World Health Organization's list of essential medicines (6, 12, 19). Due to its antineoplastic action, expressed by its ability to bind and interact with deoxyribonucleic acid (DNA) in cells inducing apoptosis, today cisplatin is predominantly used in the therapy of malignant tumors with different localizations such as testicular, ovarian, head and neck, breast, lung, esophageal, cervical (9).

Although it is essential in the fight against malignant tumors, cisplatin administration induces a number of adverse effects among which the most frequently observed are nephrotoxicity, neurotoxicity, ototoxicity, hepatotoxicity (11). The toxicity of cisplatin is due to direct binding to the DNA macromolecule, in addition to the generation of free radicals and inhibition of antioxidant enzymes, respectively the onset of oxidative stress (18).

Quercetin, one of the most studied flavonoids, is a derivative of phenylalanine. It is found in increased amounts in a variety of fruits and vegetables but is also used as a dietary supplement due to the multiple actions it possesses, such as antioxidant, anti-inflammatory and anti-proliferative (3), antidiabetic, anticarcinogenic and antimicrobial properties (17). The beneficial effects of quercitin are due to the fact that it can scavenge free radicals (4), being a potent antioxidant and to its chemical composition, it has a lipophilic structure that allows it to easily cross cell membranes (14).

Although cisplatin is a very good anti-tumour drug, the side effects are serious and there is dire need of finding a way to reduce toxic effects. Considering all, this study was designed to test the antioxidant effect of quercitin on cisplatin-induced hepatotoxicity.

Materials and methods

The study was performed on 21 adult white mice obtained from the Biobase of the University of Medicine and Pharmacy Victor Babes Timisoara. The mice were housed in plastic cages under environmental conditions of 25±20°C and 12h light/12h dark. The animals had access to food and water ad libitum.

The animals were randomly divided into four experimental groups, namely

- Group I (C) - control group (injected with 1 ml of saline i.p.), distilled water ad libitum,
- Group II (E1) - injected i.p. with 20 mg/kg cisplatin,
- Group III (E2) - injected i.p. with 20 mg/kg cisplatin and quercitin 500 mg/L.

Cisplatin and quercitin were administered once a week for 6 weeks.

Quercetin (Quercetin 500 mg, Solaray, USA) was dissolved in 10 ml of distilled water and ethanol in a 4:1 ratio for 10 minutes, then distilled water was added to 1 litre, the final concentration was 500 mg/L. The quercetin solution was prepared daily to avoid precipitation.

The experimental protocol was approved by the Ethics Committee of the University of Agricultural Sciences and Veterinary Medicine of Banat "Regele Mihai I of Romania" in Timisoara No. 136/2021.

The animals were treated in accordance to national and international laws regarding the protection and welfare of animals used in scientific purposes (5).

Results and discussions

The histological structure of the liver in the control group was normal. In histological sections, the liver shows hexagonal structural units called hepatic lobules. At the center of each lobe is the central vein (Fig. 1), towards which converge cords of liver cells, called hepatocytes. These are polygonal cells, with more than four sides, which are organized in cords (Fig. 2) facing the central vein. An increased number of hepatocytes, about 25%, are binucleated cells. Hepatocyte nuclei are spherical and centrally arranged.

Between the cords of hepatocytes are blood vessels, sinusoidal capillaries, whose walls are made up of endothelial cells and Kupffer cells. Kupffer cells are macrophages, large, branched cells with an increased number of lysosomes. They filter and phagocytose inert particles, bacteria, cellular debris and damaged erythrocytes.

The hepatic lobules are surrounded by perilobular connective tissue, detached from the liver capsule. Branches of the hepatic artery, portal vein and bile duct are located in the connective tissue between the liver lobules.

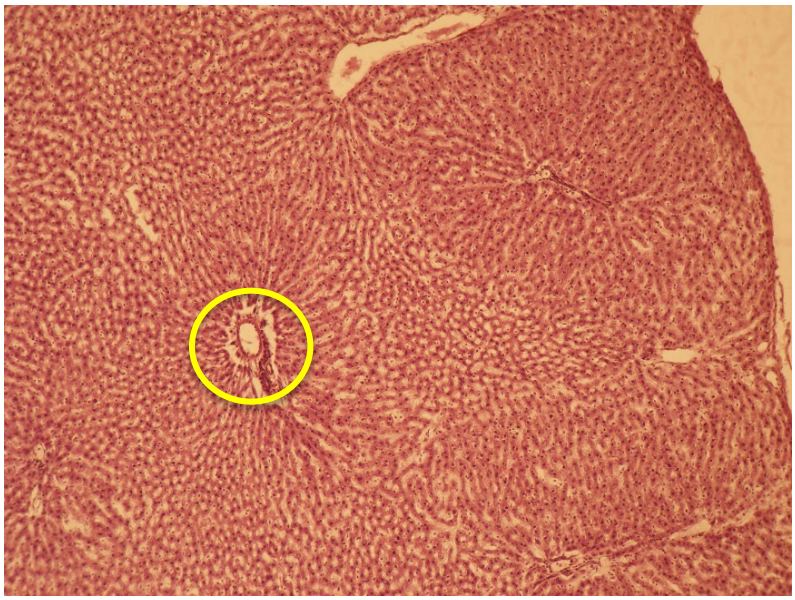

Fig. 1. Liver - control: normal appearance (central vein), H.E. stain, ob. 10X

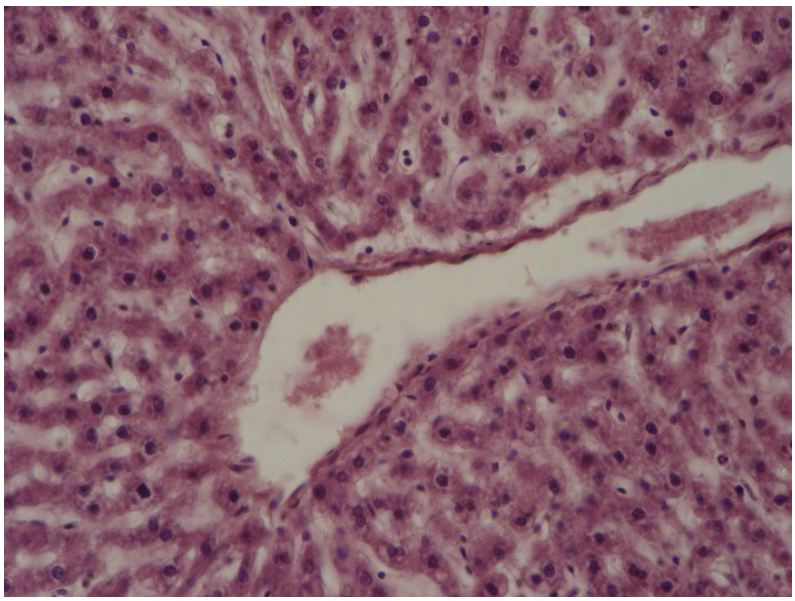

Fig. 2. Liver - control group: normal appearance: central vein and hepatocyte cords, H.E. stain, ob. 40X

Microscopic examination of the liver from E1 group treated with cisplatin revealed localized vacuolar hydropic degenerative phenomena (Fig. 3, 4). Thus, hepatocytes affected by hydropic degeneration were enlarged in volume, with clear cytoplasm where small vacuoles and centrally located nuclei were observed. Some hepatocytes showed pycnotic nuclei and karyolysis.

It is known that hepatocytes accumulate significant amounts of cisplatin, so hepatotoxicity is attributed to cisplatin storage in hepatocytes (8), which will induce the occurrence of structural changes.

Increased diameter of hepatic blood vessels associated with congestion has also been observed (Fig. 5, 6), as demonstrated by other authors [Avci et al., Mohnsen et al].

Cisplatin, due to its accumulation in hepatocytes, is responsible for cell membrane destruction and leakage of liver enzymes into the extracellular space, eventually leading to necrosis (Fig. 6). As demonstrated, all these structural changes will adversely affect the function of hepatocytes and thus the liver (1).

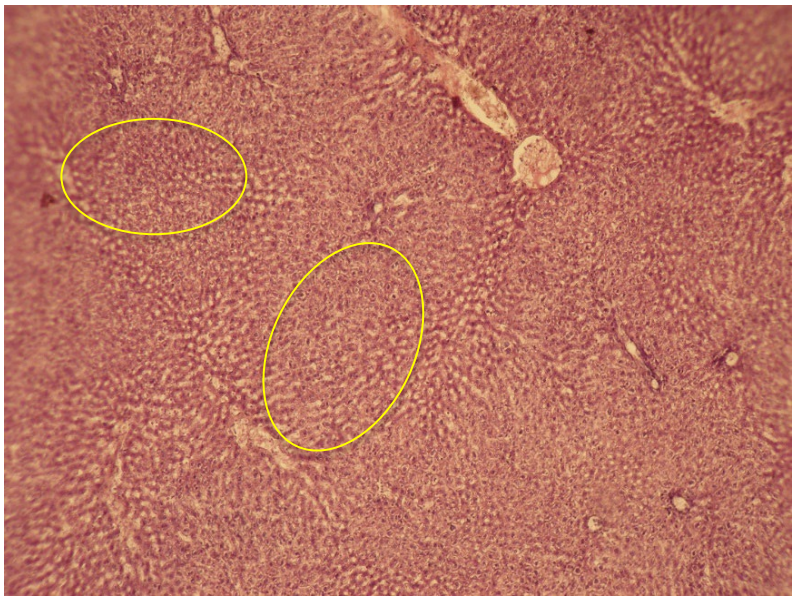

Fig. 3. Liver - E1 group, degenerative lesions - vacuolar hepatocytes, H.E. stain, ob. 10X

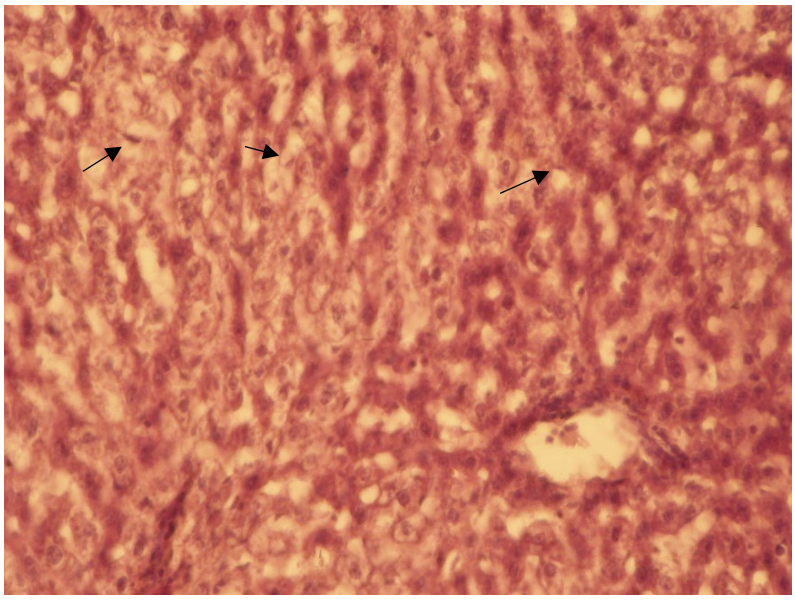

Fig. 4. Liver - E1 group: vacuolar hepatocytes with pyknotic nuclei, H.E. stain, ob. 10X

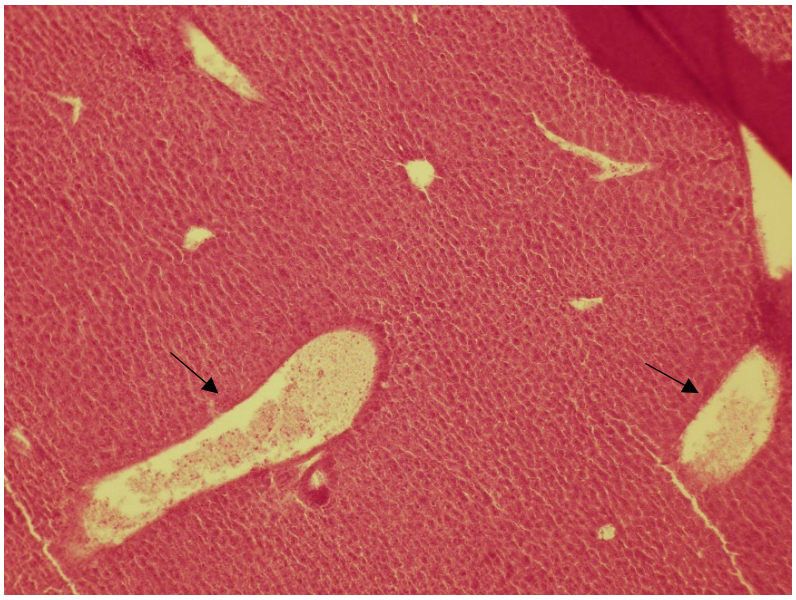

Fig. 5. Liver - E1 group, vascular dilations, H.E. stain, ob. 10X

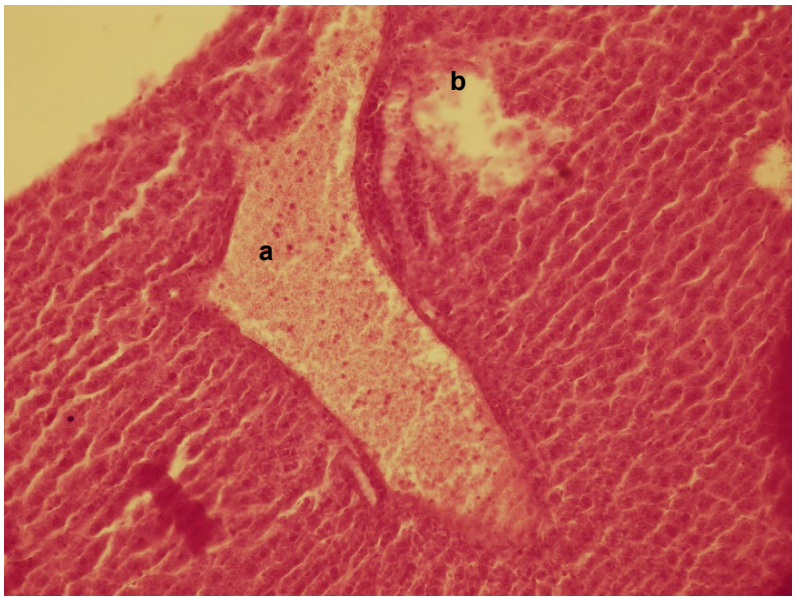

Fig. 6. Liver - E1 group: a. vascular dilation associated with congestion, b. area with necrosis, H.E. stain, ob. 20X

The liver structure of the E2 group, treated concomitantly with Cisplatin and Quercitin, is similar to that of individuals in the control group. Hepatic lobules are evident, centerd by the central vein. The cell cords are oriented towards the central vein, composed of hepatocytes, most of which are normally polygonal in appearance, with centrally arranged spherical nuclei (Fig. 7).

There are few areas of hypertrophied, optically hollow hepatocytes with peripherally arranged nuclei (Fig. 8).

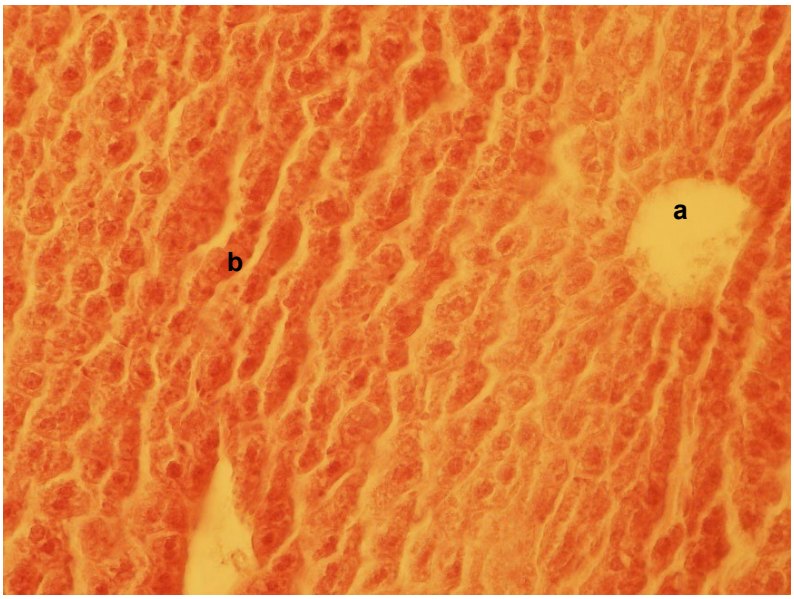

Fig. 7. Liver - E2 group, a. central vein, b. cords of hepatocytes, H.E. stain, ob. 40X

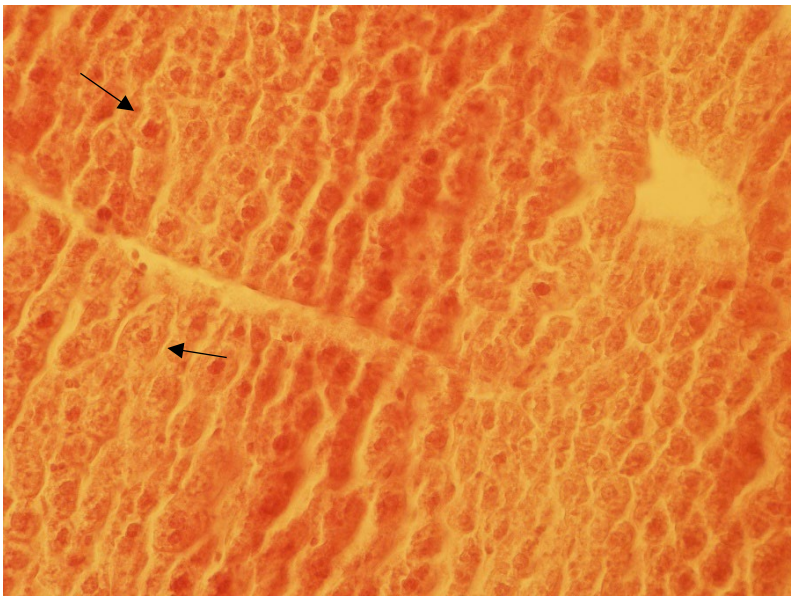

Fig. 8. Liver - E2 group: hypertrophied hepatocytes, H.E. stain, ob. 40X

Also, the diameter of the blood vessels is not enlarged; and the blood vessels do not show congestion (Fig. 9).

Areas of necrosis, evidenced in individuals of group E2, are not observed in case of concomitant administration of cisplatin with Quercitin. The absence of cisplatin-induced changes is explained by Quercetin, a flavonoid present in fruits and vegetables, with multiple unique biological properties, including antioxidant effect (13).

