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„REGELE MIHAI I" DIN TIMIŞOARA

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SAMPLING IN VETERINARY ONCOLOGY - A REVIEW

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Summary

Oncology, the study of tumors and cancer, is an expending field in veterinary medicine, with an important impact for both researchers and clinicians. Tumors (benignant or malignant) are for sure one of the biggest challenges for the veterinary clinician (cancer is the worldwide leading cause of death in pets, being responsible for the death of 47% of dogs aged over 10 years). The most important step when it comes to these conditions is a correct and comprehensive diagnosis. The practitioner plays a big part in the success of this: by evaluating the patient, the tumor itself and by sampling it, so the pathologist can determine the exact nature of the tumor and its behavior. The most common diagnostic tools are cytology (using fine needle aspiration (FNA) to collect samples) or histopathology (using biopsy tools to collect samples). Sampling is not a difficult procedure at all, but it has some rules, to be accurate and precise. Sedation or anesthesia may be necessary. The risks should be evaluated by the veterinarian and discussed with the owner; but, in all cases, sampling should not be stressful or painful for the animal. FNA is a technique that involves a thin hollow needle (23-25 gauge) that is inserted in the mass, to remove (withdrawn) a small sample of cells (as the tissue sampled is small, it is possible that the specimen obtained is not sufficient for a confident diagnosis to be made). A biopsy involves the use of a larger needle (or an excision) which removes a solid block of tissue and is therefore slightly more invasive. Since a core of solid tissue is removed, the specimen is excellent and typically is sufficient for a confident diagnosis. The goal of this paper is to make a small guide (using the latest research and the specific literature) for the veterinary clinicians, on how to do a correct and efficient sample in cases of tumors (or suspicion of).
Keywords: veterinary oncology, FNA, biopsy.

Oncology, the branch of medicine concerned with the prevention, diagnosis, treatment, and study of cancer is one of the most important and studied fields in both human and veterinary medicine. Oncological diseases have a major impact on researchers, clinicians and, of course, patients. Talking about veterinary medicine, the owners are very much involved; it is vital to have a good and trustworthy relationship with the owners, as it is for them to understand their pet’s disease, prognosis, and treatment options (2, 25).

Tumors, either benignant or malignant, are seen on a large scale in the general practice. More than this, almost 30% of all diagnosed tumors are those of the skin; so, the tumors are easily seen by the owner and the animal is brough in for a consultation. Further, the owners will be very sensitive to the subject at hand, as it is something they can sometimes see / touch and it has a visual and emotional impact on them (5).

Considering that cancer is the worldwide leading cause of death in pets (studies suggest that one in four cats or dogs will die because of cancer or cancer-related disease), it is only fair to assume that the way clinicians deal with these cases is vital. Of course, a thorough clinical examination, as well as a comprehensive evaluation of the patient is a must, and it should be the first step. But, when dealing with an oncological disease, a correct diagnosis is the key (19).

First, it is important to establish the gross characteristics of the suspected tumor. Second, and the most crucial step is to do a sample (taking a small part or quantity intended to show what the whole is like) in order to have a definitive diagnosis. At the most basic level, differentiation of neoplasia from non-neoplastic disease is likely to greatly increase the chance of therapy success; also, differentiation of benignant from malignant tumors is an essential requirement for optimizing the treatment of the individual cancer patient and for providing the client with an assessment of likely cancer behavior and likely prognosis (22).

Choosing the correct sample technique for any tumor type can present as a challenge to the clinician when trying to optimize the cost/benefit ratio of the extent of any investigations into the tumor prior to treatment.

There are several options when it comes to sampling a tumor; when choosing it, the clinician has to take into consideration a number of things. This article aims to be a small guide for the practitioners for when they have to deal with such situations, so they can opt for the technique that's most suitable considering their case, and to do it in a correct manner, so the pathologist can issue a conclusive diagnosis.

Materials and methods

Studies and research that were conducted on the subject at hand (key words: veterinary oncology, sampling, fine needle aspiration, biopsy, diagnosis) as well as other publications, were selected (using PubMed, Web of Science, Google Academic) and used for this review.

Results and discussions

In this review paper, the authors want to briefly explain the most common sampling techniques options, when dealing with a suspected tumor: cytology (smear, fine-needle aspiration - FNA) and surgical biopsies, for histological examination (punch, needle core, grab/pinch, incisional, excisional).

Proper techniques for collection and preparation are essential to secure high-quality diagnostic samples. Most clinics are equipped with the necessary supplies for collecting cytologic samples from various tissues, body cavities, and mucosal surfaces. These supplies typically include hypodermic needles and syringes, scalpel blades and handles, propylene urinary catheters, bone marrow aspiration needles, cotton swabs, clean glass slides, marking pencils, and collection

vials and tubes (including tubes with ethylenediaminetetraacetic acid - EDTA and plain sterile tubes). For the aspiration of internal lesions, especially those guided by ultrasonography or computed tomography, longer spinal needles and extension sets (used to connect the spinal needle to the aspirating syringe) prove useful. Additionally, cytologic specimens can be derived from tissues collected during surgical biopsy procedures (2, 14, 20).

Anesthesia, local or general, is necessary in most cases. When performing it, the clinician should take into consideration the general state of the animal, the age, any coexisting diseases, just like in any other situation that requires this procedure. Even if sampling can be a fast procedure, the animal should not be subject to any trauma, pain, or stress. Reluctant patients should always be anesthetized to ensure the safety of both the medical staff and the patients themselves (2).

Cytology techniques

Cytology plays a crucial role in veterinary oncology, providing valuable diagnostic information about tumors in animals. Examining tissue samples cytologically is a swift and uncomplicated technique, demanding minimal equipment and is readily executable in a general practice environment (11, 23).

Undoubtedly, cytology stands as a valuable diagnostic technique in investigating neoplasia, but it is crucial to acknowledge its limitations. Cytology often falls short of providing a definitive diagnosis for tumors. For instance, in certain solid tumors, neoplastic cells may not exfoliate adequately, hampering the availability of sufficient cells for a conclusive diagnosis. This limitation is notable in tumors that produce substantial stromal components, such as fibromas or osteomas (1, 2, 27).

Cytology is generally unsuitable for grading most tumors, as grading primarily relies on evaluating the relationship between neoplastic cells and surrounding normal tissues. Other factors, such as the mitotic rate, degree of inflammation, and necrosis, crucial for grading, are challenging or impossible to assess through cytological examination alone. Certain tumors, like mammary tumors, exhibit a highly intricate architecture, necessitating the examination of histological tissue sections for a diagnosis. In such cases, cytology proves less beneficial as it may struggle to differentiate between various types of mammary tumors (2, 3).

An important thing to keep in mind is that it is crucial to prevent cytological specimens, especially smears, from encountering formalin fumes. Exposure to formalin significantly diminishes the intensity and microscopic characteristics of typical cytological stains, making the examination of smears nearly impossible (4).

Impression smears

Impression smears can be conducted either with the mass in its original position or using biopsy specimens obtained from the mass. The steps involved:

- Gently pat the cleanly incised surface dry with gauze (sterility depends on

whether the impression smear is taken in situ or not) to eliminate excess blood, which could otherwise overwhelm the cytology preparation;

- If the mass is in its original position, lightly dab a clean glass slide onto the cut surface. If a biopsy specimen is used, dab the cut face onto a slide and allow it to air dry (3, 5).

To enhance the probability of achieving a diagnostic preparation, it is advisable to prepare multiple slides. If the tissue's surface area is limited, multiple impressions can be accommodated on a single slide (4).

Fine-needle aspiration

Fine-needle aspiration is the preferred cytological technique in situations necessitating a provisional diagnosis before resorting to incisional/excisional biopsy. It is particularly advantageous when an aspirate can be obtained through imaging-guided techniques, eliminating the need for surgical intervention. This is notably applicable, for instance, when sampling an intra-abdominal mass under ultrasound guidance (1, 5).

- Prepare the skin accordingly. In most cases, skin preparation may only involve wetting the fur with alcohol and does not require clipping. However, when there is a potential need for culturing the FNA sample, it is advisable to clip and prepare the skin as one would for a surgical biopsy. This precaution helps minimize the risk of sample contamination with cutaneous microorganisms. Additionally, for aspirating a fluctuant mass, it is similarly prudent to take further steps in skin preparation to decrease the likelihood of contaminating the fluid within the mass or cavity. This becomes especially crucial when dealing with a fluid-filled structure in proximity to a joint or body cavity. For example, in the aspiration of a perineal mass that might indicate a perineal hernia, where there exists a potential for communication between the mass and a body cavity, taking these precautions is highly important;
- Prepare a needle, 20-25 G, beginning with 1-inch 23 G, and a syringe, 5-10 ml. Introduce the needle into the mass and reposition it with a stabbing action, repeating this motion three to five times. This can be achieved either by creating negative pressure through pulling back on the syringe plunger or without applying negative pressure. It's worth noting that in certain situations, excessive negative pressure during the aspiration process can lead to cell damage, potentially diminishing the diagnostic effectiveness of the sample;
- Release any pressure on the syringe plunger and withdraw the needle and attached syringe from the patient. Disconnect the syringe from the needle hub, where any aspirated cells will be located. Air is drawn into the syringe, and then reattach the syringe to the needle hub;
- Position a clean slide on a horizontal surface and firmly press the plunger down to expel the contents from the needle/needle-hub onto the slide. A

single FNA may suffice to create two or three slide preparations;

- To create smears and ensure the preparation is not overly thick for microscopic examination, place a second clean slide at right angles on top of the first. Smoothly and gently smear out to the edge without applying excess pressure. Avoid exerting additional pressure beyond what is necessary to move the slide over the one beneath, as excessive force can lead to extensive rupture of aspirated cells, rendering the preparation challenging to interpret;
- Allow the smears to air dry before submission or in-house staining (3, 5, 6, 10).

Surgical biopsy techniques

Examining a biopsy sample through histological methods stands as the most precise approach for diagnosing cancer and is likely to yield a more conclusive diagnosis compared to relying solely on cytological diagnosis. Generally, larger tissue specimens are acquired, facilitating the observation of neoplastic cells within the context of surrounding tissue architecture. This approach offers the advantage of potentially identifying invasive features, such as the invasion of blood vessels or lymphatics, and may permit tumor grading. In many instances, submitting a smaller tissue sample for examination increases the likelihood of the tissue sections being non-representative or the pathologist facing challenges in making an accurate diagnosis (20, 21, 28).

The choice of technique for biopsy will be contingent upon the size, location, and characteristics of the suspected tumor. Deliberate consideration should be given to the biopsy procedure to guarantee the collection of a representative tissue sample, all while avoiding the potential for local tumor dissemination or jeopardizing future therapeutic interventions (4, 8).

Punch biopsy

A punch biopsy is appropriate for obtaining samples from superficial lesions, such as those on the skin or any external relatively shallow tumors. It can also be employed during laparotomy procedures for organs like the liver or spleen. The procedure involves the following steps:

- Preparation: clip and surgically prepare the site. Infiltrate local anesthetic into the area to be sampled;
- Biopsy Process: position the circular blade of the punch onto the surface of the lesion. Rotate the punch under gentle pressure to the required depth, collecting a cylindrical specimen within the punch. Closure of the wound may necessitate one or more sutures.

This technique is particularly well-suited for obtaining tissue samples in a controlled manner, ensuring precision and minimal invasiveness, especially for lesions situated on or near the skin's surface. Additionally, it proves effective when exploring deeper organs during surgical procedures like laparotomy (28).

The punch biopsy method may not penetrate deeply into the lesion, requiring caution to ensure the collection of representative tissue, especially in cases with necrosis or inflammatory tissue (e.g., oral tumors). Moreover, when obtaining a sample from a subcutaneous mass by going through the skin, it is crucial to prevent the punch biopsy instrument from merely filling with skin. In such cases, it is advisable to make a small incision to facilitate the instrument's reach to the mass. This precaution ensures that the collected sample accurately represents the target tissue, enhancing the diagnostic reliability of the biopsy (3, 10, 18).