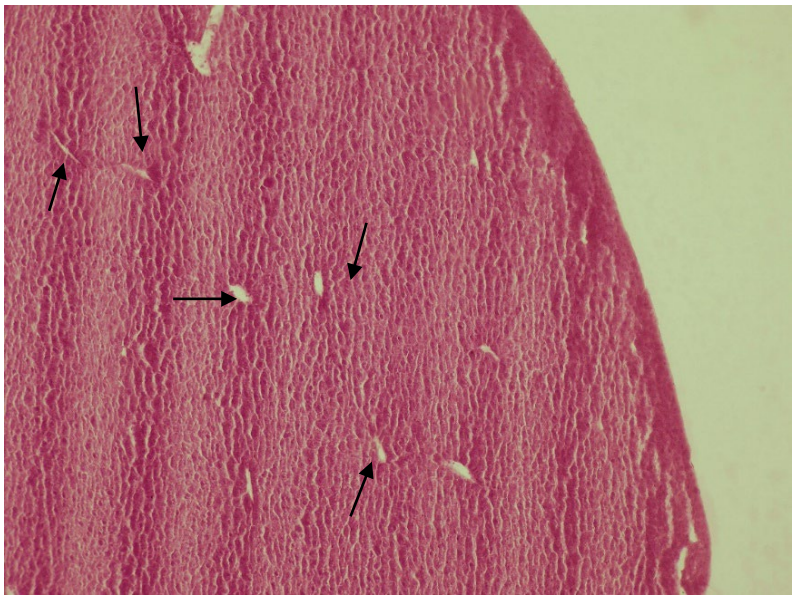

Fig. 9. Liver - E2 group, blood vessels of normal caliber, H.E. stain, ob. 10X

Some experimental animal studies have stated that the antioxidant effects of quercetin decrease oxidative damage to tissues such as the brain and heart (7). It is well established that natural antioxidants are usually harmless to the human body. They are molecules that prevent early ageing by blocking the catastrophic effects of free radicals, many diseases and chain reactions (16).

Conclusions

Cisplatin administration for six weeks also resulted in degenerative liver phenomena. Hepatocytes became hypertrophied due to the accumulation of vacuoles in the cytoplasm and the nuclei of some cells underwent pyknosis and karyolysis. Hepatocyte necrosis was observed in some areas. Structural alterations also extended to the blood vessels, with ectasia and vascular congestion.

In the group administered cisplatin concomitantly with quercetin, the liver architecture is almost normal. The lesions described in the hepatocytes are reduced, almost absent, blood vessels with normal appearance and diameter.

Taking into account these considerations and the fact that quercitin is a flavonoid with demonstrated antioxidant effect, it can be stated that the negative effects of cisplatin administration on the liver, which may occur during antitumour therapies, can be significantly reduced by the combined use of quercitin.

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HYPERTROPHIC OSTEOPATHY DUE TO INTRATHORACIC METASTASES SECONDARY TO PRIMARY BONE CANCER: A CASE REPORT

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Abstract

Hypertrophic osteopathy is a rather rare condition seen in dogs and cats. The exact pathophysiological mechanism of this condition is not fully understood, although changes in pulmonary function have been suggested to cause an increase in peripheral blood flow via a neural pathway; this leads to new bone being formed by the periosteum on the cortices of distaly located long bones. This condition is considered to be a paraneoplastic syndrome caused by intrathoracic space-occupying masses, although other non-neoplastic conditions or abominal mass-effects were also reported as causes. Hypertrophic osteopathy typically affects all four limbs, patients being presented for swollen limbs and lameness. Radiographically, periosteal thickening due to new bone formation located bilaterally or on all four limbs is the characteristic sign. The periosteal reaction is palisading, nodular or spiculated, with no signs of osteolysis being seen. Bony changes commence distally and progress proximally. The current paper presents the clinical signs and evolution, radiographical changes and computed tomography findings in a 7-year-old female Boxer, as well as a short bibliograhical review.

Keywords: *hypertrophic osteopathy, intrathoracic metastases, primary bone cancer, radiology, computed tomography.*

This condition is also known as pulmonary osteoarthropathy, hypertrophic pulmonary osteopathy, acropachia etc. (4, 13).

Hypertrophic osteopathy (HOP) is a rare condition, considered to be secondary to intrathoracic neoplasia (4, 13), but it can also be associated with other conditions such as granulomatous lesions, megaesophagus, patent ductus arteriosus, dirofilariasis, cardiopulmonary diseases, pulmonary abscesses, abdominal neoplasia, blastomycosis, spirocercosis, intrathoracic foreign bodies etc. (2, 5, 6, 12, 13).

The exact pathophysiology of this disease remains unclear. Changes in pulmonary function are believed to enhance peripheral blood flow, leading to congestion of connective tissue. This increased peripheral blood flow is thought to be mediated by neural mechanisms (4). The periosteum reacts by generating new bone on the cortices of the metacarpals, metatarsals, and other long bones, which may appear either diffuse or nodular (4).

Radiographs can be used for examination of the limbs, as well as for identiying underlying and primary conditions in the thorax. A comprehensive physical examination is crucial when evaluating affected animals (4).

Materials and methods

The patient was a 7-year-old female Boxer presenting initially at Vetpoint Vest Arad for swollen and painful limbs, decreased appetite and activity; during a follow-up, couching was also reported by the owner.

Radiographs of the forelimbs and thoracic cavity were taken.

After reviewing the radiographs, the patient was referred to the Faculty of Veterinary Medicine from Timisoara for a Computed Tomography (CT) study of the thorax and forelimbs.

A Siemens Somatom Definition AS 64 slice CT scanner was used to scan the patient, under general anesthesia (Fig. 1).

Anesthesia was performed using a combination of medetomidine (20-40 μg/kg) and propofol (0.5 – 1.5 mg/kg). Recovery was obtained with atipamezol (at 5 times the dose of medetomidine).

Both native and contrast studies were conducted and images were obtained using multiplanar reconstruction (MPR) at 0.6 mm slice thickness, and 3D volume rendering technique (VRT). Ultravist 370 (Iopromide, Bayer), at a dose of 1 ml/kg, was the contrast medium of choice used for the contrast enhanced CT scans.

Fig. 1. Siemens Somatom Definition AS 64

Results and discussions

While this condition affects mostly dogs, it was also reported in cats (3, 7, 9, 10, 14).

HOP can affect dogs of any breed or size; however, since it is most often linked to neoplasia, it is typically observed in older animals (4, 6).

Dogs are typically brought in due to lethargy, reluctance to move, and swelling in the distal extremities, the onset of which can be either sudden or gradual (4).

The affected limbs are painful, swollen and warm (4); stiffness may also be present (6). Since this condition is secondary to other underlying diseases, it is important to identify the primary causes. (4). Other signs caused by the primary condition may be present (6).

Radiographic examination reveals bilateral, symmetrical periosteal reaction on the phalanges, metacarpals, metatarsals, and as the disease progresses, more proximal: on the radius, ulna, tibia, fibula as well (4, 6, 11).

Radiographs in this dog revealed bilateral, symmetrical, thick, layered and palisading periosteal reaction on all of the long bones of all limbs, which is specific for HOP, as well as a lytic lesion on the right humerus (Fig. 2).

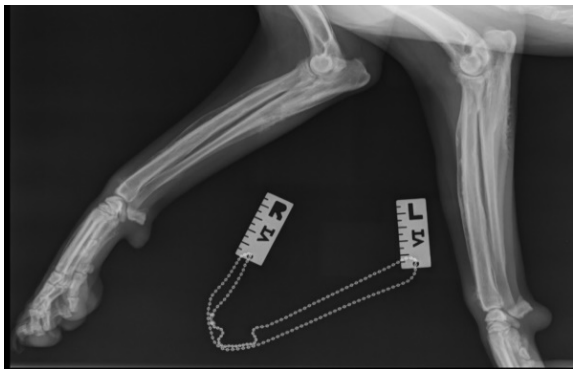

Fig. 2. Medio-lateral and latero-medial radiograph of both antebrachiums: bilateral and symmetrical periosteal reaction on the radius and ulna.

Thoracic radiographs revealed a large soft tissue mass in the cranio-ventral aspect of the chest, increasing the likelihood of intrathoracic neoplasia (Fig. 3).

Upon evaluating the MPR images, changes specific for HOP were easily identified on the long bones (Fig. 4).

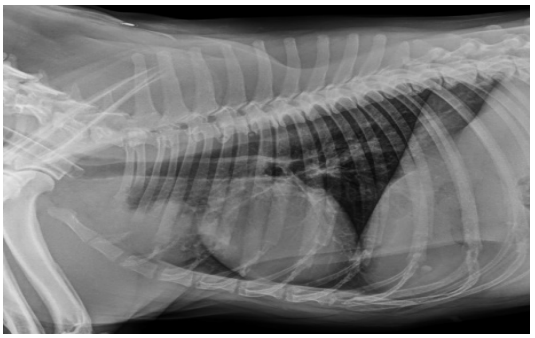

Fig. 3. Lateral view of the thoracic cavity: large, soft tissue opacity mass in the cranio-ventral part of the thoracic cavity

Fig. 4. Sagital view of the left antebrachium: smooth, solid periosteal reaction that is mostly paralel to the cortices of the radius and ulna

The contrast enhanced CT study confirmed a large, heterogeneous mass in the cranial mediastinum, thought to be metastatic lymphadenopathy (Fig. 5); several other small pulmonary masses were also identified (Fig. 6).

A large mixed-type bony mass was also identified in the proximal metaphysis and proximal part of the diaphysis of the right humerus (Fig. 7).

Bony changes found on the CT are usualy similar to those found on survey radiographs (15). CT is also useful in identifying more subtle changes or lesions, since there is no superimposition.

Soft tissue swelling is usually present, as is in this case, with no evidence of cortical lysis (6).

HOP needs to be differentiated from bone tumors (4). In rare cases, other bones (eg. carpi, tarsi, axial skeleton) may be involved, but not the articular margins of long bones, eventhough osteophytes can occur (6).

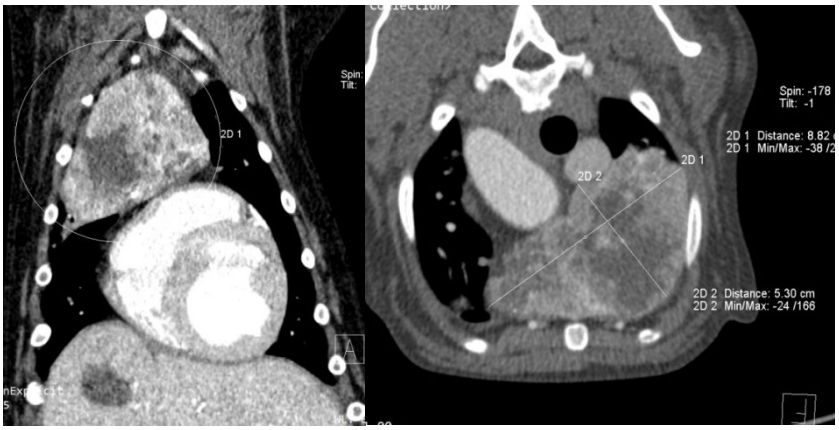

Fig. 5. Dorsal view (left) and transverse view (right) of the thoracic cavity: large heterogenous mass in the cranial mediastinum

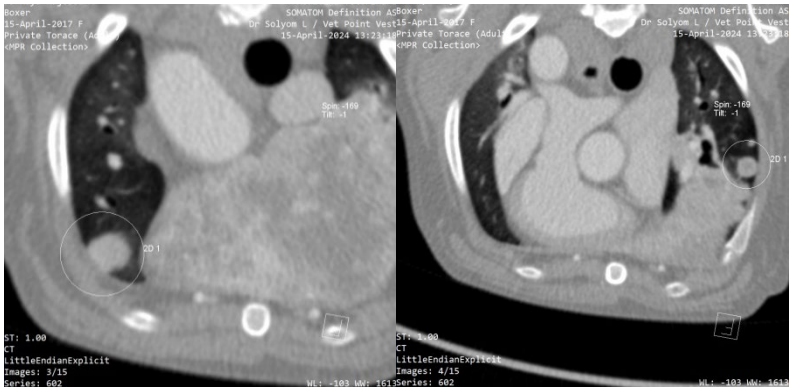

Fig. 6. Transverse views of the thoracic cavity: small, well-defined, soft tissue nodules located in both lungs, suggestive for metastases (circles)

Based on the changes observed in the proximal humerus, the diagnosis was established as being primary bone tumor, most likely osteosarcoma.

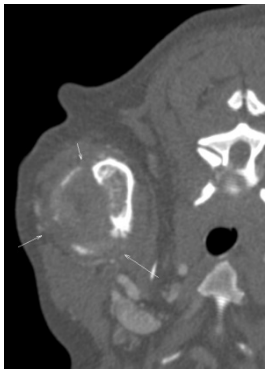

Fig. 7. Transverse CT of the proximal humerus revealing periosteal reaction and osteolysis of caudal and lateral cortices (arrows)

Osteolysis accompanied by irregular periosteal reaction, Codman's triangle and mild soft tissue swelling were noted in the proximal right humerus, findings which are suggestive for primary bone neoplasia – Fig. 8 (6).

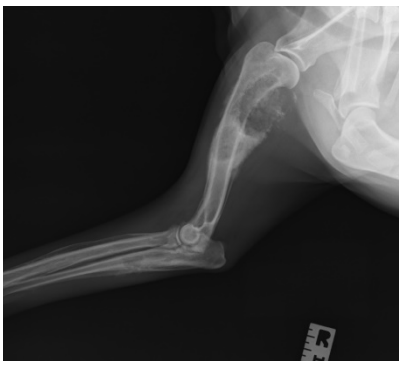

Fig. 8. Medio-lateral view of the right forelimb showing the humeral tumor

Primary bone tumors involving the appendicular skeleton usually appear in the metaphysis. Osteosarcoma being the most common primary bone tumor in canine patients (more than 80%), and with its occurence in the forelimb being „away from the elbow", this was the main suspicion (6).

The remainder of the lesions – the large mediastinal mass and other pulmonary nodules – were considered metastases from

the humeral lesion, as primary bone tumors frequently metastasize to the lungs (6), and with this, the HOP was considered to be secondary to the intrathoracic masses.

Biopsy of the masses was declined by the owners, who eventually chose humane euthanasia based on the advaned stages of the primary condition.

Treatment should based on and focused on the primary condition (4); regression of bony changes may happen after addressing it (1, 8).

Prognosis is also based on the possibility of complete resolutin of the main condition (4).

Conclusions

Clinicians whener facing with patients with radiographic signs of hypertrophic osteopathy should focus on identifying the primary cause.

CT is superior to other conventional imaging modalities in identifying the primary cause of hypertrophic osteopathy.

Acknowledgements

The authors declare that they have no conflict of interest. The case was initially seen at Vetpoint Vest Arad, for which the Faculty is grateful for referring and the collaboration.

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CRYPTORCHIDISM – AN IMPORTANT MALE REPRODUCTIVE PATHOLOGY IN DOGS AND CATS

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Abstract

Cryptorchidism, a disorder of sex development (DSD), is a developmental abnormality characterized by the fail of descent of one or both testes to the scrotum, which has significant consequences for male health. With this bibliographic study, we aim to systematize the latest information on the possible genetic, epigenetic, and other causes of the occurrence. Other topics detailed are on testicular morphogenesis, clinical types of cryptorchidism, the main risks to male health - testicular tumors, such as seminomas and Sertoli cell tumors, or testicular torsion; types of paraclinical investigation (ultrasound, serum Anti-Müllerian hormone level, molecular biomarkers).The incidence among dogs and cats is discussed along with treatment, prevention and medical perspectives on reducing the incidence. Cryptorchidism is considered to be a hereditary condition, with an increased number of familial cases in some dog breeds. Genetic studies suggest that several gene mutations are implicated in the failure of the process of testicular migration. Recent genomic and epigenomic data suggests possible biomarkers associated with cryptorchidism. This condition not only affects male's health, but also limits their access to breeding and pedigree.

Keywords: *cryptorchidism, male genital tract, testes migration, biomarkers, epigenetic modifications.*

Cryptorchidism is a developmental abnormality characterized by the inability of one or both testicles to descend into the scrotum. The retained testes may remain in the abdomen or become stuck in the inguinal canal, can impair testicular steroidogenesis and spermatogenesis (20), causing health problems such as infertility, increasing the risk of testicular tumors, and torsion, with an incidence rate of 0.8-10% (3, 26). If only one testis is affected, this condition is referred to as *unilateral cryptorchidism*, as opposed to monorchidism, a term used to describe individuals who have only one testis, regardless of its position (23). A cryptorchid testis can be located on any portion of its migration path from its initial upper abdominal position to its normal scrotal position when no abnormality is present. However, it is not very clear if this condition it's given by one of these two hypotheses: a) abnormal position of the testes resulting increased temperature that may affect the testicular cells, and therefore rising the risks of cancer, or b) the genetics and environmental factors influence the development of the male reproductive system *in utero*, leading both to cryptorchidism and testicular cancer (26).

Testicular morphogenesis

To understand the mechanism of cryptorchidism, it is necessary to describe the morphogenesis of the testes and the stages of their migration to the final position. The gonadal stage begins immediately after the chromosomal stage and in the presence of Y heterosome, the testes will be formed. The place of development of future gonads is the cranial pole of the mesonephros, and their evolution takes place in two phases. In *the undifferentiated stage* of gonad development, the primordial germ cells (PGS) must migrate from the yolk sac to the gonad precursor region to form the pregonad or the gonadal primordium. In dogs this process take place on day 28 and in cats when the embryo is 10 mm (11, 16). *The sexual phase* is influenced by the predominance of sexualization genes and the reception of target tissues. The hallmark of testicular differentiation under the influence of the Y gonosome is the appearance of Sertoli cells, mesenchymal cells, which aggregate to surround the PGC cells to differentiate into seminiferous cords. At the same time, Leydig interstitial cells, also mesenchymal cells at their origin, appear around the sexual cords. Into 2-3 days after they arrival, fetal Leydig cells produce testosterone and perhapse insulin-like peptide 3 (INSL3). If at the begining testosterone is produced constitutively, later GnRH and LH will regulate the process. The dominant gene for male pathway development is *SRY* gene (sex determining region of the Y gene). *SRY* gene is a member of the *SOX* family of transcription factors, it is placed in the terminal region of the short arm of the Y chromosome, TDF (testis determining factor) region. The gene consists of a HMG (high mobility group) box that is highly conserved and a region outside the HMG box that is species-specific and variable (1). As it can be seen in Fig.1, the descending of testes take place in some distinct phases.

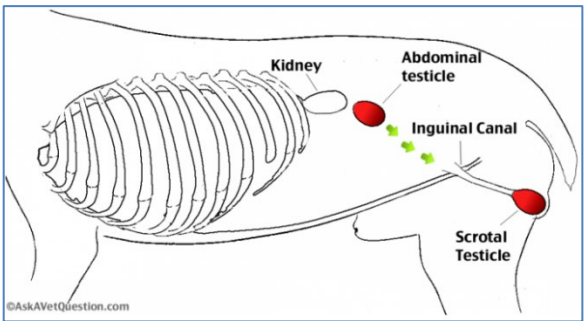

Fig. 1. Representation of the migration trajectory of the testes (30)

The three main phases of descenting of testes are:

- the enlargement of the testes and the regression of the suspensory ligament, which is the cranial suspensory ligament of the testis (derived from the diaphragmatic ligament of the mesonephros); also gubernaculum, with mesenchymal cell origin from the muscle fibers of the abdominal wall, caudally attached to the testes- drives migration of the testes to the internal ring of the inguinal canal (20);
- transabdominal descending, to the level of the inner opening of the inguinal canal; mainly controlles by androgens, leading to testicular descent into the scortum through the inguinal canal (20);
- transinguinal descending and trans-scrotal phases are mediated by testosterone and genito-femoral nerve (11, 26);

The transabdominal descending is mainly controlled by the *INSL3* hormone and its receptor *RXFP2*, while for the transinguinal descending, the control in under testosterone and its receptor *AR*, and involves the guidance of the gubernaculum testis (11, 20, 24, 25).

Etiopathogenesis. Genetic and epigenetic factors

Cryptorchidism is a condition suspected to have a genetic cause. To demonstrate the existence of a genetic component, it is first necessary to observe a significant number of familial cases compared to the general population. Suspicion of a hereditary component to cryptorchidism initially arose from a study of families of dwarf Schnauzer dogs, which included cases of cryptorchidism. In addition, it was noted that among cryptorchid dogs, many were from matings with a high degree of inbreeding (18). Another recent study analyzed weaning data for 11.230 puppies from 12 purebred dog breeds, reporting a much higher incidence of cryptorchidism at 12 months of age or later from matings between putative carriers (i.e., parents of previously identified cryptorchids) compared to the incidence in the general population, 24.1% versus 2.1% (7). To establish with certainty a genetic component, it is necessary to demonstrate that this high number of familial cases is due to genetic factors.

Current hypothesis holds that multiple genes with low penetrance are involved in cryptorchidism, and their association may lead to the development of this condition, influenced by 'modifier' genes and environmental factors (14, 18, 23).

INSL3 (insulin like factor 3) gene and its receptor, *RXFP2* (relaxin family peptide receptor 2) gene, have been intensively studied in recent years in humans and mice because of their role in testicular migration (20). A high and specific expression of *RXFP2* has been detected in various tissues involved in testis development, and *RXFP2* is up-regulated by *SOX9*, a gene important for testis formation (4). Studies have confirmed that serum levels of *INSL3* can serve as an indicator of testicular condition in horses. Specifically, *INSL3* levels provide insight into the position of the testes, whether descended or undescended (20).

A study of 22 cryptorchid and 56 healthy small-breed dogs analyzed differences in *INSL3* mRNA expression and tissue *INSL3* concentrations. The testes were categorized into three categories: normal scrotal testis, undescended testis in unilateral and bilateral cryptorchid dogs, and scrotal testis in unilateral cryptorchid dogs (8). It is already proven that *INSL3* plays a key role associated with cryptorchidism among multiple species (25). mRNA expression of *INSL3* was significantly higher in scrotal testis, and *INSL3* peptide concentrations showed a similar pattern. However, the total amounts of *INSL3* mRNA and peptide per undescended testis were significantly lower due to their low weight (8). In dogs, it is plausible that cryptorchidism is primarily associated with a loss-of-function mutation in the *RXFP2* gene rather than in

INSL3. Mutations in *INSL3* are unlikely to be related to inguinal canal entrapment, a common form of cryptorchidism in dogs, as *INSL3* does not regulate transinguinal migration (25). For *RXFP2*, the concentrations and amount of mRNA in undescended testes were almost zero compared to normal scrotal and scrotal testes in cryptorchid dogs. The results suggest that although *INSL3* is expressed in undescended testes, overall expression may be reduced due to lower testicular weight. In addition, the *RXFP2* gene is almost not expressed in undescended testes, but is active in scrotal testes of healthy and cryptorchid dogs, but the absence of *RXFP2* expression in adults does not imply its absence in the fetal and neonatal period (25).

There is evidence that global depletion of INSL3 or RXFP2 results in cryptorchidism in animal models. However, *RXFP2* expression has not been detected in adult cryptorchid dogs, but this does not exclude its presence in the prenatal and neonatal period (13).

Studies in humans suggest a role for the *ESR1* (estrogen receptor ESRα) gene in testicular migration and cryptorchidism (5, 10). However, in dog studies, the associations have not been as clear. In a study of 40 cryptorchid dogs and 68 healthy dogs (Miniature Dachshund and Chihuahua) no associations between SNPs in the 3' region of the *ESR1* gene and cryptorchidism were observed. Another study showed an increased immunoreactivity of ESRα protein in cryptorchid testis compared to scrotal testis in one-month-old German Shepherd dogs (12). In scrotal testis, ESRα was detected only in Leydig cells, but in cryptorchid testis, it was also present in Sertoli cells, suggesting a specific expression pattern (12). Another study did not identify ESRα immunoreactivity in adult testis, indicating that the expression of this protein might be limited to a window of time in early life (22).

Cryptorchidism and aging in dogs are related to certain miRNAs (cfa-miR-148a, cfa-miR-497, and cfa-miR-1841), which suggests that these miRNAs could be used as biomarkers for cryptorchidism (15). MicroRNAs, epigenetic regulators of gene expression, were observed to be dysregulated in canine cryptorchid and seminoma-affected testes. One example is the downregulation of miR-302c-3p, which suggests an increase in estrogen receptors. Increased estrogen exposure can have an impact on the development of gonads (21).

Possible genetic biomarkers for cryptorchidism in dogs can be considered hypomethylation in CpG site from 5'-flanking region of *INSL3,* and a SNP in the 5' flanking region of *RXFP2* gene. These two genes have a crucial roles in the transabdominal migration of the testes (20). A recent genetic study identified a massive gene expression alteration (about 8,000 genes) in dogs with unilateral inguinal cryptorchidism. The cause of this large change in the transcriptome can be found in aberrant DNA methylation and histone H3 (H3K9) acetylation patterns (24).

The main genes associated with cryptorchidism seen in dogs and other species are seen in Table 1.

Table 1

Genes associated with cryptorchidism

Gene	Function	Species	References
INSL3	Hormone that regulates the migration of the testes trans-abdominally	Human Dog	(8, 12, 20, 25)
RXFP2	Receptor of INSL3. Also regulates the migration of testes trans-abdominally	Human Dog	(12, 20, 25)
HMGA2	Non-histone chromosomal protein that regulates gene transcription.	Dog	(25)
MMP9	Enzyme that it is responsible for remodeling and transcription of the extracellular matrix	Dog	(25)
ADAMTS20	Enzyme that it is responsible for remodeling and transcription of the extracellular matrix	Dog Rat	(12, 25)
MID1IP1	Interacting protein. Regulates microtubule depolymerization.	Dog	(25)
KIT, KITLG	Tyrosine kinase receptor and ligand. They mediate the proliferation, migration, and cell survival of germ cells.	Human	(25)
ESR1	Estrogen receptor. Regulates the transcription of multiple genes involved in growth, metabolism, and sexual development.	Human Mice	(5, 10, 13, 25)
MED12	A subunit of the CDK8 subcomplex, it modulates Mediator-Polymerase II interactions, thereby regulating transcription initiation.	Human	(25)

UCN	Urocortin, a corticotrophin-releasing hormone (CRH)-related peptide; role in the autocrine/paracrine regulation of prostatic function, possible role in the regulation of spermatogenesis, sperm motility and testosterone releasing.	Dog	(14)
AMHR2	anti-Müllerian hormone type II receptor, protein receptor present in the Müllerian duct mesenchyme	Dog	(19, 29)

AMHR2 mutations are known to cause Persistent Müllerian duct syndrome (PMDS), a type of XY, DSD, in miniature Schnauzer that predisposes dogs to unilateral or bilateral cryptorchidism (19, 29).

Anatomical causes

Cryptorchidism is a defect in testicular descent, and anatomical abnormalities may prevent this process (10). Persistent cranial suspensory ligament - this abnormality prevents proper translocation of the testis from the abdomen (18).

Incorrect positioning of the gubernacular bulb. If the gubernacular bulb guides the testis in the wrong direction, the testis will not reach the inguinal canal (23). Disproportion in size between the testicle and the inguinal canal - in small dogs, larger testicles relative to the canal may have difficulty in transinguinal migration (23).

Endocrine causes

Endocrine factors play a key role in cryptorchidism. Testosterone is crucial for testicular descent, and any dysregulation can lead to cryptorchidism (10). Testosterone insufficiency or androgen insensitivity can prevent complete migration of the testis into the inguinal or scrotal area, leading to abdominal or inguinal cryptorchidism.

Environmental causes

Certain environmental factors are associated with cryptorchidism, especially in humans. Prenatal exposure to estrogen can inhibit the secretion of *INSL3* and androgens, crucial factors in testicular descent. Phthalates and pesticides - studies in rodents have shown that these chemicals, having estrogenic or antiandrogenic effects, can inhibit testicular descent (25, 27). Analgesics such as paracetamol, ibuprofen and acetylsalicylic acid - at high doses, these drugs have antiandrogenic effects similar to phthalates, but there are no equivalent studies in dogs.

Clinical types

Several types of cryptorchid testis are recognized in mammals: *spontaneously descended testicle*: a testicle that was initially not present in the scrotum, but has subsequently descended spontaneously, *retractile testis*: a palpable testicle, situated slightly above the scrotum, close to the physiologic position, of normal size and shape, it can be pulled into its normal position, but later returns to its original position. *Sliding testicle* is the condition when a testicle situated in the inguinal canal which can be pulled towards the base of the scrotum, but immediately returns to the inguinal position once the tension is released. *Truly undescended testis*: a testis that has stopped migrating between the retroperitoneum and the scrotum. *Ectopic testis*: a testis that has passed through the external inguinal ring but has not reached the scrotum, being found in aberrant positions such as the contralateral scrotum, femoral or perineal region (2).

Cryptorchidism in dog can be described according to the location of the retained testicle as *abdominal, inguinal* and *prescrotal,* according to the number of testicles affected as *unilateral* or *bilateral*, and according to the affected body side as *right* or *left.* In cats, cryptorchidism can be also categorized as *unilateral* or *bilateral* and depending on the location of the testis, as *intra-abdominal*, *inguinal*, *or pre-scrotal*.

The main risks to male health of cryptorchidism

A cryptorchid animal is predisposed to various disorders. First, studies have shown that the cryptorchid testis has multiple histologic defects and bilateral cryptorchid animals have no fertility (23). Blockade of spermatogenesis, reduction of the seminiferous epithelium and significant histologic alterations may be associated with heat stress, which affects Sertoli cell function (3, 9).

There is also evidence that cryptorchidism increases the risk of testicular tumors, 23 times higher for seminomas and 16 times higher for sertolinomas, and these tumors occur earlier in the animal's life (15). The main types of testicular tumors that can occur are seminoma and mixed germinal-stromal tumors, and less commonly Sertoli and interstitial cell tumors (9, 17). These tumors can cause feminization syndrome and bone marrow suppression, potentially fatal (15). Cryptorchid animals are also more prone to testicular torsion because the cryptorchid testis, especially in the abdominal position, exhibits greater mobility.

Cryptorchidism is frequently associated with other genetic defects, such as umbilical and inguinal hernias or orthopedic defects, including hip dysplasia and patellar luxation

(23), tarsal deformity, supranumerary kidney with uretheral hypoplasia, a shortened or kinked tail, tetralogy of Falllot, microphthalmia and upper eyelid agenesis. In view of these consequences and the suspicion of a hereditary origin, cryptorchidism is considered a reproductive eliminating defect, so affected animals are denied pedigree and the right to breed as a purebred.

Diagnostic

The descent of male gonads is essential for male fertility in most mammalian species. When gonads fail to descend normally, this can interfere with testicular hormone production and spermatogenesis, as well as increase the risk of malignancy. Cryptorchidism is the most frequent sex development disorder (DSD) in dogs, affecting an estimated 3.3% to 6.8% of the population. Evidence of a hereditary component comes from its repeated appearance within certain families, particularly Miniature Schnauzers, and the overrepresentation of several breeds among those affected, including Chihuahuas, Boxers, German Shepherds, Yorkshire Terriers, Staffordshire Bull Terriers, Shih Tzus, Pomeranians, Miniature Poodles, Miniature Dachshunds, Cairn Terriers, Maltese Terriers, and Siberian Huskies (20).

Diagnosis begins most of the time with a *physical examination*. The scrotum can be *palpated* to check whether or not both testicles are present (3). Testes are normally descended by 10 days of age in dogs but may be difficult to palpate in young pups due to their small size and the presence of scrotal fat therefore a diagnosis is usually not confirmed until 10 weeks of age. There is also the opinion that the diagnosis can only be confirmed in dogs over 6 months of age, as there is still the possibility, unlikely of course, but still possible, that the testis may descend without any external intervention. However, after 6 months this is no longer possible because the inguinal ring closes permanently at this age.

The inguinal testicle is palpable and does not necessarily require an ultrasound scan to locate it, unlike the abdominal testicle where a more detailed examination is required (3). Careful palpation of the scrotum and the region of the inguinal canal can be an accurate indicator, but care must be taken not to mistake fat or a lymph node for a retained testicle, which is quite common. If it is a testicle, it should be able to move freely and it should have a palpable epididymis attached to it (26). To localize undescended testicles or to confirm the presence of the testicles, an imaging method, such as ultrasound or radiography (X-ray) may be used.

Radiographic assessment of the male reproductive tract is indicated in dog with cryptorchidism. Survey abdominal radiographs may also identify cryptorchid testes that have undergone malignant transformation and are enlarged, retroperitoneal lymphadenopathy of the iliac lymph nodes in dog with metastatic testicular tumors.

Though, the "gold standard" in the diagnosis of this disease is *ultrasound diagnostics* with a sensitivity of 95-100%. However, the effectiveness of this technique can vary depending on whether the testis is located in the inguinal canal or within the abdomen. If the testis is retained in the abdomen, it may be smaller in size and situated in a wider range of locations within the abdominal cavity, making detection more challenging. As a result, ultrasonography tends to be more effective in identifying testicles retained in the inguinal canal. If an ultrasound successfully detects a testis retained in the abdomen, it should exhibit a structure similar to that of a scrotal testis, unless complications are present (26).

Advancements in diagnosing cryptorchidism in dogs include distinguishing bilaterally cryptorchid dogs from castrated ones through *rectal prostate palpation* or *hormone assays*. Intact dogs have larger, easily palpable prostates and show elevated serum testosterone levels after gonadotropin-releasing hormone stimulation (14). A study conducted on 20 dogs identified benign prostatic hyperplasia in 32.8% of cases (17).

A study investigated the use of anti-Müllerian hormone (AMH) as a diagnostic marker for cryptorchidism in dogs. The research included 10 bilaterally cryptorchid dogs, seven intact dogs, and seven castrated dogs, all over six months of age and mostly toy breeds. Results showed that serum AMH levels were significantly higher in both cryptorchid and intact dogs compared to castrated dogs. Similar results were observed also by (6).

For diagnosing cryptorchidism in cats, examining the penis for the presence of spines is also effective. These spines, which depend on testosterone, tend to atrophy within six weeks after castration. If they are still present after the operation, this means that one of the testicles has probably not been removed correctly and may influence the cat's behaviour and show signs of aggression or frequent urination like an un-neutered cat. In these cases, the best thing to follow the intact ductus deferens to the small testis adjacent to the pubic symphysis to locate undescended testis (3).

Transcutaneous ultrasonography is helpful to confirm findings for a cryptorchid testis under skin in the inguinal region and an transabdominal ultrasonography to locate an abdominal cryptorchid testis.

Treatment

Reducing the occurrence of cryptorchidism should involve minimizing the exposure of pregnant females to environmental agents that are estrogenic, anti-androgenic, or harmful through other mechanisms, based on evidence that concentrations of harmful substance can be altering fetal develpoment.

Cryptorchidectomy is the preferred treatment for cryptorchidism, as affected dogs should not be used for breeding due to the risk of passing the condition to their offspring. It is also essential to address the increased risks of testicular torsion, testicular neoplasia, and impaired spermatogenesis, even when cryptorchid testes still produce testosterone. The surgical approach to locate and remove the retained testicle varies depending on its position. Treatment primarily aims to reduce the risks of neoplasia and heritable defects, as cryptorchid males may still be fertile. Consequently, castration is the most common treatment option for cryptorchidism. The retained testicle can be removed by a laparoscopic or laparoscopic-assisted cryptorchidectomy with two ports is an appropriate surgical procedure to treat cryptorchid cats with intra-abdominal testes, with all benefits of minimal invasion surgery. Traditionally, intra-abdominal testes were removed through a conventional celiotomy or minimal laparotomies with the use of a spay hook, with potential complications such as trauma of the abdominal structures. If it's a case with intra-abdominal testes, a laparotomy for a caudal midline approach is advised most of the time. A study made on nineteen cryptorchid cats showed that laparoscopic cryptorchidectomy is an appropriate procedure to treat cryptorchid cats, with all benefits of the minimal invasive surgery, such as a better visibility of abdominal structures, shorter hospitalization times, lower morbidity, less surgical site infections, and most importantly, minimized surgical pain and discomfort (28).

Two commonly used incisions for removing abdominal testes are the parapreputial skin incision and the paramedian abdominal wall incision. The paramedian incision is often less desirable due to higher risk of muscular bleeding and limited access to the abdominal cavity. In contrast, a parapreputial skin incision with a midline abdominal wall approach is generally preferred, as it provides clearer visibility and reduces bleeding, though it requires more extensive tissue dissection. If the testicle is difficult to locate, the midline incision can be extended, allowing access to any area between the kidneys and the inguinal ring, with minimal tissue trauma.

In human medicine a possible surgical treatment is *orchiopexy,* define as surgical placement and fixation of the cryptorchid testis into the scrotum, but in veterinary medicine is not considered a tratment option due to the hereditary origin of cryptorchidism.

In some cases of cryptorchidia, tumours may develop if that case lasts too long. Therefore, a conservative approach should be regarded with caution and a close follow-up of the preserved testes is recommended (25).

Concerning the possibility of hormonal treatment for cryptorchidism cases, it's not that common in animals. Only few studies have been made, and it needs furthers researches about this subjects for more information about it. Only one controlled study has examined the efficacy of medical treatment with GnRH. This study included 10 puppies with an undescended testis at 49 days. Five were treated with buserelin (GnRH) and five served as a control group. The testis descended in 80% of the treated cases compared with 20% of the controls, the latter corresponding to the spontaneous descent observed in some puppies by 6 months. Despite these results, medical treatment is not recommended because of the hereditary nature of the condition and the associated risks (testicular tumours, torsion of the spermatic cord), although in human medicine, hormonal therapy is more used, even though the surgical treatment is still on choice (26). Overall, dogs with cryptorchidism should not be included in breeding programs. Effective control of this condition involves removing affected dogs, as well as ideally their dams and sires, from breeding lines. Bilateral castration is the recommended treatment.

Conclusions

Cryptorchidism is a disorder of sex development, with genetic, epigenetic and environmental causes. Because genetic factors are the main factor in dogs, with the help of recent research in genomic and epigenomic data, some possible biomarkers have been established. For abdominal cryptorchidism, possible biomarkers can be considered hypomethylation in CpG site from 5'-flanking region of *INSL3,* and a SNP in the 5' flanking region of *RXFP2* gene, due to the fact that these two genes have a crucial roles

in the transabdominal migration of the testes. Certain miRNAs (cfa-miR-148a, cfa-miR-497, and cfa-miR-1841) could be used as biomarkers for cryptorchidism, these epigenetic regulators of gene expression were observed to be dysregulated in canine cryptorchid and seminoma-affected testes. Another biomarker, but this time for the testicular degeneration and impaired spermatogenesis, that is seen in cryptorchid testes, is the serum level of AMH. Elimination or reduction of environmental factors that have a estrogenic or anti-androgenic effect can help in reducing the incidence of cryptorchidism.

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RETROSPECTIVE STUDY ON PREVALENCE AND RISK FACTORS IN LOWER URINARY TRACT DISEASE IN CATS

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Abstract

Feline Lower Urinary Tract Disease (FLUTD) is a term used to describe a spectrum of disorders affecting the ureters, urinary bladder, and urethra, manifesting in symptoms like haematuria, pollakiuria, stricture, and urethral obstruction. Understanding the multifactorial causes of FLUTD is essential for developing effective treatment plans. Therapeutic management includes relieving symptoms, treating the underlying cause, and preventing future episodes. This requires a comprehensive approach, including dietary changes, creating a stress-free environment, and sometimes using medications. This study aimed to evaluate FLUTD prevalence in cats, considering age, gender, breed, and environmental factors, and highlight associated laboratory findings. This study examined medical records from cats diagnosed with FLUTD at the University Veterinary Clinics (CVU) from the Faculty of Veterinary Medicine, Timisoara. The highest prevalence was found in European breed cats aged 3-5 years, with males being predominant. Idiopathic cystitis emerged as the predominant condition, with pollakiuria as the primary clinical sign. Additionally, renal biochemical parameters were frequently elevated above the upper reference values. This study provides valuable insights into the epidemiology of FLUTD and emphasizes the need for thorough diagnostic methods to achieve the best treatment outcomes.