Needle core biopsy

A needle core biopsy is suitable for extracting small tissue cores from solid soft tissue lesions that can be precisely located and secured for sampling. This technique is applicable with ultrasound guidance, particularly for sampling intra-abdominal and certain intrathoracic lesions. The procedure involves the following steps:

- Preparation: clip and surgically prepare the skin over the lesion. Administer sedation to the patient. Infiltrate local anesthetic into the skin and soft tissue in the region of the lesion;
- Biopsy Process: the 'Tru-cut' biopsy needle is commonly used for this procedure. In manually operated types, after introducing the needle into the lesion, the central core is advanced further into the lesion and rotated to collect tissue in the specimen notch. The outer sleeve is then advanced, trapping the tissue in the notch. The needle is withdrawn, and it is opened for the collection of the biopsy sample.

This technique is particularly valuable for obtaining precise and targeted tissue samples from solid lesions, offering a controlled and effective means of diagnosis. The use of ultrasound guidance enhances the accuracy of the biopsy, especially when dealing with lesions in challenging anatomical locations (3, 10, 16).

Grab/pinch biopsy

A grab or pinch biopsy is suitable for collecting samples from mucosal surfaces, such as those in the respiratory, alimentary, and urogenital systems. This technique is commonly employed in conjunction with endoscopy, allowing for visualization of the surface to be sampled. Biopsy cups on endoscopes are typically used to collect mucosal samples through a biting action. General anesthesia is often necessary for this procedure.

While providing a relatively non-invasive means to access hollow organ systems, the drawback of the grab or pinch biopsy technique lies in the superficial nature of the collected samples. These samples may not be truly representative of the pathology affecting the organ. The technique's limitations stem from the nature of the biopsy method and the size of the biopsy cup, especially in confined spaces, where the obtained samples can be extremely small. This diminutive size can pose challenges in achieving a meaningful histopathological diagnosis (17, 22, 28).

Incisional biopsy

An incisional biopsy involves excising a wedge-shaped segment of tissue using a scalpel. This method yields larger tumor samples, enhancing the accuracy of the diagnosis. Incisional biopsies are recommended under the following circumstances: when both core needle and punch biopsies have been unsuccessful in obtaining a representative tissue sample; when a larger tissue sample is required, especially in the case of a necrotic or ulcerated tumor; in situations where an exploratory laparotomy allows for the removal of a more substantial tumor piece (28).

Anesthesia and wound closure with sutures or staples are necessary for the procedure.

It's important to note that the use of electrocautery should be avoided before the complete removal of tissue biopsies to prevent thermal artifacts that could affect the diagnostic accuracy of the samples. The incisional biopsy method provides the advantage of larger tissue specimens, contributing to a more precise and reliable diagnosis (4, 17).

Excisional biopsy

Excisional biopsy involves the complete removal of a tumor without presurgical analysis of the tumor type. This method is preferred for small, slowly growing cutaneous tumors, splenic tumors, and pulmonary tumors. In these cases, standard treatment protocols are often applicable, irrespective of the specific tumor type (17, 28).

However, for all other tumor types, it is advisable to consider non-excisional biopsies initially. Histopathologic tumor classification through non-excisional biopsies allows for a pretreatment diagnosis, which, in turn, may facilitate the development of a customized treatment protocol based on the specific characteristics of the tumor. This approach enables a more tailored and targeted treatment strategy for a broader range of tumors (17, 22).

When sending tissue samples, it is preferable to submit the entire sample in formalin whenever possible. This approach allows for the comprehensive assessment of the entire tumor specimen, including surgical margins. In the case of complex specimens, it's essential to mark tissue orientation if deemed significant. If only part of a large tissue mass needs to be sent, send representative portions from the periphery, avoiding areas of hemorrhage or necrosis. Maintaining a high formalin volume-to-tissue volume ratio (10:1) is crucial, and efforts should be made to avoid forcing large samples into small containers (4, 15).

When submitting only part of the tumor, avoid sending necrotic or hemorrhagic areas as these areas may lack sufficient cellular detail for an accurate diagnosis. For very small samples like endoscopic biopsies, it is imperative to prevent introduction of artifacts during specimen collection. Crush artifacts, which obscure cellular detail, can occur when nuclei and cytoplasm are squeezed out of damaged cells. Additionally, avoid artifacts caused by cautery, especially at critical

tumor margins (14, 15).

Providing additional information about the case, including signalment, lesion location and orientation (where appropriate), and any relevant clinical details enhances the accuracy of the diagnosis. This information enables the pathologist to offer a precise diagnosis and provide prognostically useful details such as tumor grade, presence of vascular/lymphatic invasion, and extent of surgical excision. In cases of diagnostic uncertainty, the pathologist may suggest further assays, such as different histochemical stains or immunohistochemical staining, to aid in clarification (8).

There is a substantial body of literature cautioning clinicians about the risk of seeding tumor cells, known as needle tract metastasis (NTM), or potentially inducing distant metastasis through biopsies in companion animals. A recent meta-analysis revealed that while NTM is an exceedingly rare occurrence, there may be a potential risk of biopsy-induced seeding of tumor cells into the biopsy tract. However, it is essential to note that there is no evidence supporting the hypothesis of biopsy-induced malignancy in the available literature within the fields of human or veterinary oncology. To date, only seven reported cases exist in the literature documenting needle tract metastases or tumor seeding in companion animals (9, 15).

To ensure a proper diagnosis and to understand the disease and the state of the animal, the following complementary investigations can be made: computer tomography, magnetic resonance imaging, echography, radiography, positron emission tomography, nuclear scintigraphy. Diagnostic imaging is essential in the diagnosis, clinical staging, and evaluation of response to therapy of cancer patients (12, 13).

Conclusions

Pets are living longer, healthier lives thanks to advances in veterinary medicine, preventive care, and nutrition. As the lifespan of pets increases, however, so does their risk for developing cancer. Dogs and cats have higher age adjusted incidence rates for many kinds of cancers than do humans. For example, dogs are 35 times more likely to get skin cancer than are humans. They suffer from 8 times the amount of bone cancer and 4 times the amount of breast cancer. Cancer is one of the major causes of morbidity in veterinary patients, and surgical removal of tumors one of the most common surgical procedures performed by veterinarians (4, 7, 19).

Obtaining tissue samples and information before treatment allows for the best possible outcome. Knowing if a tumor has metastasized beyond the primary location can enable planning for more extensive surgery or for multimodal treatment with surgery, chemotherapy, and/or radiation therapy. By understanding tumor behavior and the route by which a specific tumor type may spread, oncologists can recommend the most valuable staging test for that cancer (9).

Early detection and consultation with a veterinary cancer specialist leads to positive outcomes in most cases. Cancer is not just one disease; there are hundreds of different tumor types, and they all respond uniquely to treatment. Even with the most aggressive cancers, veterinarians can help to provide comfort, pain control, and support for the owner and for the pet.

The process by which these samples are collected, coupled with the information supplied by the clinician to the pathologist, can significantly improve or potentially hinder the accuracy of the diagnosis derived from those samples. The pathology report is key in determining prognosis, therapeutic decisions, and overall case management and therefore requires diagnostic accuracy, completeness, and clarity. Successful management relies on collaboration between clinical veterinarians, oncologists, and pathologists (14, 23, 24).

The annual physical examination plays a key role in detecting neoplasia, either directly via palpation or through paraneoplastic syndromes, which may be found on routine blood and urine analysis. The physical examination allows detection of possible tumor presence and identification of concurrent conditions that may influence the treatment plan.

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IN DEPTH REVIEW OF FELINE PANLEUKOPENIA VIRUS

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Summary

Feline panleukopenia virus (FPV), a highly contagious and deadly pathogen affecting cats, is the causative agent of feline panleukopenia. FPV is a single-stranded DNA virus that belongs to the *Parvoviridae* family. The virus is resilient in the environment, and its eradication is being a real challenge. Despite the significant progress in understanding FPV, ongoing research is focused on improving diagnostic methods, developing novel vaccines, and gaining deeper insights into the virus' genetic diversity and evolution. Our review research is focused on virus epidemiology, immune response, clinical manifestations, pathophysiology, diagnosis and prevention as well as molecular genetics with the latter being the most promising; highlighting the importance of continuing research efforts in combating this deadly virus. We tried to analyze the host as a main source of interest regarding the infective process. In the same time, we tried to answer in questions like: "*Why is the virus only affecting cats?"* or "*What other treatment could be used?*" and most importantly "*Can the contact with the host itself be stopped?*". The review research provided useful insight about cytotoxic factors released by T cells and also about TfR1 and TfR2 expression regarding their role in the infection. In order to conclude, our work was meant to deeply understand the mechanisms behind the action of this deadly pathogen as well as speculate new perspectives regarding the approach medical science could have towards it.

Keywords: panleukopenia, felines, future perspective, TfR1, TfR2.

The feline panleukopenia virus (FPV) from *Parvoviridae* family, *Carnivore protoparvovirus 1* (CP) is a deadly pathogen affecting a wide range of felines. It primarily targets rapidly dividing cells, leading to severe lymphopenia and a myriad of clinical symptoms, including anorexia, vomiting, fever, diarrhea, and even neurological signs. The virus can be transmitted through direct contact with infected cats or their contamined environment making it a real threat to the feline population.

Diagnosis of FPV typically involves blood count analysis, clinical signs and molecular analysis, such as PCR. Timely and accurate diagnosis is pivotal in managing and preventing outbreaks and vaccination is the most effective counter measure to date.

Studying this pathogen is important for the veterinary practice as it has shown its capacity to express mutations. One example would be the *Canine Parvovirus* (CPV) variant that appeared in 1978 as a mutation of FPV which was isolated in tissue culture (from cats) for the first time in 1964, but cases of the illness were reported even way back in 1920; 1940 respectively. To this date, FPV as a

singular viral entity remains stable compared to its mutation, CPV, which further evolved in various antigen variants (2a, 2b and 2c) by genetic recombination (28).

The present work aims to deeply understanding the virus both structurally and pathologically as well as the infection itself in order to theorize possible ways of combating this deadly viral entity.

1. Epidemiology

In Australia, cases of feline panleukopenia since the 1970s were at an all-time low. In the year 2014, the virus has emerged again and more outbreaks have taken place between 2015 and 2018. In New Zealand and in the United Arab Emirates, the virus has reappeared after decades of low activity in 2016-2017 and 2018, respectively (4). Distribution of the cases is detailed in Fig. 1 and Fig. 2.

The occurrence of feline panleukopenia virus in such countries, where the use of the FPL (Feline panleukopenia) vaccines is widespread has raised a couple of questions as to what are the factors that caused the outbreak.

It is recommended to vaccinate cats of 8-9 weeks of age and 3-4 weeks later with a consecutive booster 1 year afterwards. The third vaccination is supposed to be given at 16-20 weeks of age, especially to kitten from places with a high infection pressure. Additional boosters should be administered at intervals of 3 years (32). Modified live virus (MLV) vaccines may act through interferon release to produce rapid protection but, they do have some risks besides the known side effects (sarcomas, even though very rarely). Infections of other nature, especially with the immunosuppressive retroviruses like *Feline Immunodeficiency Virus* or *Feline Leukemia Virus* can prove problematic to say the least in the case one owner would wish to vaccinate their cat. Also, pregnant queens or neonatal kitten (4-6 weeks) should not receive vaccinations in order to avoid encephalitis and cerebellar damage (31).

In the above-mentioned outbreaks, data and clinical samples have been collected to evaluate the epidemiological factors that are related to the outbreaks and vaccination status. The data necessary for analyzing the reason why the virus has emerged again in Australia has been collected from a national online companion animal disease surveillance-reporting database and from the medical records of animal shelters or veterinary hospitals where the outbreak has taken place. In New South Wales it has been discovered that two of five shelters did not vaccinate cats under their care. The information received from 610 diagnosed cats has revealed that 528 (87%) of them had never been vaccinated or had an unknown vaccination history. Out of the 82, sixty-five (79%) cats that have received at least one vaccination were <16 weeks of age at the time of their last vaccination (4). In this case it is safe to assume that a causing factor was the lack of vaccinations in the shelters.