Keywords: *feline lower urinary tract disease, idiopathic cystitis, cats, pollakiuria.*

Feline lower urinary tract disease (FLUTD) is a complex and multifactorial condition that includes a variety of disorders affecting the lower urinary tract, including the ureters, urinary bladder, and urethra, in domestic cats. The term "FLUTD" describes these conditions without specifying the underlying cause, making it a clinical diagnosis rather than a single disease entity. The most common signs of FLUTD are frequently observed in affected cats and include haematuria (blood in the urine), pollakiuria (increased frequency of urination), dysuria (painful urination), and, in more severe cases, urethral obstruction. These clinical manifestations can significantly impact the cat's quality of life, leading to discomfort, distress, and, in extreme cases, life-threatening complications (15).

The pathophysiology of FLUTD is diverse, with several potential etiologies ranging from metabolic disorders to infectious agents and from traumatic injury to neoplastic growths. Some of the most common causes include urinary tract infections (UTIs), bladder stones (uroliths), feline idiopathic cystitis (FIC), and urethral obstructions. Metabolic conditions, such as diabetes mellitus and hyperthyroidism, can also predispose cats to urinary tract problems, while trauma from accidents or surgery can directly compromise urinary tract function. Furthermore, genetic predispositions are thought to play a role in developing certain FLUTD forms, with some breeds exhibiting a higher incidence of urinary issues. Neoplastic conditions, although less common, can present as a potential cause of FLUTD, with tumours such as transitional cell carcinoma affecting the urinary bladder or urethra (14, 15).

In some cases, the exact cause of the lower urinary tract symptoms remains unclear despite extensive diagnostic workups. These cases are categorized as "idiopathic FLUTD," where no definitive aetiology can be identified. Feline idiopathic cystitis (FIC) is the most common manifestation of idiopathic FLUTD and is believed to have a multifactorial origin involving environmental stress, neurogenic inflammation, and abnormalities in bladder tissue. The pathogenesis of FIC, while not fully understood, is thought to involve an interplay of environmental factors, urinary tract microbiota, and the cat's inherent stress response mechanisms. Idiopathic forms of FLUTD often present as recurrent episodes with varying severity and can be challenging to manage, mainly because effective treatment options are still being investigated (14, 16).

The diagnosis of FLUTD involves a comprehensive approach, including a detailed history, clinical examination, and diagnostic tests such as urinalysis, urine culture, radiography, and ultrasonography. These tools allow veterinarians to rule out or identify potential causes such as infections, stones, or tumours. In cases where no definitive cause is found, further tests may be conducted to explore other potential underlying factors, such as metabolic disorders, trauma, or the possibility of idiopathic disease. Although treatment varies depending on the diagnosis, options can include medical management, dietary changes, surgical intervention, or

pharmacologic agents to reduce inflammation, manage pain, and prevent further urinary tract obstructions (10, 11).

One of the challenges in managing FLUTD is its recurrence, particularly in cases where an idiopathic cause is suspected. Managing stress, which is a well-recognized trigger for FIC, is crucial in preventing future episodes. This involves addressing both environmental and behavioural factors, such as providing a safe and enriched living space for the cat, ensuring access to clean litter boxes, and minimizing conflicts between cats in multi-cat households. For many affected cats, long-term management strategies are necessary to minimize the impact of recurrent episodes of FLUTD (5).

Regarding epidemiology, studies have shown that FLUTD can affect cats of all ages, breeds, and genders. However, certain risk factors appear to be more prevalent in specific groups. Male cats, particularly those who are obese or have anatomical predispositions such as a narrow urethra, are at a higher risk for urethral obstruction. Breed-specific trends have also been identified, with certain breeds, such as the Persian and Siamese, being more susceptible to urinary tract issues. Age is another critical factor, with younger and middle-aged cats being more commonly diagnosed with FLUTD, although elderly cats may present with similar symptoms due to concurrent metabolic diseases or neoplastic processes (5, 9).

Given the varied nature of FLUTD, understanding its prevalence and risk factors across different populations of cats is essential for developing targeted prevention and management strategies. The interplay of genetic, environmental, and physiological factors contributes to the complexity of this disease, and ongoing research is critical to unravelling the underlying mechanisms that predispose cats to develop FLUTD. It is also important to emphasize the role of early diagnosis and intervention in preventing severe complications such as renal failure, urethral rupture, and sepsis, all of which can arise from untreated or poorly managed urinary tract diseases (12, 16).

The present study aimed to evaluate the prevalence of feline lower urinary tract disease depending on age, gender, and breed and to highlight laboratory findings in cats presented for consultation with various lower urinary tract pathologies.

Materials and methods

The medical records of 2010 cats presented to the University Veterinary Clinics (UVC) at the Faculty of Veterinary Medicine, Timisoara, between 2020 and 2023 were reviewed. The following data were collected for each cat: breed, age, gender, living environment, clinical signs, and laboratory findings. Out of these records, 84 cats were diagnosed with feline lower urinary tract disease (FLUTD), presenting various lower urinary tract pathologies. Blood samples were analyzed at the Laboratory of Functional and Metabolic Explorations, University of Life Sciences, Timisoara. Complete blood counts (CBC) were performed using flow cytometry with an automated haematology analyzer (ProCyte Dx – IDEXX). The blood biochemical parameters, including albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), total protein, creatinine, urea, cholesterol, and triglycerides, were measured using standard methods with an automated biochemistry analyzer (Rx Daytona+ - Randox).

Results and discussions

In this study, the prevalence of FLUTD in cats within University Veterinary Clinics (UVC) is 4.17%, with 95.83% of the feline population unaffected. Depending on the geographical area, FLUTD among cats brought for consultation in veterinary hospitals and clinics is estimated to be 1.5-2.2% (7, 11, 12). These results indicate that FLUTD is a relatively low-prevalence condition in the studied population, highlighting the importance of targeted diagnostic and therapeutic strategies for this specific subgroup of affected cats (Fig. 1). The dataset indicates a marked difference in the prevalence of FLUTD between males (82%) and females (18%) (Fig. 2). These results are similar to those found in a clinical trial conducted in Norway, in which 80% of the cats with LUTD were male (14). This aligns with the established understanding that male cats are more susceptible to urinary blockages due to their narrower urethras, predisposing them to complications like urethral obstruction. However, while less common in females, the condition may manifest as non-obstructive forms of FLUTD (10). These findings highlight the need for targeted monitoring and preventive measures in male cats. Also, in the study by Buffington et al. (1), they identified cats with increased nervousness and aggression among the risk factors associated with clinical signs of lower urinary tract disease. Therefore, one might speculate that male cats have more difficulty adapting to indoor housing and that gender may increase the risk of FLUTD (1, 2).

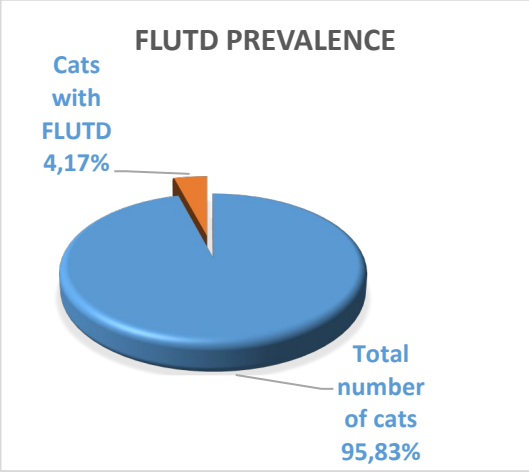

Fig. 1. FLUTD prevalence

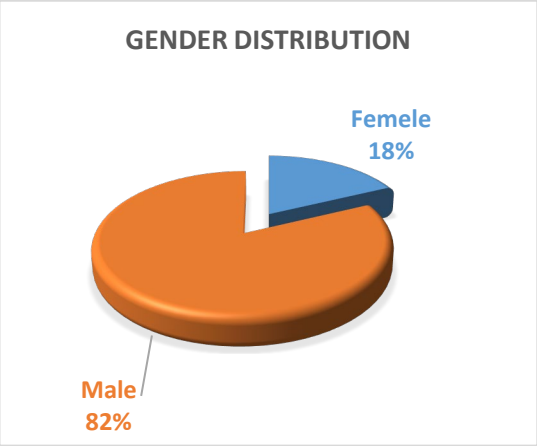

Fig. 2. Gender distribution

Regarding the breed structure of the cats diagnosed with FLUTD, it was found that European breeds exhibit the highest prevalence at 44%, followed by Persian cats at 33%, and British Shorthairs at 23% (Fig. 3).

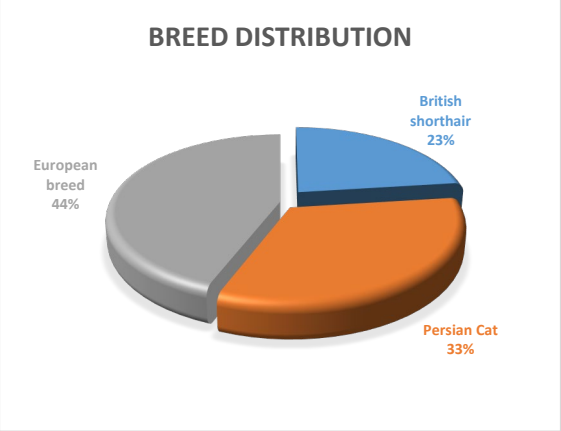

Fig. 3. Breed distribution

These findings suggest that European breeds may possess intrinsic risk factors, possibly genetic or environmental, that predispose them to FLUTD. Persian cats are also notably affected, likely due to a combination of genetic predisposition, stress sensitivity, and dietary influences. While exhibiting a lower prevalence, British Shorthairs still represent a substantial percentage of affected individuals, indicating that FLUTD is a concern across various breeds. The results of the present study are similar to those of a study conducted in Poland, in which mixed-breed cats showed an increased risk of FLUTD. In contrast, other studies have reported a positive association between Persian or other long-haired cat breeds and FLUTD. In the United States and Canada, purebred cats, including Russian Blues, Himalayans, Persians, Abyssinians and Manxes, showed an increased risk of FLUTD. The variability in the results is likely due to the popularity of particular breeds of cats in a particular area at different times. This variability indicates that in areas where pedigreed cats are more common, they become more frequently affected (7, 12). Age-related patterns indicate that FLUTD cases peak in cats aged 3-5 years (46%), followed by 1-2 years (27%), 6-8 years (18%), and 9-16 years (9%) (Fig. 4). Epidemiologic studies in recent years have shown that idiopathic cystitis is by far the most common cause of FLUTD in cats aged 1-10 years, accounting for 60-70% of all cases of FLUTD (6, 12, 14). These findings suggest that younger to middle-aged cats are at the highest risk, possibly due to diet, stress, and activity levels. The relatively lower prevalence in older cats may result from survivorship bias or the prominence of other age-related health conditions.

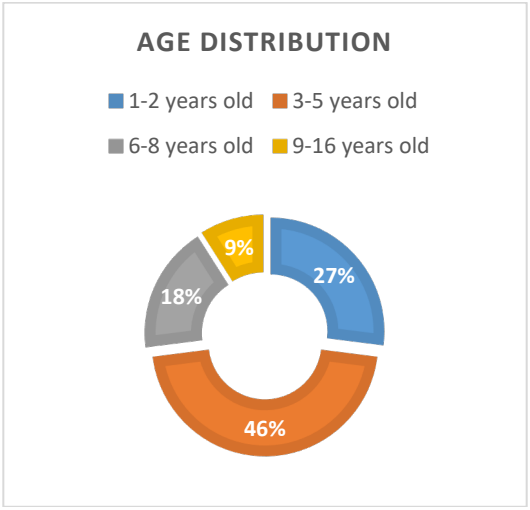

Fig. 4. Age distribution

Idiopathic cystitis emerges as the most prevalent FLUTD disorder (55%), followed by obstructive FLUTD (18%), bacterial cystitis (13%), and urolithiasis (13%) (Fig. 5).

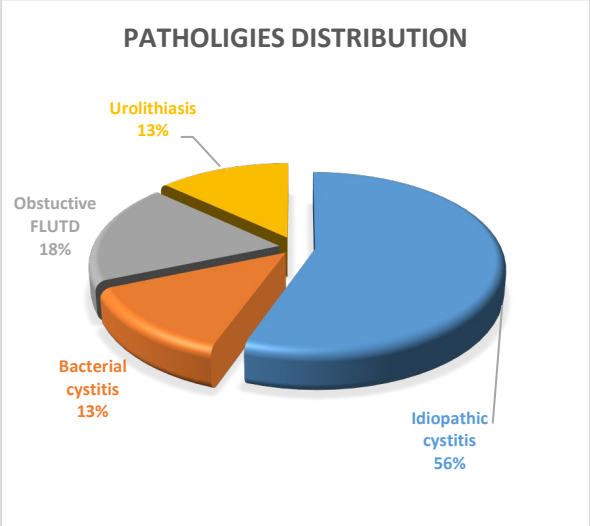

Fig. 5. Pathologies distribution

The dominance of idiopathic cystitis reflects its multifactorial aetiology, often involving stress and environmental factors. The results of the present study agree with reports in the literature, which reveal that feline idiopathic idiopathic cystitisaccounts for more than 50% of the causes of FLUTD (6, 7, 9, 12, 14). The most common symptoms include pollakiuria (70%), hematuria (56%), dysuria (54%), inappropriate urination (40%), and vocalization during urination (37%) (Fig. 6).

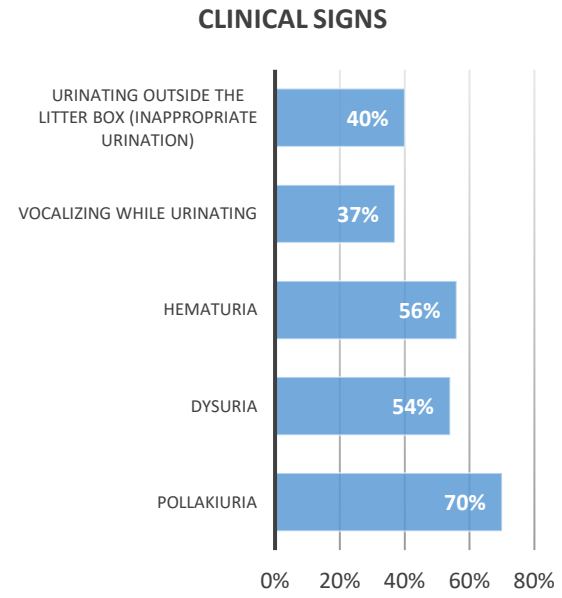

Fig. 6. Clinical signs

These findings indicate that FLUTD is often painful and distressing for cats, leading to noticeable behavioral changes. Early recognition of these symptoms by pet owners and veterinarians is crucial for timely diagnosis and treatment, potentially preventing severe complications like urethral obstruction. The reported frequency of crystalluria in FLUTD varies considerably, and in the study by Sævik et al. (14), the number of cats with crystalluria was considerably higher in the subsample with urethral plugs. Furthermore, Kruger et al. (6) suggest that males with feline idiopathic idiopathic cystitis (FIC) and crystalluria are at higher risk of urethral plug formation and subsequent urethral obstruction. Differentiating between obstructive FIC and urethral obstruction by urethral plugs can be difficult. The study by Gerber et al. (4) found that 55% of cats with idiopathic cystitis had urethral obstruction (4, 13).

The third cause of FLUTD in this study was bacterial cystitis, identified in 13% of cases. Some studies and textbooks report that bacterial cystitis is uncommon in cats, unless the defenses are compromised due to concurrent disease. Evidence suggests that in cats less than 10 years of age with signs of FLUTD, less than 2% of cases have been associated with a urinary tract infection (1, 13). In contrast, several more recent studies have reported a prevalence of 11% to 15.9% of bacterial cystitis as a cause of FLUTD (3, 4, 14).

Urolithiasis was the condition with one of the the lowest prevalence in cats with FLUTD in this study. In the majority of cats, uroliths in the bladder were composed of ammonium magnesium phosphates (struvites) and calcium oxalates. In other studies, the prevalence of urolithiasis as a cause of FLUD ranged from 11% to 21% (5, 12, 14, 16).

Indoor cats make up the majority of FLUTD cases (68%), followed by outdoor (17%) and mixed-lifestyle cats (15%) (Fig. 7).

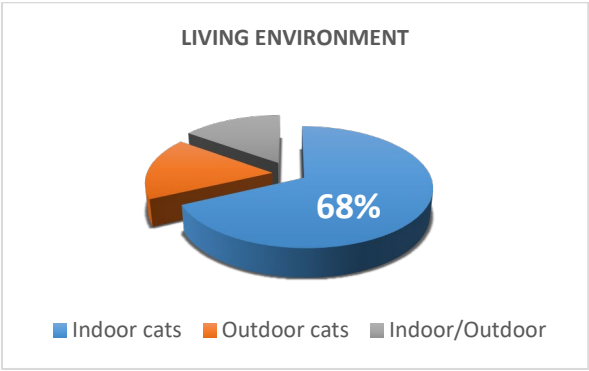

Fig. 7. Living environment

This prevalence among indoor cats may be linked to limited physical activity, environmental stressors, or dietary differences compared to outdoor cats. These results emphasize the need for environmental enrichment and stress management in indoor settings, such as providing sufficient play opportunities and minimizing sudden changes in the household. For example, Lue et al. (8) reported that owners with the strongest bond with their pets kept their pets indoors. Furthermore, owners with the strongest bond with their pets were more likely to require a higher level of medical care for their pets. It is also likely that cat owners who stay strictly indoors spend more time with their cat and that this allows them to more closely observe signs that indicate illness and seek veterinary care when they deem necessary (8).

The urea levels vary significantly across different FLUTD conditions in cats. Idiopathic cystitis shows the lowest mean urea level (30.23 mg/dL), followed by bacterial cystitis (35.32 mg/dL), while urolithiasis (42.01 mg/dL) and urethral plugs (59.5 mg/dL) exhibit progressively higher levels (Fig. 8). The marked elevation in cats with urethral plugs likely reflects severe urinary obstruction and subsequent azotemia. In contrast, milder increases in bacterial cystitis and urolithiasis suggest localized inflammation or partial obstruction.

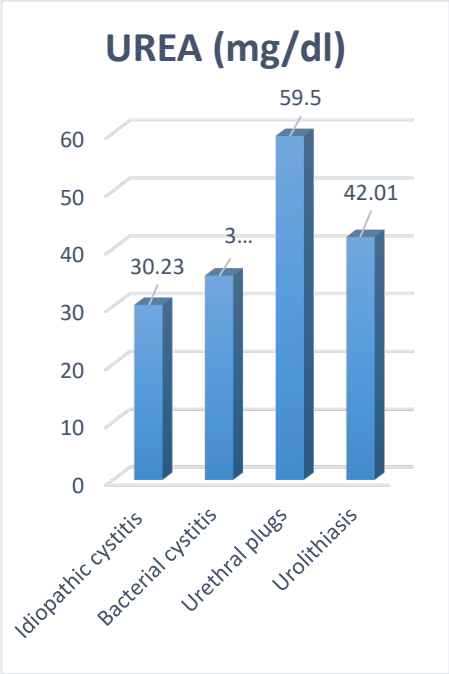

Fig. 8. Mean serum urea concentration values according to FLUTD cause

The creatinine levels across FLUTD conditions in cats are highest in cases of urethral plugs (2.3 mg/dL), reflecting severe obstruction and compromised renal filtration. Cats with urolithiasis show moderately elevated creatinine levels (1.7 mg/dL), while cats with idiopathic cystitis (1.3 mg/dL) and bacterial cystitis (1.1 mg/dL) present the lowest values, likely due to minimal systemic impact on renal function (Fig. 9)

These results underscore the role of creatinine as a marker for detecting significant urinary obstructions, with elevated levels highlighting more critical conditions such as urethral plugs or severe urolithiasis.