In Wellington, New Zealand, out of 399 symptomatic cats, 365 total cases of FPL were diagnosed in the two outbreaks that have occurred (in December 2016 and January 2018). Out of that number, 32% of cats were unvaccinated, 58% had

received one vaccine, 8% had been vaccinated two times and 2% had been three times vaccinated. The median time since the last vaccination was about 7 days and the one since shelter admission was also 7 days. Data regarding the recovery rate for vaccinated cats in this outbreak is not available but it is known they presented clinical symptoms (4).

In Dubai around the same time there were fourteen recorded cases of feline panleukopenia. All of the cats were strays or recently adopted strays. All of them, except one, were unvaccinated. However, the vaccinated cat was of 18 months age old and had a full vaccination history (4). According to this data, vaccination alone is not a full-proof method of assuring owners of the survivability of their cats. This fact is raising the need for antiviral studies more than ever.

Another interesting fact is related to the regional characteristics of the outbreaks. Data could be incomplete as other unreported outbreaks could have occurred in different regions during that time but based on the information at hand, factors like the population's wealth, temperature and cat population definitely affects the infection rate. The degree to which it does so could constitute the topic for future studies as information of this nature can increase awareness and improve prevention.

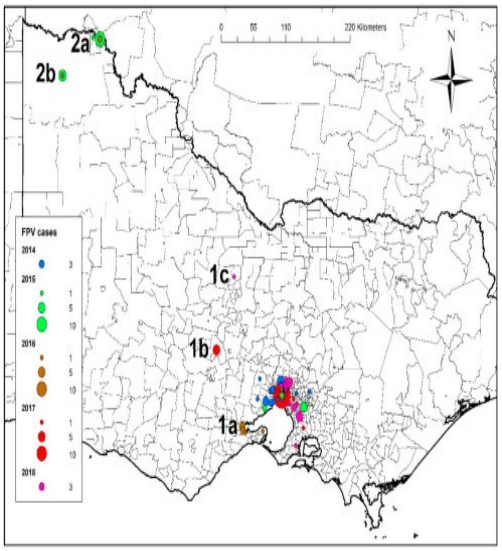


Fig. 1. Regional/Timeline distribution of cases that occurred in Australia between 2014 and 2018 (4)

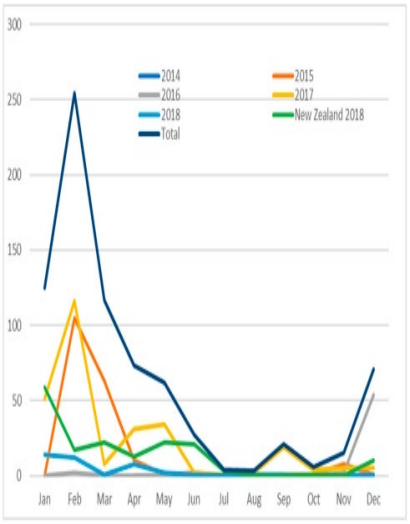


Fig. 2. Number of cases/month during outbreaks per each year in Australia and in New Zealand for 2018 respectively (4)

2.Virus Genetics

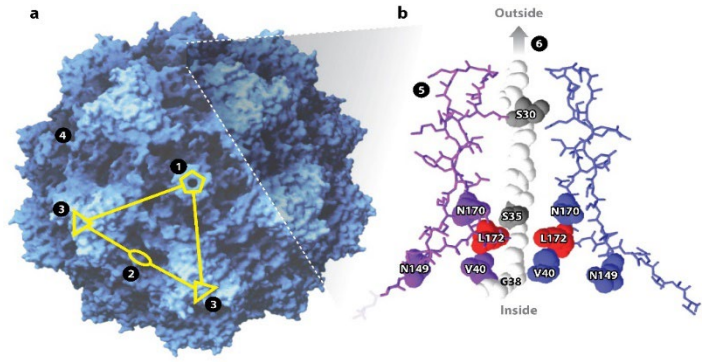
2.1. Molecular structure of the virion

The capsid presents itself with an icosahedral symmetry made of 60 viral proteins. Studies on FPV's viral capsid are scarce but there is some research made on the capsid of MVM (Minute virus of mice) which is a virus that belongs to the same genus (thus it's morphology must resemble the one of FPV). In this virion's case the protein sequence, through its icosahedral symmetry creates two-, three- and five-fold symmetry interactions which highlights it's topological characteristics which are:

- depressions at the twofold axis which mediate the virus tropism through glycan mediated interactions
- elevations at the threefold axis which are involved in interactions both mediated by receptors or glycans
- cylindrical projections surrounding the fivefold axis that are surrounded by small depressions without known functions (19).

Furthermore, the cylinders around the fivefold axis' are formed through juxtapositioning of the antiparallel β-hairpins of each viral protein (VP) to form a small channel that connects the environment from outside of the virion with the one inside the virion. Biochemical and genetic studies highlighted the role of this channel to be the internalization and externalization of the viral DNA (Fig. 3) (8).

The most important proteins within the capsid's structure are VP1 and VP2. VP2 occupies approximately 90% of the capsid being a smaller but more abundant protein which also includes the C-terminus zone of the capsid while VP1 is a larger protein but less abundant, having a N-terminus unique sequence which constitutes a phospholipase (PLA2) that could be responsible for the escape of the virion from the late endosome during endocytosis (21).



Cotmore SF, Tattersall P. 2014.
Annu. Rev. Virol. 1:517–37

Fig. 3. A-MVM capsid and the fivefold cylinder as well marked by the asymmetrical structure within the 5 fold axis; B-Structural representation of the cylinder wall (8)

2.2. The virus nucleotide sequence

The feline panleukopenia virus has a small genome single stranded DNA of 4,983 Kb which encodes for the synthesis of viral proteins (VP1 and VP2) and for at least 1 non-structural protein (NS1, even though in CPV and MVM the virus encodes for NS2 as well). Through an experiment in which viral DNA of FPV was cloned there were 2 ORFs (Open Reading Frame) discovered. The right one from nucleotide 258 to 2271- 2013 bases (which encodes for VP1 and VP2) and the second from 2358 to 4533- 2175 bases (which encodes for NS1). These sequences being in frame 3 on the complementary strand. One additional smaller ORF of 264 bases was found in frame 2 on the complementary strand (1970 to 2234) (20).