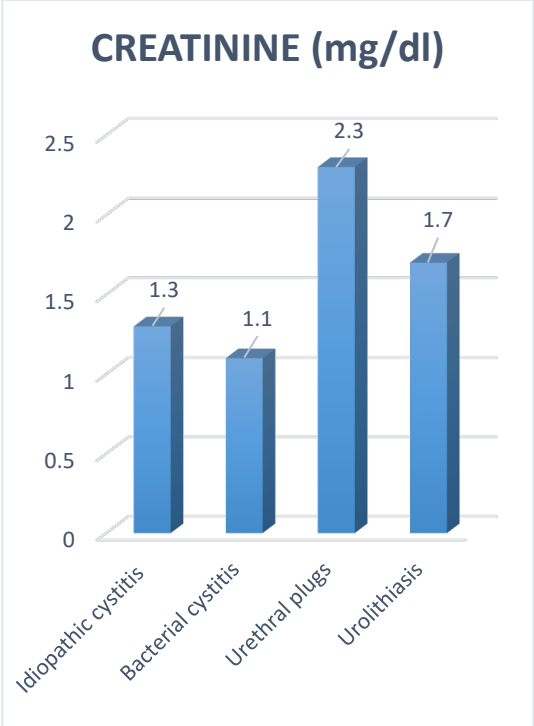

Fig. 9. Mean serum creatinine concentration values according to FLUTD cause

Conclusions

In this study, idiopathic cystitis was the most common cause of FLUTD, followed by urethral plugs and urolithiasis.

Clinical signs based on which FLUTD can be suspected are pollakiuria, haematuria and inappropriate urination behaviour.

In obstructive form of FLUTD, there is a risk of acute renal failure of post-renal cause resulting from blockage of urine flow through the urethra.

Male gender and indoor living environment were the main risk factors for developing FLUTD.

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DIFFERENCES AT MOLECULAR LEVEL BETWEEN BOS TAURUS L. AND BUBALUS BUBALIS L.

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Abstract

This study investigates molecular differences between *Bos taurus* (domestic cattle) and *Bubalus bubalis* (water buffalo), with a focus on the protein profiles of their milk and their genetic material. The research addresses the growing issue of food fraud in the dairy industry, where higher-value products like buffalo or sheep milk are sometimes replaced with lower-cost cow milk. Through the use of DNA barcoding and protein fingerprinting, we aimed to create a reliable identification method for detecting species in dairy products. DNA barcoding involves amplifying conserved short DNA sequences, allowing for species-specific identification, while protein analysis highlights unique patterns in the milk of these species. For the protein analysis, we used microfluidic electrophoresis, which provided high-resolution separation of milk proteins, and DNA sequencing to compare mitochondrial COI gene regions between the two species. The results demonstrated distinct differences in protein composition and genetic sequences between Bos taurus and Bubalus bubalis. Specifically, major deletions and single-nucleotide polymorphisms (SNPs) were observed, with a sequence similarity of approximately 87.39%, sufficient for species differentiation. Additionally, protein profiling revealed variations in casein and whey proteins, which influence cheese yield and quality. These findings highlight the potential of combining DNA and protein analysis as a practical tool for ensuring product authenticity and preventing fraudulent substitution in the dairy industry. This approach offers significant benefits for both consumer safety and quality control in food production, especially in regions with high-value dairy markets.

Keywords**:** *DNA barcoding, milk protein fingerprinting, Bos taurus, Bubalus bubalis*.

Today's food market faces a serious challenge: ensuring that products are authentic and safe. With food fraud cases on the rise and consumers increasingly demanding high-quality items, it has become harder to verify that products are truly what they claim to be (7, 9). Dairy products—especially milk and milk-based foods like cheese and yogurt—are particularly vulnerable (3, 17). Higher-value dairy products, like milk from sheep, buffalo, or goats, are sometimes fraudulently replaced with cheaper cow's milk, undermining the quality and misrepresenting the product (12). These practices don't just hurt consumer trust; they also impact producers who are following quality standards (1, 18). In some cases, fraud goes even further, with milk being adulterated with added substances such as reconstituted milk powder or even melamine, which can artificially increase protein readings but poses health risks (16, 20).

To help address these issues, DNA barcoding has emerged as a promising tool (8). This technique relies on sequencing a short DNA segment that's unique to each species (5, 19). By matching this DNA "barcode" to known sequences, it's possible to identify the species in the product, even in mixed or processed food items (11). DNA barcoding is especially valuable for animal-derived products like milk. One reason for this is that mitochondrial DNA, often used in barcoding, remains stable through processing, such as heat treatments (4, 13). This means that the species origin can still be detected even when proteins are degraded by food processing methods, something traditional protein-based methods struggle with (6, 10).

This study zeroes in on milk from *Bos taurus* (domestic cattle) and *Bubalus bubalis* (water buffalo), analyzing the molecular differences through DNA and protein profiling (14). Since buffalo milk is typically more expensive and often substituted with cow's milk, DNA barcoding offers a practical way to identify and prevent this kind of substitution (14, 18). Unlike some other testing methods, DNA barcoding works effectively on both fresh and processed dairy products, making it ideal for catching fraud in a range of dairy items (12, 19).

DNA-based approaches have clear advantages over protein-based methods when it comes to food authenticity (8, 20). DNA is more stable than protein and less likely to degrade through food processing, which makes it a more reliable marker for detecting the original species source (4, 13). For this study, certain DNA regions were selected because they show just the right amount of variation to tell cattle and buffalo milk apart, even after processing (10). This approach can be especially helpful in verifying authenticity for regionally protected foods (1, 15). For example, European cheeses like Greek Feta or Italian Mozzarella di Bufala must meet specific requirements for both ingredients and origin (7). Tools like DNA barcoding can help ensure that these standards are met, protecting both the value of these products and the consumer (12).

Beyond single-product verification, DNA barcoding can also be scaled up thanks to high-throughput sequencing and bioinformatics platforms, making it possible to run these tests on a larger scale (9, 17). Key markers, such as the COI gene, are well-suited for barcoding because they're highly conserved within species but vary enough across different species to be effective identifiers (5). As more people seek premium, authentic dairy products, the need for quick, reliable verification methods is only growing (3, 19).

While protein-based methods still have their place, DNA-based techniques tend to be more sensitive (10, 20). This sensitivity makes them useful for catching both accidental and deliberate cases of adulteration, such as mixing in cow's milk with buffalo milk (14). In this study, optimized DNA extraction methods for dairy products were also explored, recognizing that milk presents some specific challenges (13). Its high fat content, microbial presence, and other factors can make it difficult to extract clean, high-quality DNA. By refining these extraction methods, it's possible to ensure accurate species identification, even for products that are complex, multi-ingredient, or heavily processed (3, 17).

The potential for DNA barcoding goes beyond just dairy products. DNA-based identification systems are becoming important across various food sectors, especially as health and food safety standards continue to tighten (9). One of the key advantages of DNA barcoding is its ability to confirm raw material sources and to detect adulteration, giving both consumers and producers a reliable way to verify food contents (12). This capability is particularly important for people with dietary restrictions or sensitivities to certain types of milk (20).

However, while DNA barcoding holds great promise, it's not yet a routine part of testing in many production lines due to cost constraints (7, 18). The current cost of DNA sequencing is relatively high, so for now, it's mostly used for high-value products or in cases with a strong reason to suspect fraud (1). Still, as technology advances, the costs are likely to drop, which could make DNA barcoding a routine part of quality control for a wider range of food products (5, 19). With larger reference databases of genetic sequences, more affordable diagnostic tools, and integration into existing safety protocols, DNA barcoding could become a standard method for verifying food authenticity and preventing fraud (10).

To sum it up, DNA barcoding stands out as a promising tool in food biotechnology, particularly for verifying the quality and authenticity of dairy products. As this technology becomes more widely used, the food supply chain is expected to become more transparent, allowing producers to maintain quality standards and providing consumers with greater confidence in their purchases (12). With continued progress, DNA barcoding has the potential to significantly reduce food fraud, build consumer trust, and support fair competition for producers who prioritize quality in an increasingly competitive market (19, 20).

Materials and methods

Biological Samples

The study involved milk samples collected from two species: *Bos taurus* (domestic cattle) and *Bubalus bubalis* (water buffalo). Samples were gathered from certified farms in Timiș County, Romania. Each sample was collected in sterile Eppendorf tubes, doubled for backup, and immediately placed on ice. Once transported to the lab, samples were stored at -20°C until analysis.

Primers Used

The study employed specific primer pairs (dgLCO and dgHCO) to amplify DNA sequences of approximately 700 base pairs. These primers were selected based on their effectiveness in amplifying target regions essential for species differentiation.

Primers used:

dgLCO – F GGTCAACAAATCATAAAGAYATYGG

dgHCO – R TAAACTTCAGGGTGACCAAARAAYCA

PCR Analysis

Polymerase Chain Reaction (PCR) analysis was conducted using a Surecycler thermocycler (Agilent Technologies). The PCR mix contained a KapaRobust Hot Start 2X kit, with 20 pmol of each primer and 1 µl of DNA template, adjusted to a final volume of 25 µl with distilled water. Standard conditions were used, following literature guidelines: an initial denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 20 seconds, annealing at 55-60°C for 20 seconds, and extension at 72°C for 1 minute. The final extension was performed at 72°C for 3 minutes.

Milk Protein Microfluidic Electrophoresis

Freshly collected milk samples were initially stored at -20°C, then thawed and thoroughly mixed before analysis. Samples were centrifuged at 3000 RPM for 15 minutes to remove fats, leaving a clear milk phase for analysis. About 1 ml of fat-free milk was transferred to Eppendorf tubes. Aliquots of 100 µl were then diluted 1:20 with deionized water. Protein separation was performed using the Agilent Bioanalyzer 2100 and Protein 80 kit, which applies microfluidic capillary gel electrophoresis combined with laser-induced fluorescence for precise protein detection.

The gel-dye mixture for electrophoresis was prepared by filtering 650 µl of gel matrix, then adding 25 µl of blue dye, and vortexing for a uniform mixture. The processed samples and molecular weight markers were loaded on the chip, and protein migration times were recorded and analyzed by Agilent's software, yielding molecular weight and concentration data for individual proteins.

Agarose Gel Electrophoresis

For DNA analysis, agarose gel electrophoresis was employed to separate nucleic acids based on size. This method utilized a horizontal gel apparatus with wells formed using a comb. Agarose (0.7 g/100 ml for genomic DNA; 2 g/100 ml for PCR products) was dissolved in TEA buffer, cooled to approximately 50°C, and combined with ethidium bromide. Once solidified, the gel was placed in a TEA-filled tank for electrophoresis. DNA samples, stained with migration dye, were loaded into the wells and subjected to 80-100 V for roughly two hours. Post-electrophoresis, DNA bands were visualized under UV light, photographed, and analyzed with VisionWorks software.

Results and discussions

This study applied DNA sequencing to identify milk from *Bos taurus* (cattle) and *Bubalus bubalis* (water buffalo), offering a precise tool for distinguishing species by examining milk samples. DNA sequencing serves as a powerful method in food traceability, especially for dairy products, where it can detect any accidental or deliberate mislabeling. This technique has become the preferred choice in food analysis, replacing older methods like ELISA and chromatography, thanks to its sensitivity and the stability of DNA, which remains intact through food processing.

For this study, the mitochondrial COI gene was targeted, as it is a common marker in animal species identification. By using PCR, a 700-base-pair fragment of this gene was amplified from each milk sample, creating a clear genetic profile unique to each species. The sequences obtained matched known reference sequences for *Bos taurus* and *Bubalus bubalis*, proving the effectiveness of this DNA marker in identifying the milk source accurately.

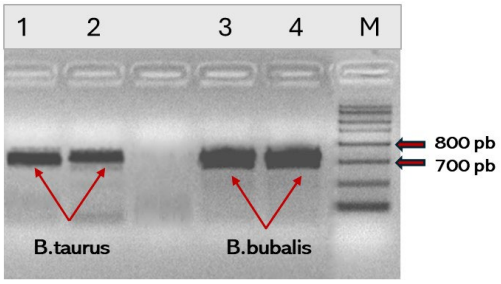

Fig. 1. Sequencing gel for *B. taurus* and *B. bubalis*

The DNA sequences from each sample revealed distinct patterns: *Bos taurus* DNA aligned closely with other bovine samples in the BOLD database, achieving a match percentage of around 99.84% across various cattle breeds. Similarly, the *Bubalus bubalis* sequence showed a 99.32% match with buffalo reference sequences, clearly distinguishing it from cattle DNA. However, it also revealed a close relation to other buffalo-like species, such as the yak (*Bos grunniens*), with a similarity of about 95%.

When comparing the DNA sequences of cattle and buffalo, notable genetic differences surfaced. The T-Coffee software highlighted an overall similarity of 87.39%, reflecting the close relationship between these species. However, significant distinctions—two major deletions, a smaller single-nucleotide deletion, and around 85 unique single-nucleotide substitutions—confirmed their genetic distinction. These differences, especially in the COI gene, suggest a reliable way to differentiate between cattle and buffalo milk, offering a valuable tool for ensuring milk authenticity in the food industry.

CLUSTAL W (1.83) multiple sequence alignment

```
B.bubalis    AACCGCTGATTATTCTCAACCAATCATAAAGATATCGGCACCCTATACTT
B.taurus     CA----------------------------------------------TAAAGATATTGGTACCCTTTATCT

B.bubalis    ACTATTTGGTGCCTGGGCCGGCATAGTAGGAACAGCCCTAAGCCTGCTAA
B.taurus     ACTATTTGGTGCTTGGGCCGGTATAGTAGGAACAGCTTTAAGCCTTCTAA

B.bubalis    TTCGCGCTGAATTGGGTCAACCCGGAACCCTGCTCGGAGATGACCAAATC
```

B.taurus TTCGCGCTGAATTAGGCCAACCCGGAACTCTGCTCGGAGACGACCAAATC

B.bubalis TACAACGTAGTTGTAACTGCACACGCATTTGTAATAATCTTCTTTATAGT
B.taurus TACAACGTAGTTGTAACCGCACACGCATTTGTCATAATCTTCTTTATAGT

B.bubalis AATGCCAATTATAATTGGAGGGTTCGGTAATTGACTAGTTAATCTAATAA
B.taurus AATACCAATCATAATTGGAGGGTTCGGTAACTGACTTGTTCCCCTAATAA

B.bubalis TTGGCGCCCCCGACATAGCATTCCCCCAGATAAATAATATAAGCTTCTGA
B.taurus TTGGTGCTCCCGATATAGCATTTCCCCGAATAAATAATATAAGCTTCTGA

B.bubalis CTCCTTCCCCCCTCCTTCCTACTACTTCTAGGGTCATCCATAGTTGAGGC
B.taurus CTTCTCCCCTCCCTCATTCCTACTACTCCTCGCATCCTCTATAGTTGAAGC

B.bubalis TGGGGCAGGAACAGGTTGAACCGTGTATCCCCCTTTAGCAGGTAACCTGG
B.taurus TGGGGCAGGAACAGGCTGAACCGTGTACCCTCCCTTAGCAGGCAACCTAG

B.bubalis CCCAGGCAGGAGCCTCAGTAGACCTAACCATTTTCTCTTTACACTTGGCA
B.taurus CCCATGCAGGAGCTTCAGTTGATCTAACCATTTTCTCTTTACACTTAGCA

B.bubalis GGTGTCTCCTCAATTTTAGGAGCTATTAATTTTATTACAACAATTATTAA
B.taurus GGAGTTTCCTCAATTTTAGGAGCCATCAACTTCATTACAACAATTATCAA

B.bubalis TATAAACCCAACCGCAATGTCA-AATGCCAAACCCCTCTATTCGTGTGAT
B.taurus CATAAAGCCCCCGCAATGTCACAATACCAAACCCCTCTATTCGTATGAT

B.bubalis CCGTAATAATCACCGCCGTATTACTGCTCCTTTCACTTCCTGTACTAGCA
B.taurus CCGTAATAATTACCGCCGTACTACTACTACTCTCGCTCCCTGTATTAGCA

B.bubalis GCTGGCATTACAATGCTACTAACAGATCGAAATCTAAATACAACCTTCTT
B.taurus GCCGGCATCACAATGCTATTAACAGACCGGAACCTAAATACAACTTTCTT

B.bubalis TGACCCGGCAGGAGGAGGGGACCCTAACCTATACCAACACTTATCTTGAT
B.taurus CGACCCGGCAGGAGGAGGAGATCCTATTCTATACCAACACTTATTCTGAT

B.bubalis TCTTTGGACACCCCGAAGTCTATATTCTTATTCTACCTGGGTTTGGAATA
B.taurus TCTTTGGACACCCCGAAGTCTATATTTTAATCTTACCTGGATTTGGAATA

B.bubalis ATCTCTCATATTGTAACTTACTACTCAGGGGAAAAAGAACCATTCGGATA
B.taurus ATCTCTCATATCGTAACCTACTACTCAGGAAAACGAAGAACCATTCGGATA

B.bubalis TATAGGAATAGTTTGAGCTATAATATCAATCGGATGTTTAGGGTTCATCA
B.taurus TATGGGAATAGTTTGGGCTATAACGTCAATCGGATTTCTAGGTTTCATCG

B.bubalis TATGAGCTCACCACATATTCACAGTCGGAATAGACGTCGATACAC----------
B.taurus TATGAGCCCACCATATATTCACTGTCGGAATAGACGTCGACACACGAGCC

B.bubalis ----------
B.taurus TACTTCACATCAGCCACTATAATTATTGCTATTCCAACCGGGGTAAAAGT

B.bubalis ----------GGG
B.taurus CTTCAGCTGATTGGCAACACTTCATGGAGGT

The study also investigated the protein profiles of milk from both species, focusing on major milk proteins. Microfluidic chip electrophoresis was used to analyze these protein fractions, which showed clear differences in both the molecular weight and concentration of key proteins between *Bos taurus* and *Bubalus bubalis*.

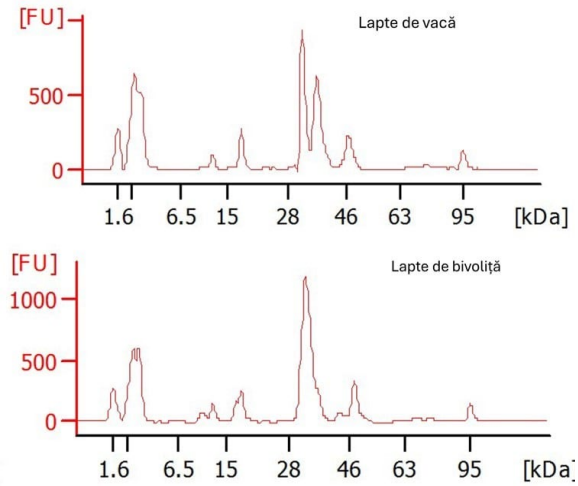

Fig. 2. Graphs representing the molecular weight of proteins in the studied milk

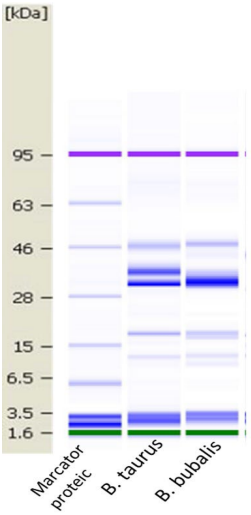

Fig. 3. Milk protein gel electrophoresis

For example, beta-casein in cattle milk had a molecular weight of 32.4 kDa, compared to 33 kDa in buffalo milk. Protein concentrations varied as well; beta-casein reached 1470 ng/µl in buffalo milk versus 638 ng/µl in cattle milk. These variations, especially in protein concentration, indicate that protein profiling can help distinguish between these two types of milk. However, this method is only reliable with fresh, unprocessed samples since protein structures tend to degrade during food processing.