On the viral strand there were also 2 ORFs of smaller dimensions. One made of 420 bases (from 911 to 1331) and 306 bases respectively (from 2009 to 2315) also found in frame 2 (7). The DNA was measured in map units (M.U.); the 3' end being considered M.U. 0 and the 5' end being considered M.U. 97 (20).

In the viral genome there are 8 TATAA promotor sequences but only 3 of these are specific for the gene expression in a eukaryotic cell (located at M.U. 4, 30 and 39). Also, the genome consists of only 2 AATAAA sequences necessary for the addition of polyA at the 3' end of the mRNA (at M.U. 31 and 94) with the mention that the second is followed immediately by a stem-loop structure of 23 nucleotides. It is assumed that because of the secondary associated structure this is the zone for the polyadenylation of all the mRNA transcribed from the viral genome. This theory also stands on the fact that the sequence located at M.U. 31 appears in the middle of the left ORF. Having only a sequence responsible for the adenylation, the synthesis of three distinct proteins is possible because of the splicing phenomenon. Introns being placed between nucleotides 527 to 1998 and from 2274 or 2310 to 2383. According to it's genome, the NS1 protein contains 668 amino acids, the VP1 contains 727 amino acids and the VP2 contains 584 amino acids (20).

2.3. Binding site and TfR description

The virus binds to TfR1 on the residue situated on position 221 (Leu). TfR is a type 2 transmembrane homodimeric receptor and each monomer of this receptor is made out of 3 domains: Protease like, helical (which is near the membrane) and the apical domain (Fig. 4).

The role of the apical domain besides the ligand properties for the virus described in the present work are insufficiently studied but what it is known for now is that this receptor is made for the internalization of iron bound transferrin (holo-tf). Holo-Tf binds to the helical and protease like domains and is competitive with another protein which is called HFE (hemochromatosis protein) which is closely bound to the protease-like domain. In the context of the present study, it is important to mention that the receptor also has 3 N-glycosilation sites for cats (different from dogs where there are 4). The experimental change of these sites as well as the change of the amino acid sequence in the apical domain have a strong impact over the infection which can allow for total new perspectives in future research and

development of antiviral strategies (12).

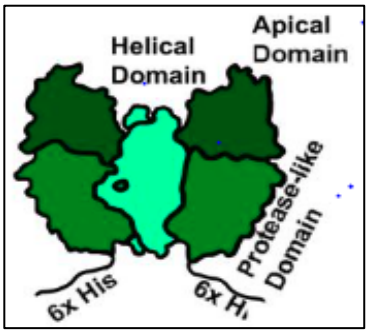


Fig. 4. Visual representation of the transferrin receptor (6)

2.4. TfR1 and TfR2 differences

The organism has 2 different TfR receptors: TfR1 and TfR2. Hypothethically, a simple inactivation of the TfR1 could solve the infection problem if this protein would not be involved in the homeostasis of the organism through its control over the intracellular levels of iron. However, the protein HFE also plays a very important role in this physiological mechanism. Mutations regarding HFE and TfR2's expression resulted in hemochromatosis caused by a hypersecretion of hepcidine (which is a protein that blocks iron absorbtion by destroying the protein responsible for iron absorbtion in the small intestine- ferroportin- or it can act by sequestrating iron in the liver). Both TfR1 and TfR2 are able to bind HFE. The mechanism through which mutations can cause these pathologies is believed to be due to the fact HFE is in complex with TfR1 at a low iron saturation, but if the iron levels rise, HFE is replaced by Holo-Tf on TfR1 and binds to TfR2. Scientists believe that HFE in complex with TfR2 or another protein triggers a cascade of events which result in hepcidine hyperexpression. However, TfR2 is a transferrin receptor, meaning that it is also capable of internalizing iron in the cell via clathrin mediated endocytosis. Thus, studies that focus on the genes that encode for these 2 proteins (TfR1 and TfR2) and the proteins themselves (Fig. 5 and 6) can determine the structure differences in their different domains (protease-like, helical and apical) as well as in their glycosylation sites to determine the affinity of the virus for TfR2 (16).

Fig. 5. TfR1 protein alignment. Generated using www.uniprot.org (35)

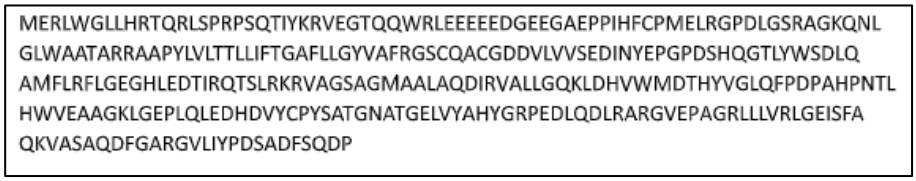


Fig. 6. TfR2 protein alignment. Generated using www.uniprot.org (35)

2.5. Virion entry

The virion mutations that appeared for the CPV-2b and CPV-2c are found on the 426th position so this could be the main binding site of the virus (13).

The infection model follows a clathrin mediated endocytosis pathway (6). The study of the virus' entry can be made with the help of fluorescent microscopy over the "Rab proteins" used as markers. These are basically GTP-ases associated with different stages of the endosomes. Rab5 is associated to the early endosome, Rab7 to the late endosome and Rab11 to the recirculatory endosome (13).

These studies suggest that after the virion exits the late endosome (presumably with the help of PLA2), it reaches the nucleus with the help of microtubules from the cytoskeleton. Not all viral particles manage to go through the nuclear membrane because of the invaginations at this level, but the ones that do are ready to initiate replication (Fig. 7) (13, 6).