Overall, these results demonstrate that DNA sequencing and protein profiling are both effective for distinguishing cattle and buffalo milk. DNA analysis, particularly of the COI gene, proves to be a highly reliable approach to species identification in dairy products, supporting efforts to ensure food authenticity and reduce fraud. Protein profiling, while useful, is more limited by the impact of processing on protein structure. Combined, these findings underscore the importance of using both genetic and proteomic tools to enhance traceability and authenticity in the dairy industry.

Conclusions

The findings from this study underscore the practicality and precision of DNA barcoding for species identification, especially when dealing with complex biological materials like milk. By successfully amplifying a specific gene sequence (CO I) with the primer pair dgLCO and dgHCO, the study highlights DNA barcoding's effectiveness in reliably distinguishing between species. This process, coupled with data analysis through the BOLDSYSTEMS database, showcases how molecular identification can be applied even in challenging matrices where traditional methods may fall short. This advancement makes DNA barcoding a highly relevant tool for food quality control, ensuring that the authenticity of species can be verified even in processed dairy products.

Moreover, the study introduces protein profiling as a valuable complement to DNA barcoding. By examining the electrophoretic profiles of milk proteins, this approach offers an additional layer of accuracy when verifying species origin. The protein profiling technique enables a quantitative comparison of milk from different species, adding another means to confirm authenticity in food products. Together, DNA barcoding and protein profiling form a robust framework that could be standardized for more reliable species identification, supporting efforts to maintain integrity within the food industry.

These combined methods have meaningful implications for consumer trust and product transparency. In a market increasingly concerned with food authenticity, the ability to confidently verify the origin of dairy and meat products protects consumers from potential misrepresentation and reinforces ethical practices within the industry.

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PROTEIN FINGERPRINT OF COW MILK AS REVEALED BY MICROFLUIDIC ELECTROPHORESIS TECHNIQUE

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Abstract

The aim of this study was to evaluate the protein content and identify the protein profile of cow milk using microfluidic electrophoresis, following the methodologies described in two previously published studies. Five cow milk samples were analyzed using the Agilent 2100 Bioanalyzer with the Protein 80 kit, a microfluidic-based system that offers rapid and automated separation and quantification of milk proteins. The primary objective was to determine if this method could reliably replicate the results obtained in previous research under our laboratory conditions. Our findings confirmed that the method successfully separated and quantified the major milk proteins, including caseins (αS1-casein, β-casein, and κ-casein) and whey proteins (α-lactalbumin and β-lactoglobulin). The electropherogram patterns closely matched those reported in earlier studies, with clear resolution of the individual protein fractions. The protein identification and concentration data obtained for each sample were consistent with the results found in the referenced studies, further validating the effectiveness of the microfluidic electrophoresis technique in protein analysis. We proved that the microfluidic electrophoresis technique is a fast, reliable, and reproducible method for analyzing cow milk proteins in our laboratory. The ease of use and automated nature of the Agilent 2100 Bioanalyzer system make it an excellent alternative to traditional SDS-PAGE techniques, providing high-resolution protein separation with minimal sample preparation and reduced analysis time. The results demonstrate that this method can be successfully applied in routine milk protein analysis for quality control and research purposes.

Keywords: *microfluidic electrophoresis, cow milk proteins, protein fingerprinting, milk quality analysis.*

Milk is a vital biological fluid, rich in nutrients and bioactive compounds, and has been a staple in human diets for millennia. Among its key components, proteins hold a central role due to their nutritional value, functional properties, and bioactive potential. Milk proteins are categorized into two primary groups: caseins, which constitute approximately 80% of the total protein, and whey proteins, which make up the remaining 20% (5, 7). Accurate analysis of these proteins is essential for various applications, including evaluating milk quality, detecting adulteration, ensuring regulatory compliance, and determining suitability for specialized food applications (1, 6, 9).

Traditional techniques for milk protein analysis, such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and liquid chromatography, have been the cornerstone of protein characterization for decades (4, 8). These methods allow for the separation and quantification of individual protein fractions, providing valuable insights into the molecular composition of milk (5, 7). However, despite their efficacy, these techniques have notable limitations. They often require labor-intensive sample preparation, extended analysis times, and expertise in manual operation, making them less practical for high-throughput applications or routine analysis (11, 13).

Recent advancements in analytical technologies, particularly microfluidic electrophoresis, have revolutionized protein analysis by offering rapid, automated, and highly reproducible methods (9, 14). Notably, Skelte G. Anema highlighted the potential of "lab-on-a-chip" microfluidic SDS electrophoresis technology for milk protein separation and quantification (3). This approach significantly reduces sample preparation times while delivering precise results, making it an attractive choice for protein analysis**.** Building on such innovations, the Agilent 2100 Bioanalyzer, equipped with the Agilent Protein 80 kit, exemplifies this technological leap. This system uses microfluidic-based separation technology and automated protocols specifically designed for high-resolution protein profiling (2, 9, 20).

The Agilent Protein 80 kit enables the precise separation of proteins in the molecular weight range of 5–80 kDa, making it ideal for analyzing the major milk proteins, including caseins (αS1-casein, β-casein, and κ-casein) and whey proteins (α-lactalbumin and β-lactoglobulin) (5, 18). The Agilent 2100 Bioanalyzer provides several advantages over traditional methods. Its integrated microfluidic chips and pre-programmed protocols allow for rapid sample processing with minimal preparation, significantly reducing analysis time while maintaining high accuracy and reproducibility (1, 9, 13). By automating many of the steps traditionally performed manually, such as gel preparation and staining, the system minimizes user error and variability, making it an

attractive option for routine use in both research and quality control settings (7, 13, 22).

The accurate characterization of milk proteins is particularly important due to the functional and nutritional roles these proteins play. Caseins, such as αS1-casein, β-casein, and κ-casein, are critical for forming milk micelles, which influence the texture, stability, and processing behavior of dairy products (8, 10). Whey proteins, including α-lactalbumin and β-lactoglobulin, are valued for their high digestibility, balanced amino acid composition, and bioactive properties, such as antioxidative and immunomodulatory effects (6, 9, 18). Additionally, understanding the protein profile of milk is vital for detecting allergens and tailoring products to consumer needs, including individuals with specific dietary restrictions (21).

Several studies have demonstrated the effectiveness of the Agilent 2100 Bioanalyzer and its associated kits in analyzing complex protein mixtures, including milk proteins (9, 13, 17). By adhering to the Agilent protocol, researchers have consistently achieved high-resolution separation and accurate quantification of milk proteins, with results comparable to or exceeding those obtained through SDS-PAGE (2, 5, 13). The integrated software provided by Agilent further simplifies data analysis by automatically generating electropherograms and quantifying protein concentrations based on predefined parameters (14, 18, 23).

Proteomic approaches, including those leveraging microfluidic electrophoresis, have also played a significant role in advancing our understanding of milk proteins. The ability to identify and quantify multiple proteins simultaneously provides a comprehensive picture of milk's molecular composition, essential for food quality analysis and product innovation (15, 16, 22). This level of detail also aids in monitoring the impact of processing methods on protein integrity and functionality (16, 25).

This study builds on these findings by utilizing the Agilent 2100 Bioanalyzer and the Agilent Protein 80 kit to evaluate the protein content and profile of cow milk samples. The primary objective is to validate the reproducibility and reliability of this microfluidic electrophoresis method under controlled laboratory conditions and to compare its performance with results reported in prior research. By leveraging the standardized Agilent protocol, this study ensures consistency and accuracy in milk protein analysis, demonstrating the suitability of this approach for routine applications (20, 24).

The results of this study highlight the advantages of microfluidic electrophoresis, including its speed, reproducibility, and ease of use. The high-resolution separation capabilities and automated nature of the Agilent 2100 Bioanalyzer, coupled with the optimized design of the Agilent Protein 80 kit, make it a superior alternative to traditional SDS-PAGE techniques (17, 22). By confirming the method's reliability under laboratory conditions, this study reinforces its value as a robust and reproducible tool for routine protein profiling in milk and establishes its broader applicability in quality control, product development, and nutritional research (9, 19).

Materials and methods

Collection and Preservation of Milk Samples

Milk samples were sourced from five *Bos taurus* cows on a local farm, with great care taken to collect them in sterile containers. Following collection, the samples were transferred into 2 ml Eppendorf tubes to maintain sterility during transport to the laboratory. Upon arrival, the samples were promptly frozen at -20°C to ensure the stability of biomolecules critical for subsequent analysis.

Pre-Analytical Processing

Before analysis, the frozen milk samples were thawed to room temperature. Each sample underwent a five-minute homogenization process using a vortex mixer to ensure uniformity. Subsequently, 1 ml of each sample was split into two equal portions, each placed in separate 1.5 ml microcentrifuge tubes. One portion was subjected to centrifugation at 3500 rpm for 10 minutes to facilitate the separation of milk components. This process resulted in a clear stratification, with a lipid layer forming at the surface and somatic cells settling as sediment at the bottom. The lipid layer was carefully removed to produce defatted samples. Both defatted and untreated samples were diluted in a 1:10 ratio with deionized water to prepare them for analysis according to the manufacturer's instructions.

Protein Analysis Protocol

The characterization of milk proteins was carried out using a microfluidic electrophoresis approach, implemented with the Agilent 2100 Bioanalyzer. The Protein 80 Kit, specifically designed for this platform, included all the necessary reagents and components, such as analytical chips, gel matrix, sample buffer, dye, protein ladder, and molecular weight markers covering a range of 1.6–95 kDa.

Sample Preparation and Electrophoresis

Milk samples were prepared by combining 4 µl of milk extract with 6 µl of protein ladder and

2 µl of denaturing buffer in a 0.5 ml microtube. These mixtures were denatured by heating at 95°C for 5 minutes. After cooling, each tube was briefly centrifuged, and 84 µl of deionized water was added to ensure proper dilution. The priming of the analytical chip was performed using gel solutions supplied with the kit.

Once prepared, 6 µl of each sample and the protein ladder were loaded into individual wells on the chip. The chip was then placed into the Agilent 2100 Bioanalyzer, where the automated system ensured correct placement of electrodes and initiated the analysis.

Data Collection and Analysis

The entire process of electrophoretic separation and automatic data integration was completed in approximately 30 minutes. The upper and lower molecular weight markers were used as internal standards to ensure precise calibration, while the protein ladder served as an external reference for molecular weight determination. The Agilent 2100 Expert software facilitated seamless data acquisition and analysis, delivering highly reproducible and accurate results that confirmed the system's reliability for protein profiling in milk samples.

Results and discussions

The protein profiling of cow milk was successfully carried out using the Agilent 2100 Bioanalyzer with the Protein 80 kit. The microfluidic electrophoresis technique effectively separated and quantified major milk proteins, including caseins (αS1-casein, β-casein, and κ-casein) and whey proteins (α-lactalbumin and β-lactoglobulin).

Electrophoretic Analysis

As illustrated in **Figure 1**, the electropherogram results appear as a virtual ELFO gel. The distinct lanes for each milk sample and the reference protein ladder demonstrate high-resolution separation of protein fractions. The automated system provided consistent results across all five milk samples, confirming the reliability of this method under laboratory conditions.

Comparative Quantitative Analysis

The protein quantification data align closely with previously published studies. **Table 1** compares the molecular weights of the identified milk proteins from the current study with established values reported in the literature. The measured values for α-casein, β-casein, κ-casein, β-lactoglobulin, and α-lactalbumin displayed minimal deviations, confirming the accuracy of the method.

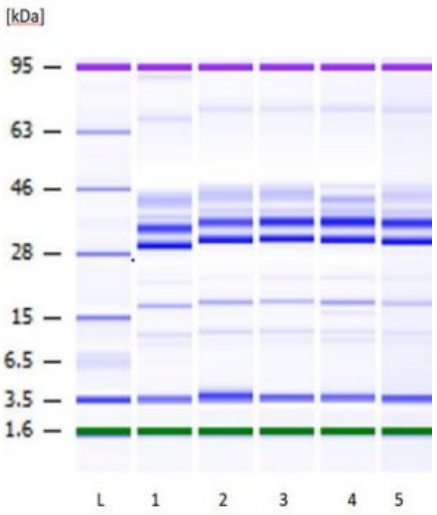

Fig. 1. Virtual ELFO gel: L-Protein ladder, lanes 1-5 milk samples

The Agilent 2100 Bioanalyzer demonstrated clear advantages over traditional SDS-PAGE methods. The automated process significantly reduced the sample preparation and analysis time while maintaining high reproducibility. Electropherograms generated by the system were consistent across replicates, minimizing user error and variability.

The use of microfluidic electrophoresis provided unique insights into the subtle variations in protein composition among the milk samples. The system's sensitivity enabled the detection of minor protein fractions that might be overlooked by conventional methods. This capacity to resolve detailed protein profiles is particularly valuable for assessing milk's nutritional and functional qualities, which can be influenced by factors such as lactation stage, diet, or breed.

Furthermore, the streamlined workflow of the Agilent 2100 Bioanalyzer aligns well with the increasing demand for rapid and efficient analytical techniques in the dairy industry. By reducing analysis time and human intervention, this method supports high-throughput applications, making it suitable for large-scale quality control operations. These attributes position microfluidic electrophoresis as a transformative tool for both research and industry, fostering advancements in dairy science and product innovation.

Table 1

Quantitative Milk Protein Analysis: Agilent, Literature, and Current Findings

Milk Protein	Agilent Standard, 2008 [kDa]	Skelte G. Anema, 2008 [kDa]	This study [kDa]
α-casein	37	36	36.5
β-casein	33	29	32
κ-casein	46	42	46.5
β-lactoglobulin	18	18	18
α-lactalbumin	12	12	13
Total Protein g/L	33	N/A	32.88

The accurate characterization of milk proteins using this method supports its application in various fields, including milk quality control, allergen detection, and the development of specialized dairy products. The high-resolution protein profiling provided by the microfluidic electrophoresis technique is invaluable for monitoring processing impacts and ensuring product consistency.

These findings validate the utility of the Agilent 2100 Bioanalyzer as a reliable and efficient tool for routine milk protein analysis, with broad implications for research and industrial applications.

Conclusions

This study demonstrated that microfluidic electrophoresis, implemented using the Agilent 2100 Bioanalyzer with the Protein 80 kit, is an efficient and reliable method for analyzing cow milk proteins. The technique successfully identified and quantified major milk proteins, including caseins (αS1-casein, β-casein, and κ-casein) and whey proteins (α-lactalbumin and β-lactoglobulin), with results that closely aligned with previously published data.

The high-resolution electropherogram patterns and consistent protein concentration measurements reaffirm the validity of this approach for milk protein analysis. The findings highlight the significant advantages of this method over traditional techniques such as SDS-PAGE, including automation, reduced sample preparation, shorter analysis times, and excellent reproducibility.

The study further establishes the suitability of the Agilent 2100 Bioanalyzer for routine applications, such as quality control and research in dairy science. This method provides a valuable tool for advancing the analysis of milk protein composition, offering a robust alternative for laboratories seeking efficient and accurate protein profiling techniques.

Future studies could expand on these findings by applying the method to other milk sources or testing its efficacy under varied processing conditions to explore broader applications in dairy research and industry.

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PREVALENCE OF GASTROINTESTINAL PARASITES IN DONKEYS FROM ALBA COUNTY – PRELIMINARY RESEARCH

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Abstract

Due to their strength, endurance and docility, donkeys are frequently used for certain household jobs, being easy to handle by women and children. But, like horses, they are susceptible to various parasitic infestations, many common to the two species. This study was carried out in 5 nearby localities in Alba County: Ocna Mureș, Unirea, Lunca Mureșului, Noșlac and Fărău, on 9 donkeys. Digestive strongyles, roundworms, *Strongyloides westeri*, anoplocephalid tapeworms and pinworms were identified. The highest prevalence was obtained in digestive strongyles (88.88%), followed by anoplocephalid tapeworms (33.33%). Roundworms, pinworms, respectively *Strongyloides westeri* had the same prevalence, of 22.22%. The highest intensity of parasitism was reported in digestive strongyles, with an average EPG of 870.83. Not all the mentioned species were found in all localities.
***Keywords**: prevalence, parasites, donkeys, Alba County.*

Born from the foam of the sea, horses were, along with dogs, the most beloved creatures. They have always represented wealth and power. But, from the Bucephal to the Godolphin Barb or from the Catalan to the recently publicized Delta horses, all horses harbour large parasitic communities (a veritable biodiversity) in their digestive tract, among which the cyathostomins (small strongyles) are the most important, both in number as well as pathogenicity (7, 8, 9, 13, 16, 17).

Despite all the financial efforts carried out during the prophylactic and therapeutic campaigns, the extensiveness of parasitism, both in farms and in the households, reaches significant levels throughout the world. It seems that, according to the statistics, the most significant damages are due to gastrointestinal nematode infestations, which "although they do not kill the animal, they destroy the farm" (4, 5, 15).

Studies on donkeys are not very numerous. If there are some mentions of parasitism in the foreign specialized literature (1, 2, 3, 11, 12, 14, 18), in Romania the studies are rare and the information scarce.

This study aimed to identify intestinal parasites in donkeys from the northeast of Alba County.

Materials and methods

Farms and animals

Fecal samples were collected from 9 donkeys from five localities (Ocna Mureș, Unirea, Lunca Mureșului, Noșlac and Fărău) within a radius of 10 km around the locality of Ocna Mureș in central Romania (Fig. 1). Their age ranged from 11 months to 18 years.

Data on herd health management were requested from the owners, including information on the administration of treatments in the last few years.

Fecal samples

Fresh faecal samples were collected immediately after defecation, which were packed in plastic bags and labeled. The samples (from the nine individuals) were collected during March and May 2023. To highlight the *Oxyuris equi* infestation, transparent adhesive tapes were used.

The samples were analyzed by the McMaster quantitative method, with a sensitivity of 50 eggs per gram of faeces (EPG) in the Laboratory of Parasitology, the Faculty of Veterinary Medicine Timișoara (Table 1).

Fig. 1. Location of the localities studied in Alba County (black circle)

Table 1

Coproscopic prevalence of various intestinal helminths identified in donkeys from Alba County

Locality	MU	Digestive strongyles.	Roundworms	*Strongyloides westeri*	Cestodes	Pinworms**
Ocna Mureș (n = 3)	No (%)*	3	1	-	1	-
	OPG	833,33	150	-	150	-
Unirea (n = 2)	No (%)*	2	-	1	1	1
	OPG	650	-	150	100	++
Lunca Mureșului (n = 1)	No (%)	-	-	-	-	-
	OPG	-	-	-	-	-
Noșlac (n = 1)	No (%)*	1	1	-	-	1
	OPG	800	200	-	-	++
Fărău (n = 2)	No (%)*	2	-	1	1	-
	OPG	1200	-	250	150	-
TOTAL (n = 9)	**No (%)***	**8 (88.88)**	**2 (22.22)**	**2 (22.22)**	**3 (33.33)**	**2 (22.22)**
	OPG	**870.83**	**175**	**200**	**133.33**	**++**

* at least two species of parasites in some individuals; ** identified with adhesive tape.

Results and discussions

Of the nine investigated donkeys, only eight (88.88%) were parasitized. Also, polyinfestations were frequently observed.

The epizootological investigation revealed the fact that there is no effective plan for parasitological control, and when deworming is still carried out, the most used drugs are benzimidazoles.