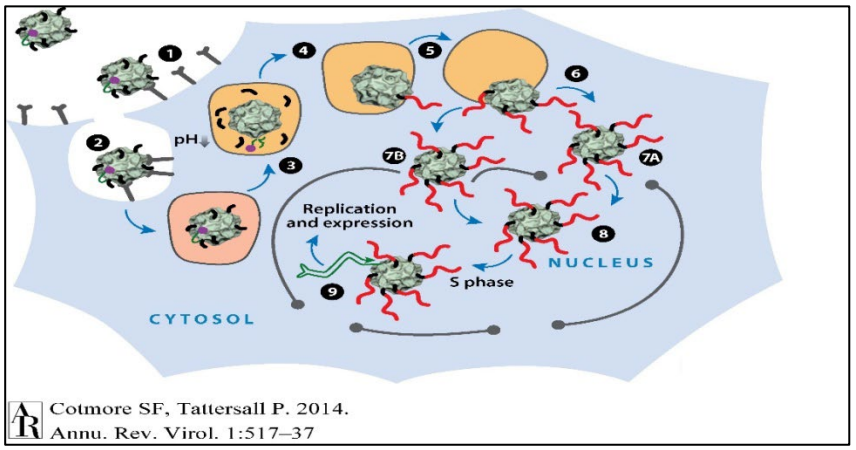


Fig. 7. Virion cell-infection via endocytosis followed by entry into the nucleus (8)

2.6. Replication

Source data to highlight the exact replication mechanism of FPV are very scarce thus in the present work MVM is the reference point again with the same arguments mentioned in the first subchapter. The virus has a particular rolling hairpin replication model. The genome has a special structure with T shaped hairpins made of highly reiterated palindromic sequences at each end of the strand. The sequence at the 3' end has -OH bound bases. It is exactly this terminal that will function as the primer for DNA synthesis. Logically, using terminals as primers would result in the loss of sequence with each replication cycle but the replication mechanism prevents this phenomenon because of a thing called "strand displacement" (3).

The first step in replication is represented by the elongation of the 3' end until it reaches the region close to the other end of the genome (where the 5' end is initially). Here, a "bending" of the DNA will take place; whether it is because of the action of the NS1 protein or because of the cell's own topoisomerases is not completely understood, yet. Consecutively, the elongation of the 5' end takes place. The nucleotide assembly then continues on a complementary model in the same circular path to the 3' end while in this region the NS1 protein with endonuclease properties nicks the strand in order to isolate the regional hairpin which will also elongate afterwards. The now elongated regional hairpin will serve as the template for the synthesis of the new hairpin. From this point, the replication has a cyclic character because of the synthesis of this concatemer (Fig. 8). Viral genomes are then excised from the concatemer with the help of the NS1 protein and then attached within the virion's newly formed or already formed capsids (Fig. 9) (3, 19).

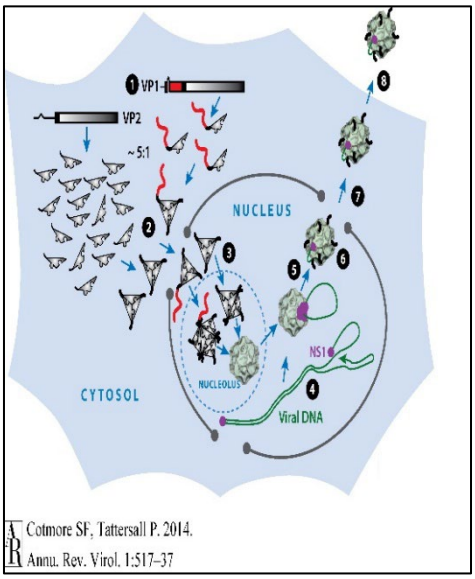


Fig. 8. Viral DNA rolling hairpin replication model (8)

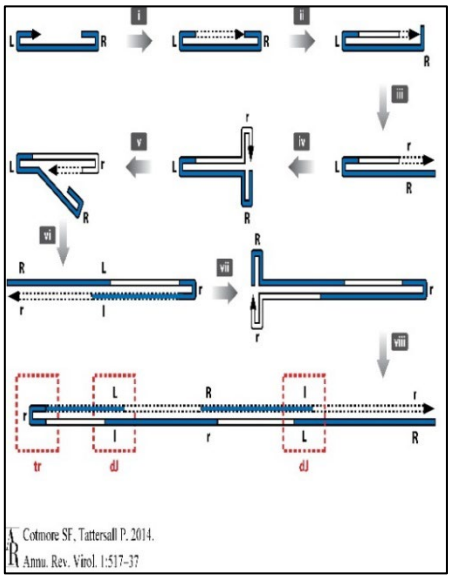


Fig. 9. Formation and exit of newly formed virions from the nucleus (8)

3.Immunology

3.1. How does it get in the host?

FPV can produce fetal and neonatal infections besides its regular infection parameters. For the felines older than 6 weeks the virus is spreading through fecal-oral contact or with infected feces, body fluids, surfaces and even fleas. It has high resistance in the environment due to its highly condensed, robust capsid. After the virus enters the body through intranasal or oral route, it starts to replicate in the oropharynx, and in 2-7 days the viremia begins, distributing the virus throughout the body. The most affected tissue is the most mitotically active (it needs cells in the S phase for viable replication) like the lymphoid tissue, bone marrow and intestinal mucosa (29).

3.2. Immune barriers and the immune response.

Before the virus can invade the body, it needs to survive the organism's defense mechanisms. The first one is with the help of cytotoxic cells. Here, a big role is played by the MHC class I (Major Histocompatibility Complex) molecules. Their major function is to present peptide fragments of endogenous antigens to the CD8 T cell. These molecules are heterodimers composed of a heavy chain and a β -2-microglobulin that are essential for the MHC I expression on the cell membrane. The chain is composed of one intracellular domain and two extracellular Ig-like domains. MHC I molecules always function as ligands, reverse signaling being demonstrated two decades ago. It plays an important role in apoptosis, activation, or function (33).