The most prevalent species were digestive strongyles (especially cyathostomins), with 88.88% (Fig. 2). Tapeworm eggs were expelled by 3 (33.33%) horses, while eggs of roundworms (*Parascaris equorum*), threadworms (*Strongyloides westeri*) and pinworms (*Oxiuris equi*) were eliminated by 2 individuals for each category (22.22% each of them).

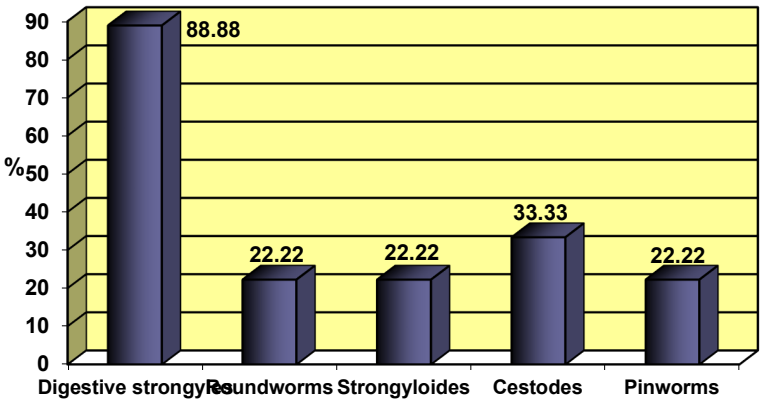

Fig. 2. The prevalence of helminths in donkeys in Alba County

Although most of the pastures in the localities included in the study are found along the Mures valley, the most infested donkeys were those from Unirea, where four categories of parasites were identified. Three categories of parasites were identified in donkeys from other three localities (Ocna Mureș, Noșlac and Fărău), while no parasites were recorded in the only donkey investigated in Lunca Muresului.

The most intensively parasitized with digestive strongyles were the donkeys from Fărău (1200 EPG), and the lowest intensity was recorded in those from Unirea (650 EPG).

A cross-sectional study carried out in Oromia Region, Ethiopia, on 395 donkeys shown the following prevalence: *Strongyle* spp. (100%), *Oxyuris equi* (10.1%), *Parascaris equorum* (23.8%), *Fasciola* spp. (0.3%), *Gastrodiscus aegyptiacus* (4.6%), *Strongyloides westeri* (47.8%) and *Anoplocephala perfoliata* (0.5%), which are different from those obtained by us (6). Some other studies reported higher prevalence for the digestive strongyles in different countries (14, 20) and a lower one in others (10, 19). Also in Ethiopia, but in the south of the country, species of digestive strongyles (48.17%), *Parascaris equorum* (11.45%), *Strongyloide westeris* (5.99%), and mixed infection (strongyles + *Parascaris* - 9.11% and strongyles + *Strongyloides* - 0.52%) were identified, with an overall prevalence of gastrointestinal nematodes of 75.26% (2).

Another study carried out in the Northeast of Portugal on 62 donkeys demonstrated a decrease in the prevalence of parasitism from 35.5% to 19.4% in herds subjected to anthelmintic treatments (14). The most prevalent species were the digestive strongyles, but also *Trichostrongylus axei* and *Parascaris equorum* were identified.

Conclusions

The donkeys from the investigated localities showed polyparasitism, in most cases, with the exception of the one in Lunca Muresului, where no parasites were identified.

Digestive strongyles were the species with the highest prevalence.

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BLOOD COLLECTION TECHNIQUES IN RATS

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Abstract

The study of blood collection techniques in rats is important in the fields like biomedical research and veterinary medicine, serving as a fundamental practice for various experimental protocols. Accurate blood sampling allows researchers to obtain critical physiological and biochemical data which is essential for the assessment of health status, disease progression, and certain pharmacokinetics aspects. Given that rats are frequently used as model organisms, it is important as researchers to orient towards methods that optimize quality of blood samples, minimize distress and ensure animal welfare. While there are many protocols that can be followed regarding blood collection in rats, the volume of blood sampled and means of restraining the rat are important. Many techniques emphasize on the easy access to blood vessels for harvesting, while others require higher skill and even more than one person to accomplish. The quality of blood samples collected is in direct corelation to the site of blood harvest but also to the animal well being when non-terminal blood collection techniques are involved. Advantages and limitations of certain protocols are discussed. The study can be useful for researchers working with laboratory rats, as well as veterinary clinicians.

Keywords: *blood collection techniques, rat, blood parameters.*

The selection of appropriate blood collection techniques in rat research is crucial to both the welfare of the animals and the quality of the data obtained. Key methods include sampling of blood from the following: tail vein, tail artery, jugular vein, saphenous vein, and femoral artery collections, each presenting unique advantages and potential drawbacks (25).

Careful consideration of these methods is essential, as the choice of technique can influence metabolic responses and hormone levels, thus impacting the overall reliability of physiological studies involving hematological assessments (1, 2).

The ethical considerations and best practices involving blood collection techniques in rats must follow certain protocols, approved by the regulatory agencies, therefore ensuring humane treatment of the animals. The necessity of minimizing animal distress and pain has garnered increasing recognition among the scientific community, emphasizing the importance of conducting thorough severity assessments prior to experimentation (13). Such an approach not only emphasizes ethical compliance but also enhances the validity of research outcomes by maintaining the well-being of the subjects involved. Consequently, researchers are urged to adopt methods that reduce suffering, such as utilizing the least invasive techniques, implementing analgesic protocols, post-operative care and refining procedural practices (22, 26).

According to the technique chosen for blood collection, an important aspect which must be considered is the apropriate training of the personel. Some techniques require more than one person (26).

Certain methods require general anesthesia of the animal, while others can be done on the concious rat, without a great impact regarding stress level (9, 26).

There are a number of recommended anesthesia protocols for the blood collection techniques used in rats, amongst which are numbered pharmacological substances that are administered either via inhalation and/or injectable anesthetics administered intraperitonially or intramuscular (15, 25, 26).

Materials and methods

An adult rat weights about 150g to 400g (4). The total blood volume of a rat is aproximately 64ml/kg body weight (9). Total blood volume is influenced by age and certain metabolic states, so older and obese rats tend to have a lower volume of blood (9, 27, 29).

Table 1 shows the permitted quantity of blood collection per sampling in correlation to the body weight of the rat according to Manoj et al. (9).

The maximum volume of blood that can be drawn from a rat should not surpass 7.5% of the total blood volume of the animal, or 4-5.3 ml/kg per week according to IACUC Guideline (Table 2) (25). It is recommended that the blood collection volume should be limited to the lower end of the range, as excessive blood drawing can result in anemia, temporary autonnomic dysfunction (orthostatic hypotension), hyperventilation and low body temperature. If the loss of blood is greater than 40% of the total blood, this could cause lethargy and even coma (14).

Table 1

Amount of blood permitted to sample from a rat according to body weight- according to Manoj et al. (9)

Body weight of the rat	Permitted blood sample volume
100-150g	0.60 – 0.91 ml
200-250g	1.1 – 1.5 ml
300-350g	1.7 – 2.1 ml

Other repercusisons of excessive blood sampling are physiological stress and sometimes even death (26, 30).

To avoid these kinds of outcomes, blood sampling should be taken no more than two weeks apart (4, 8, 13, 26). It must be mentioned that the frequency of blood collection is influeced by the volume needed and the technique used. For multiple sampling, micro needles are recommended (capillary sampling through the dorsal pedal vein) (14). Microsampling implies that the blood volume collected is up to 50µl (26).

Table 2

Quantity of maximum blood collection for single and multiple blood samplings in rats- according to IACUC Guideline (25)

Type of blood sampling	Quantity of blood harvested (ml) without fluid supplement	Quantity of blood harvested (ml) with fluid supplement
Single sample	1.7-2.1	2.5-3.2
Multiple sample	1.2-1.6 per week	1.7-2.1 every 2 weeks

Blood quality can be assessed based on certain aspects. The technique and site of blood collection play a big part in the quality of the blood samples; venipuncture failure can result in no blood collection, while an interrupted blood collection can lead to clot formation, abnormal counts for red blood cells and platelets (23).

Preparation of the blood samples for analysis is also of great importance. Inadequate storage or handling of the blood samples can lead to alteration of the total number of blood components due to hemolysis of the red blood cells (23).

When assessing blood quality of collected samples it is recommended that the blood is tested for serum potassium level and blood urea nitrogen (BUN) as those two parameters are indicators of blood hemolysis (and may wrongfully point towards renal failure) (16).

Lindstorm et al. (7) report the following normal hematologic blood parameters (Table 3).

Blood sampling techniques can be cathegorized as terminal and non-terminal procedures. Terminal procedures include:

- Cardiac puncture (25);
- Orbital plexus technique (9);
- Posterior vena cava sampling (26);
- Exsanguination (26).

Non-terminal blood collection techniques include sampling from the following sites:

- Tail vein (9);
- Tail artery (1);
- Orbital plexus (14);
- Jugular vein (26);
- Saphenous vein (9, 25, 26);
- Dorsal pedal vein (9).

It must be mentioned that along with the specified techniques classified as non-terminal, blood vessel catheterization can be used for multiple sampling. Permanent monitorization of the animal must be made, as the procedure requires anesthesia and post-operative analgesia of the animal. The appropriate needle size must be used. It is recommended that the needle size is 19-21G (this permitting up to 15 ml of blood volume to be collected) (9).

Tail vein collection allows for quick and relatively stress-free blood sampling. It yields lower volume of blood and quality compared to femoral artery techniques, which can accommodate larger blood draws (up to 8 ml) without significantly affecting the rat's physiological status (1). The procedure is done using a 23G to 26G needle (26). Before the procedure, the tail must be cleansed with antimicrobial solutions like diluted clorhexidine and then dried (1).

Table 3

Normal hematological parameters in male and female rats – Lindstorm et al. (7)

Parameter	Rat	
	Male	Female
Red blood cells (x10^6/µL)	8.15–9.75	6.76–9.2
Packed cell volume(%)	44.4–50.4	37.6–50.6
Hemoglobin (g/dL)	13.4–15.8	11.5–16.1
Mean corpuscular volume (fL)	49.8–57.8	50.9–65.5
Mean corpuscular hemoglobin (pg)	14.3–18.3	15.6–19
Mean corpuscular hemoglobin concentration (%)	26.2–35.4	26.5–36.1
Platelets (x10^3/µL)	150–450	160–460
White blood cells (x10^3/µL)	8.0–11.8	6.6–12.6
Neutrophils (%)	6.2–42.6	4.4–49.2
Lymphocytes (%)	57.6–83.2	50.2–84.5
Eosinophils (%)	0.1–0.63	0–1.96
Basophils (%)	0–0.6	0–0.4
Monocytes (%)	0–0.65	0–1.81

Noted is the fact that warming the rats' tail is crucial, this resulting in the blood vessels becoming dilated (13). Rubbing the tail from the base to the tip is not recommended as it can result in leukocytosis (13). Applying finger pressure at 5 cm from the tail tip can enhance the visualization of the blood vessel (26). When accessing the lateral tail vein, one should aim for approximately one-third of the way along the tail from the tip. If multiple samples are needed, the harvest can be done toward the base of the tail. Collecting samples from the proximal end first can lead to clots and inflammation, reducing blood flow to the distal part of the vessel (22).

Ventral tail artery is located underside the tail. The procedure is done using a a 24G or 26G needle. Considering the high blood pressure present in the artery this technique results in collection of approximately 1 ml of blood volume in one puncture (1). The schematic representation of the tail blood vessels (25) are shown in Figure 1.

Jugular vein sampling allows for collection of 0.1-0.3 ml and it requires a 23G needle. Performing jugular sampling by a single person requires a very high skill, so it is recommended that two people do this procedure. One person takes the blood sample and the other restrains and monitors the rat. Tilting the rats head at an angle exposes the small triangular patch of skin under the scapula. Shaving or trimming the area can facilitate the localization of the sampling site. This method allows for up to eight samples in 24 hours. After the sampling, gentle pressure should pe applied at the site for about thirty seconds (26).

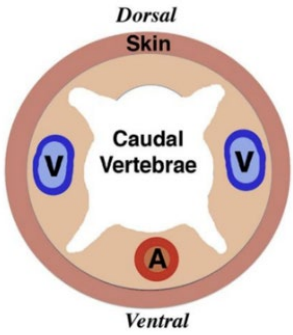

Fig. 1. Lateral tail veins (blue) and ventral tail artery (red) from the rat tail (25)

Saphenous vein is easily spotted (9), as it is localised on the lower portion of the hind leg (27). This method provides up to 0.2 ml of blood (26). The hair removal from the puncture site is not recommended, but aseptic procedures should be used. Anesthesia of the animal is not required, although to reduce stress, sedatives can be used (24). The hind leg chosen must be fixed in an extended position while applying gentle pressure above the knee joint. Blood collection requires the use of a haematocrit tube (9, 28).

Dorsal pedal vein blood sample requires the use of absolute alcohol for aseptic procedures, 23G/27G needle and a capillary tube for blood collection. The conscious animal is restrained as the chosen foot is held by the toes. The medial dorsal pedal vein is located on the plantar side of the foot (Fig. 2) (13).

Retro-orbital plexus blood sampling requires the use of sterile hematocrit capillary tubes (2-2.5 cm) or Pasteur pipettes. The sampling is done under general anesthesia of the rat. The animal is held by the scuff while pulling the skin around the eye. The capillary is inserted into the medial canthus of the eye at a 30° angle to the nose. Slight pressure is applied to puncture the tissue and the blood should flow through the tube (14).

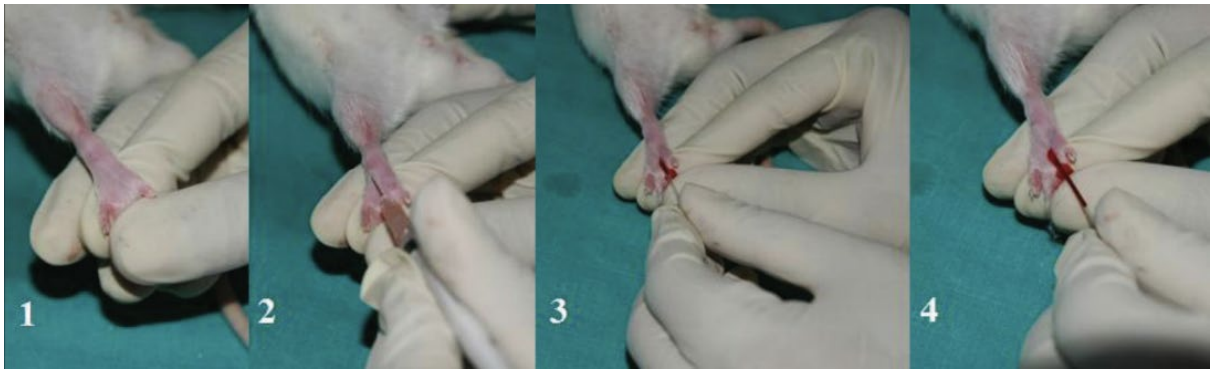

Fig. 2. Pedal vein blood collection technique according to Parasuraman and Kesarvan (13)

Great importance should be attributed to the aseptic procedures when using this technique as failure to do so can result in periorbital infection and long-term damage to the eye (16). Therefore, after sampling the orbital plexus, a sterile cotton pad is used to wipe the area punctured and stop the bleeding. Another important aspect that should be considered is the contamination with topical anesthetic if used. This is a technique that requires intensive training as even a small mistake can result in permanent damage to the eye. Following the procedure, analgesia and monitorization of the animal are required (14). Repeated blood sampling is not recommended (13, 14).

Besides the traditional method of obtaining blood samples through the orbital plexus, some authors describe a lateral approach which can be safer (Fig. 3) (16).

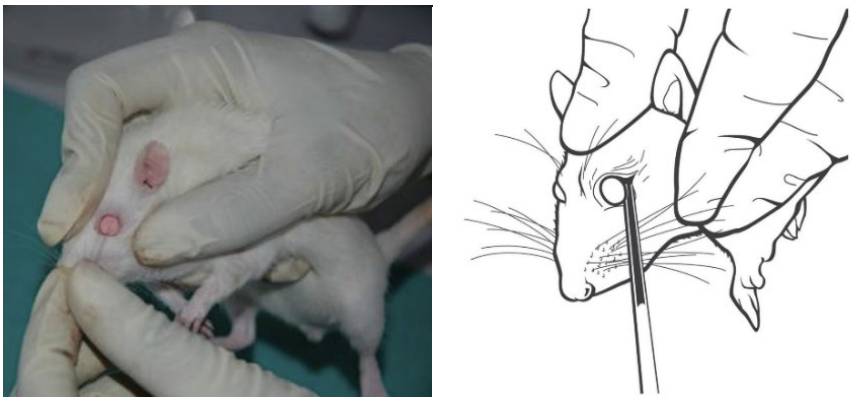

Fig. 3. Left - traditional retro-orbital approach according to Parasuraman and Kesarvan (13). Right - lateral approach of retro-orbital plexus blood sampling from rats according to Sharma (16)

Sublingual vein puncture technique for blood sampling can be used for repeated blood collection. A 23G hypodermic needle is required (21). Under general anesthesia, the tongue of the rat is pulled forward using the fingers, while the vein is punctured. It is recommended that two people participate for the sampling (one person to collect the blood while the other person holds the tongue of the animal) (26).

Results and discussions

The number of needle sticks at each attempt to harvest blood from rats should be limited to three. If more samples are needed,

then surgical catheterisation or temporary cannulation of a different blood vessel should be considered (4).

Some studies specify that depending on the site of blood collection used, there may be differences in certain blood parameters (17, 18).

It must be noted that even if the volume of blood collected is found within the recommended values, there are still some adverse effects that can occur. Amongst those, there can be mentioned:

- Bruising and haemorrhage – can result after a venipuncture (13);
- Infection at the site of the needle entry – can result because of the lack of aseptic precautions (14);
- Nerve damage – can occur in orbital puncture technique (14);
- Scarring – might occur if the animal is not restrained properly (13).

An aspect regarding blood sampling is the fluid supplementation, which can follow the procedure. The fluid replacement volume should be aprproximately equal to the amount of blood collected. The administration is done subcutaneously and the solution must be warmed beforehand. Recommended as fluid replacement solutions in rats after blood collection are Sterile Saline (0.9% NaCl) or Lactate Ringers Solution. Even so, there are certain volume limits which must not be exceeded (25).

While some of the blood collection techniques can be performed on the concious rat, others require general anesthesia of the animal. It is important to use an appropriate protocol, as there are certain anesthetics that can increase the concentration of certain hormones (19).

Lelovals et al. (2018) point towards the fact that all anaesthetic agents used (inhalatory and/or injectable) can produce increased values for aspartate aminotransferase and alkaline phosphatase (6). Common anesthetic protocols used in rats for blood collection techniques include inhalatory anesthesia, inhalatory anesthesia combined with injectable anesthesia or injectable anesthesia alone. Commonly used inhalatory agents in rats are Isoflurane and Halothane (9,15).

At the same time, some authors metion that restraining also can have effects on blood parameters. Upton et al. (18) mention that haemoconcentration coupled with calcium and magnesium elevated can be noticed after manual restraint. Novozhilov et al. (10) point towards the elevated reticulocyte and neutrophil count. Therefore it is recommended that concious rats should be restrained manually for the minimum duration necessary (26).

Certain methods dont't requrie the use of anesthetics, but sedation should be considered for stress reduction. It should also be considered the fact that some sedatives contain peripheral vasodilators, therefore doses should be low to avoid bleeding from the sampling site (26).

Light anesthesia is sometimes recommended. Sublingual vein sampling method allows for repeated harvesting. According to Donovan and Brown (1) does not have a great impact on the well being of the animal, as rats monitored after the procedure tend to increase their body weight in the next 24h (this shows the consumption of water and food).

Brown (2) mentions that tail vein blood sampling can lead to intermittent or slower collecting time compared to ventral tail artery sampling, the collection tube should contain approximately 20µl of liquid anticoagulant. The anticoagulant (e.g. EDTA) should be buffered when used, as it can alter the osmolarity and acidity of the blood sample. Those effects lead to red blood cells hemolysis and white blood cells shape distortion. The syringe or butterfly needle used should also contain a certain amount of liquid anticoagulant. This can be done by drawing and then expelling the liquid anticoagulant, therefore leaving the walls of the blood harvesting tool coated with the substance (1).

Xiao-hua et al. (20) claim that subclavian sampling offers a 95% success rate, requires less time, and avoids anesthesia, making it more efficient for large-scale studies. Charles et al. (5) in a video article claim that tail venipuncture has more advantages over subclavian techniques, such as the ability to obtain multiple samples without extensive surgical procedures, it does not require extensive training and has a reduced risk of fatality.

Advantages and disadvantages linked to each blood sampling technique discussed previously are shown in Table 4.

Table 4

Advantages and disadvantages of using different blood collection sites in rats

No.	Collection site	Advantages	Disadvantages	Source
1	**Lateral tail vein**	-No anesthesia (26) -Allows repeated sampling (26) -Easy vein access (1, 9, 26)	-Small amount of blood (26) -Animal must be appropriately restrained (1, 25, 26) - Requires the use of anticoagulants for harvesting(1)	(1, 9, 25, 26)
2	**Ventral tail artery**	-Allows repeated sampling (25, 26) -Moderate volume of blood that can be collected (1, 25)	-Animal must be restrained (1)	(1, 25, 26)
3	**Jugular vein sampling**	-Can yield large volumes of blood to be collected (25, 26) -High quality sample (20)	-Does not lend to repeated sampling (26) -Anesthesia required (25, 26) -More technical skill required (26) -Usually requires two people (26)	(20, 25, 26)
4	**Sphenous vein**	-Less stressful for the animal (25) -Repeated sampling possible (24, 25) -No anesthesia (24, 25)	-Animal must be restrained (9, 24, 25) -Requires specialized training and some specialized equipment (25, 26) -Variable sample quality/quantity (25)	(9, 24, 25)
5	**Dorsal pedal vein**	-Easy vein access -No anesthesia	-Animal must be restrained	(9)
6	**Retro-orbital plexus**	-High quality samples (3, 14, 24)	-Long time before another blood collection can be done (2 weeks) (14, 16) -Aseptic protocol must be applied (14, 16) -Post-operative care must be established (14) -Requires apropriate training of the personel performing the procedure (9, 14)	(3,9, 14,16)
7	**Sublingual vein sampling**	-Allows repeated sampling	-Requires light anesthesia -Requires two people	(21)

Conclusions

As the field of veterinary and biomedical research evolves, future directions in blood collection techniques for rats emphasize minimizing animal distress while enhancing the quality of samples.

Importance should be given to the techniques chosen for blood sampling in rats. The protocols should be well established and followed as there are many aspects that can lead to alterations in the quality of blood samples and erroneous results.

Proper training is crucial to ensure the safety and well-being of the animals during blood collection.

Certain advantages and disadvantages are linked to each technique. The researcher should choose the protocol most appropriate for the study purpose, while also considering blood sample quality, the suitable tools needed, the skills required to succesfully perform the procedure and also appropriate animal handling and post-procedure care.

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ANESTHESIA PROTOCOLS IN RATS

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Abstract

Rats are a cornerstone of biomedical research, serving as valuable models for understanding human physiology and disease. The ethical and scientific necessity of minimizing pain and distress during experimental procedures requires the reliable and effective use of anesthesia. This paper provides a comprehensive overview of commonly employed anesthetic protocols in rats, encompassing injectable and inhalant agents, monitoring techniques, pre- and post-operative care, and critical considerations for protocol selection. Understanding the nuances of rat physiology and the properties of various anesthetic agents is crucial for ensuring animal welfare, minimizing experimental variability, and obtaining robust scientific data. While inhalatory anesthesia is advantageous because of less side effects, the depth of anesthesia must also be considered. Fluid therapy during anesthesia and in the post-operative period is a relevant subject. Attention should be drawn towards pain-relief medication that include opioids and/or non-steroidal anti-inflammatory drugs and premedication with specific agents like atropine.

Keywords: *inhalatory anesthesia, injectable anesthesia, rat, post-operative care.*

The use of anesthesia in laboratory rats is crucial for both ethical and scientific reasons. Ethically, it is important to spare animals in research from unnecessary pain and suffering. Scientifically, insufficient anesthesia can lead to physiological alterations that may produce erroneous experimental outcomes, affect behavioral responses, and ultimately undermine the validity of the study (7).

The use of anesthesia in research animals is governed by strict ethical guidelines emphasizing the principles of the 3Rs: Replacement, Reduction, and Refinement. Refinement specifically aims at reducing pain and distress, as well as selecting and administering appropriate anesthetic protocols and post-operative care. Researchers have an ethical responsibility to remain informed about best practices in anesthesia and to continually seek ways to enhance animal welfare (28).

A thorough understanding of the properties of various anesthetic agents, proper monitoring techniques, and diligent pre- and post-operative care are crucial for achieving successful anesthesia and minimizing animal suffering. The choice of anesthetic protocol should be a collaborative decision between the researcher and the veterinary staff, taking into account the specific needs of the animal and the experimental design. Continuous advancements in anesthetic techniques and monitoring strategies promise to further enhance animal welfare and the quality of research using rat models (15, 20).

Selecting the appropriate anesthetic protocol for rats is a multifaceted process involving careful consideration of the procedure being performed, the animal's health status, age, strain, and the desired duration of anesthesia (8).

Anesthetic agents should also be chosen according to the type of experimental protocol. In some type of procedures, that only require a short duration or light anesthesia, inhalatory anesthetics may be more useful (12).

Materials and methods

Normal body weight of an adult rat ranges between 150g to 400g (26). Physiological normal values of core body temperature is around 37.5-38.5°C, normal heart rate range is 260 to 600 bpm, the respiratory rate range is 75 to 120 bpm, pulse oxymetry is greater than 95% (28). Some physiological parameters are affected during anesthesia in rats. The specific anesthetic protocol used not only influences the survival rate of the animal, but it can alter the experimental results. Some of the adverse effects during anesthesia in rodents can affect: blood pressure, blood oxygen saturation and cerebral metabolism (7). Monitorisation of the rats vital signs is a must during anesthesia (5). UBC Animal Care Guidelines provide certain physiological parameters values of the concious and anesthesized rat, which can be seen in Table 1.

There are three phases of general anesthesia: induction, maintainence, recovery (24). Rodents have a higher metabolic rate than other small animals, therefore when administering anesthetic agents it has to be noted that the doses needed are much higher. At the same time, the standard dose might be inneficient in certain rats or it can be excessive in others. Oh et al. (16) mention that if repeated doses are needed, no more than 10%-25% of the initial dose should be administered.

Injectable anesthetics are delivered using a needle and syringe. In rats, the appropriate

syringe needle used should be 22G needle, but can be addapted to the animals' size. The length of time that the anesthesia lasts depends on the specific drug, but it generally ranges from 20 to 30 minutes in rodents. Recovery often takes a longer time, usually much longer than the anesthesia itself (25).

Inhalant anesthetics necessitate specialized equipment to provide a precise dosage of the anesthetic, keeping the animal under anesthesia only while it continues to breathe the anesthetic gas. Animals typically experience a swift recovery from inhalant anesthetics such as isoflurane (25). Table 2 shows commonly used general anesthesia protocols in rats.

Table 1

Physiological normal values of conscious rats and rats under anesthesia – UBC Animal Care Guidelines (28)

Parameter	Value under concious conditions	Value under anesthesia
Core body temperature	37.5-38.5°C	No change stated
Heart rate	260-600 bpm	250-400 bpm
Respiratory rate	75-120 bpm	60-90 bpm
Blood oxygen saturation	>95%	No change stated

Table 2

Commonly used general anesthesia protocols in rats

Drug	Dose	Route*	Observations	Source
Acepromazine	0.5–2.5mg/kg (12, 16).	IM/SC/ PO (12, 16)	Higher doses administered only PO (12); Not recommended in preweanling animals (16).	(12, 16)
Alfaxan	20mg/kg (12).	IM (12, 16)	Used as a continuous perfusion IV (21).	(12, 14, 21)
	2–5mg/kg (12).	IV/IP (12, 16)		
Ketamine	50–100mg/kg.	IM/SC	Can be used alone for immobilisation, but yields poor muscle relaxation alone.	(12)
Ketamine+Dexmedetomidine or Ketamine+Medetomidine	75mg/kg+ 0.5mg/kg	IP	Duration up to 20-30min.	(16)
Ketamine+Xylazine	a)75mg/kg+10 mg/kg (16); b)40-60 mg/kg + 3-5-mg/kg (22).	IP (8)	Oxygen supplementation might be needed when using high doses of Xylazine (6); b) Supplement with 1/3 of ketamine dose only (22).	(8, 16, 22)
Ketamine+Xylazine +Acepromazine	a) 50 mg/kg+ 5 mg/kg+ 1 mg/kg (27); b) 31.25 mg/kg+6.25 mg/kg+1.25 mg/kg (16).	IP (16)	a) Duration of anesthesia is aproximately 30min (27); b) May not produce surgical-plane anesthesia (16).	(16, 27)
Servoflurane	a) Up to 8% in oxigen; b) 3-5% in oxygen.	IH	a) Used for anesthesia; b) Used for maintainence.	(12)

Isoflurane	a)0.5% in oxygen then slowly increase to 5% over aproximately 5 min (28); b)1-3% in oxygen (23).	IH	It is more irritative than Sarvoflurane (11); b) Used for maintainence (23).	(11, 23, 28)

*IM-intramuscular; SC-subcutaneous; PO- per os; IP- intraperitoneal; IV- intravenous; IH- inhalation

Injectable anesthesia protocols used in rats include the use of Xylazine in 5 to 10 mg/kg in combination with Ketamine administered at the dose of 40 to 90 mg/kg (intramuscular or intraperitonial) (13). Atropine is used as preanesthetic medication to reduce bronchial secretion, salivation and to protect the heart from vagal inhibition. The dose administered is 0.02 mg/kg intramuscular or subcutaneous (16).

Anesthesia machines are used to administer isoflurane, with modifications made to maintain a minimum alveolar concentration of 1.5 during procedures (19). Greenfield et al. (10), mention that inhalatory anesthesia for induction can be used through administration of the anesthetic agent on cotton balls which are placed inside an unmodified 50-mL graduated conical tube, while temporarly maining the rats' head inside.

Both subcutaneous and intraperitoneal routes of administration for some injectable anesthesia agents can be used.The subcutaneous route is recommended to be chosen between the two, as it is less invasive. Intraperitoneal administration can be associated with pain and peritoneal irritation (27).

White et al. (21) used alfaxalone solubilised in 2-hydroxypropyl-b-cyclodextrin as an anesthetic agent administered intravenously in male and female rats. Even through the compound proved to provide excellent total anethesia, there were also some side effects predominantly seen in male rats: apnea or an increase in end tidal carbon dioxide.

A Ketamine, xylazine and acepromazine cocktail can be prepared by mixing 5ml (500mg) of ketamine (100mg/ml) with 2.5ml (50mg) of xylazine (20mg/ml), along with 1 ml (100mg) acepromazine (10 mg/ml). Added should be 1.5 ml of diluent. The preparate should be stored in a cool place, away from light. This provides for a 30min anesthesia duration (18).

Another cocktail that can be prepared and administered to rats for anesthesia that lasts aproximately 30 min implyies ketamine and medetomidine. Preparation is as follows: 3 ml (300mg) ketamine (100mg/ml) combined with 2ml (2mg) of medetomidine (1mg/ml), along with 1 ml of diluent. This combination can be supplemented when needed by the administration of ketamine in a dose of 75 mg/kg (18).

During anesthesia, fluid supplementation must be provided. An exeption to this is if fluid may alter the results of the study (18). Preffered in rats is the Hartmann's solution or 0.9% saline. Replacement rate should be 10% of the calculated blood volume every 30-60min. If a great volume of blood is lost during the surgical procedure then the volume administered should be five times the estimated blood loss. Whole blood transfusion should also be considered in this situation. Flecknell (9) claims that administering 0.18% sodium chloride with 4% dextrose subcutaneously will help maintain fluid balance in the post-operative period as the route of administration provides for a slow assimilation. The dose recommended is 10-15 ml/kg (9). An important aspect regarding fluid administration during anesthesia is that liquids used must be warmed before administering (8, 20).

It is recommended that throughout the procedure, at fixed time intervals (e.g. 10min intervals) the rats breathing pattern and rate, temperature, color of extremities should be checked, so anesthetic level and heat support are adjusted according to needs (27, 28).

Anesthetic depth can be checked by the absence of certain reflexes like pedal withdrawal reflex (27). The blink reflex can be checked by gently touching the eyelid with a gauze pad, while the withdrawal reflex can be checked by pulling the hind leg out straight and pinching the foot using the thumb and forefinger (10).

Results and discussions

It should be mentioned that certain breeds of rats, like tailess rats, have issues in thermoregulation (besides other problems like spinal defects and impared balance) (12). Noted is the fact that rodents have a great body surface aria to volume ratio, therefore, a greater surface for heatloss. This makes them more prone to hypothermia (7). Heatloss during anesthesia can be prevented by the use of

heating blankets. Additionally, wrapping the rat in bubble packing can also contribute to the maintainance of the body temperature (9).

Another important aspect is the high metabolism rate, which results in: low glycogen reserves and high oxigen demands (7). Blake and Pellet (5) mention that even very short periods of oxigen depravation (seconds) can lead to central nervous system damage in rats.

Kubiak (12) specifies that the mortality rate during anesthesia in rats is up to 2.01%. Besides the previously mentioned reasons, it should be also noted that rats have fewer easily accessible veins for catheterisation and at the same time, endotracheal intubation requires much higher skill than in cats and dogs. Flecknell (9) mentions that a plastic cannula can be used as an endotracheal intubation tube in rats. In a 200-400g rat the size of the endotracheal intubation tube outside diameter should be 18–12G.

A thoroughly examination of the rat should precede the anesthetic procedure. Taken into consideration should be that a great number of rats have pre-existing respiratory diseases (12).

Some of the common infectious agents that can cause respiratory distress are: *Mycoplasma pulmonalis, Streptococcus pneumoniae, Corynebacterium kutscheri,* cilia associated respiratory bacillus, Senday virus and coronavirus (3). Factors that predispose individuals to respiratory diseases are typically associated with animal hubandry and nutrition (3). These include:

- overcrowding,
- inadequate ventilation,
- poor diet,
- the presence of aromatic or dusty bedding,
- elevated ammonia levels in the environment,
- high humidity,
- abrupt temperature changes (3).

An unspecific sign of the rat stress or suffering is the presence of red tears known as chromodacryorrhoea (5, 12). The red staining is usually found not only around the eyes, but also around the nose and shoulders, the latter being the result of the grooming behaviour of the animal (3).

Prior to anesthesia rodents should not be fasted. It is known that rats can not vomit and have a high metabolic rate, therefore, starvation of the rodent overnight can lead to dehydration (12, 28).

Ketamine, propofol and isoflurane/halothane are the most commonly used anesthetic agents for inducing and maintaining anesthesia in laboratory animals. Those can affect the carbon dioxide levels in arterial blood or exhaled air, potentially leading to respiratory acidosis (7).

Sedation onset is faster if midazolam is used in combination with medetomidine and butorphanol. Some results cited by Bellini et al. (2) suggest that medetomidine produces dose-dependent decrease of pulse rate and respiratory rate, even when not used in combination with midazolam and butorphanol (2).

Alves et al. (1) state that administering propofol (100 mg/kg) along with medetomidine (0.1 mg/kg) and fentanyl (0.1 mg/kg) intraperitoneally can be used safely as an anesthesia protocol. The recovery period is fast, while the surgical window lasts 25 min and restraint of animal 30 min (1).

Several hours of anesthesia can be achieved in rats. Kiefer et al. (11) found that in mechanically ventilated rats inducted with servoflurane, then administered a combination of propofol, ketamine and rocuronium, anesthesia can be maintained for hours.

Cicero et al (7) state that using servoflurane increases the induction time and must be used in greater concentration compared to isoflurane.

Bhatia et al. (4) while developing an appropriate ear surgery anesthesia protocol on rats, found that using ketamine and xylazine prolonged the induction and recovery period, in comparison to using isoflurane. Noted was also the mortality rate in ketamine/xylazine rat group. Benato et al. (3) mentions that servoflurane is less irritative than isoflurane when used in rat anesthesia.

Ketamine combined with xylazine are used for short duration anesthesia when applying blood collection techniques in rats. Though this protocol is commonly used, Pereira et al. (17) mention that using ketamine/xylazine as an anesthesia protocol in rats leads to altered corticosterone levels.

Certain sources also point out that atipamezole in a dose of 5mg/ml can be administered subcutaneously or intraperitoneally to reverse xylazine effect (18).

Cllahan et al. (6) studied the effect of medetomidine and ketamine induced anesthesia in pregnant and non-pregnant Wistar rats. Even through the mortality rate was not associated with pregnancy, the authors underline that higher estrogen levels can increase the sensitivity of α2-adrenoreceptors and recommend proper dosage adjustments. It is also advised that atipamezole, the antidote used for medetomidine, is at hand when using protocols involving α2-adrenoreceptor agonists (6).

During anesthesia, it should be noted the use of an ophthalmic ointment to prevent eye

dryness (9, 18). Products like Hypotears or Lubrithal Eye Gel can be used (18).

If the experimental protocol does not specifically prohibits it, analgesia of the rat should be implemented in the post-operative period (5). Usage of pain relief medication can be done preventively, with a lowering effect on maintenance anaesthesia doses (12).

Signs of the rat being in pain are: back arching, loss of balance during grooming, twitches of the back muscles. While most animals cry out when in pain, it should be considered when assessing pain in rodents that their cries are at high sound frequencies and can not be heard by humans. Analgesia protocol in rats during post-operative period can be done with opioids and non-steroidal anti-inflammatory drugs (NSAIDs). Local anesthesia can also be implemented when needed (9).

Table 3, after Fleknell (9), shows doses used in pain relief for rats.

Table 3

Suggested dose rates for non-steroidal anti-inflammatory drugs and opioids for analgesia – after Flecknell (9)

Drug	Class	Dose	Duration	Administration
Aspirin	NSAIDs	100mg/kg	variable	per os
Ibuprofen		15mg/kg	variable	per os
Ketoprofen		5mg/kg	variable	subcutaneously
Meloxicam		1mg/kg	variable	subcutaneously/ per os
Buprenorphine	Opioids	0.01-0.05mg/kg	8-12h	sbcutaneously/intr avenously
Butorphanol		1–2mg/kg	4h	subcutaneously
Pentazocine		5–10mg/kg	3-4h	subcutaneously
Meperidine		10–20mg/kg	2-3h	subcutaneously/in trapetironeally

Conclusions

Total anesthesia in rats can be achieved using inhalatory anesthetics which can be combined with injectable anesthesia agents. The duration and depth of anesthesia are variable.

Anesthesia protocol should be adapted to the research objectives and/or the procedure done on the rat. Some rats might need dose adjustments as there are certain individual differences.

To enhance the success of anesthesia in rodents, it is essential to focus on several key factors: stabilizing animal's clinical condition, preventing hypoxia, avoiding hypothermia, providing fluid supplementation and providing safe and effective analgesia.

Analgesics, similar to anesthetics, can produce a wide range of responses. Researchers must acknowledge that certain parameters should be considered indicators of analgesic efficacy with specific drugs.

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