Even if the virus cannot be seen by the immune system when it is inside the cell, said cells can show other ones what is inside of them using the MHC class I. If the cell is infected, the pieces of peptides will include proteins made by the virus. In this process a major role is played by the cytotoxic T lymphocyte that has specialized proteins called T cell receptors (TCRs) which help recognize the virus. If detection happens, the lymphocyte will release cytotoxic factors (perforin, granzymes, granulysin) to kill it. Even so, sometimes the virus is able to adapt and find ways to avoid detection of the T cell by stopping the MHC molecules to be displayed on the surface. In this case the lymphocytes cannot detect if the cell is infected or not; however, in this context, natural killers are able to detect the structures that have a reduced number of MHC class I molecules on their surface and so, they release toxic substances similar to the ones released by the CD8 T lymphocyte (15).

The cytotoxic factors are stored inside granules; perforin being a protein that makes pores in the cell membrane which facilitates the infiltration of other factors like granzymes that once inside the infected cell initiate the apoptosis. Another factor is granulysin, which directly attacks the outer membrane producing lysis. CD8 lymphocytes synthetize proteins called cytokines after interacting with the infected structures. These are interferon-G and Tumor Necrosis Factor which enhance the other immune mechanisms (1).

Cytotoxic T lymphocytes are, then, a very good way to fight viral infections and recent discoveries in synthetic immunology demand further studies. Science already made it possible for us to engineer T cells through chimeric antigen receptors (CARs) that can recognize tumor cells. CARs have two domains: one outside domain and one inside domain. The outside domain is engineered in order to recognize a specific antigen and the inside domain can trigger the release of cytotoxic factors, although really effective CARs can also stimulate proliferation of antigen stimulated T cells thus amplifying the organism’s response (27).

The immune response is also achieved with the help of interferons. These are small proteins that are able to block the ability of an infected cell to replicate. Type I IFN induces a vast quantity of proteins that limit the spread of the infection by causing problems regarding its replication mechanism. 20-50-oligoadenylate synthetase (OAS) is an enzyme induced by Type I IFN that activates latent endoribonuclease RNase L, which is involved in viral nucleic acid degradation. Another induced enzyme, protein kinase RNA-activated (PKR), a member of the eukaryotic initiation factor 2a family blocks viral RNA translation by stopping the recycling of GDP. In addition to their roles, these interferons are also signaling molecules that make the nearby cells increase the number of expressed MHC class 1 molecules (17). Interferons can also affect viruses by:

- affecting viral capsid localization through a special class of GTPases called myxovirus resistance (Mx)
- interfering with viral particle release through interferon stimulated gene 15 (ISG15) and tripartite motif (TRIM)
- expression of APOBEC3, a protein that can mutate viral DNA.

Of course, there are more types of interferons (I, II and III based on their capacity to bind to different receptors) with various subtypes. Interferons have a less "invasive" antiviral activity compared to cytotoxic factors thus these molecules might constitute better ways of fighting viruses as they had been used in therapies before (17, 23). Further interferon study could focus on finding the best interferon and way of inducing it in order to fight FPV.

Antibodies are a useful way to remove viruses from the body before the infection spreads too much. These proteins can stick to invading pathogens and specifically recognize them. By sticking to viruses, the antibodies can fulfil their job by neutralizing the virus, rendering it incapable to infect any more cells and making the virus particles bind together through a phenomenon called agglutination. This way, the virions are an easier target for the immune system (5).

Antibodies can also activate phagocytes to eliminate pathogens. An antibody that is virus bound sticks to receptors called Fc receptors, which are on the surface of phagocytic cells and trigger phagocytosis by engulfing the virus and destroying it. Activating the complement system is also done by antibodies, which eventually also promote phagocytosis (5).

4.Pathophysiology

4.1. Microscopical changes

Because the most common entry pathway of the virus is the oral route, the first tissues that face the viral particles are represented by the lymphoid tissue located at the oropharynx. These organs are known as tonsils. They have lymphoid formations which are in fact annexes of the pharynx mucosa. Structurally, they are made of conglomerations of lymphocytes covered externally by a protective epithelium layer. The component cells of these organs are predominantly: lymphocytes (T, B, NK), macrophages, mastocytes, epithelial cells, stromal, dendritic and also granulocytes (especially neutrophils). The virus affects the rapidly dividing cells the most (like leukocytes or epithelial cells), thus, the sensibility of these organs to infection is considerably high (19).

Following the virus' entry at the tonsils, the death of these formations takes place up to the point where these structures can eventually (in advanced stages of the illness) express signs of secondary necrosis which follows apoptosis. This event is also characteristic for other structures which the virus affects due to the fragmentation of biological membranes within the organelles as well as the membrane of the cell itself resulting in intracellular enzyme/protein expression within the interstitial space (30). Varying the gravity of affected cells and localization of the tissue, these can be: lactic acid, lactate dehydrogenase and TNF thus leading to elevated levels of erythrocyte sedimentation rate and C-reactive protein.

Cellular death is similar in the case of FPV to the one in CPV given the fact CPV appeared as a mutation of FPV. A study on CPV tried to highlight the cell death mechanism in this viral infection. According to it there could be a long debate over the type of cell death during the FPV infection as both apoptosis markers (caspases)

and necrotic events specific to secondary necrosis are seen. The mechanism through which the necrosis takes place is not yet fully understood but it could have something to do with the lysis of the mitochondrial membrane (22). The synthesis of viral proteins (NS1, VP1 and VP2) could inhibit the synthesis of the proteins necessary for the maintenance of the mitochondrial membrane. The most noticeable protein being ChChd3 (Coiled-Coil-Helix-Coiled-Coil-Helix Domain Containing 3). The center of oxidative phosphorylation for ATP production in a mitochondria is the crista membrane which in essence is represented by proeminences on the inner mitochondrial membrane (IM). ChChd3 interacts with other proteins in the mitochondrial membrane like OPA1 (OPA1 Mitochondrial Dynamin Like GTPase), responsible for the morphology of the crista and Sam50 (Sorting assembly machinery) which is responsible for the import and assembly of β-barrel proteins which mediate transport and biogenesis (9, 10). An event which would trigger a discrepancy in this whole mechanism would eventually destroy the mitochondrial membrane and could in turn lead to the destruction of the cell membrane because of the lack of ATP which is an event characteristic to necrosis. Another hypothesis is focused mainly on the SAM complex. It could be that even if Sam50 does not have known channel translocase activity, this protein is essential for the internalization of granzymes which target the mitochondria. The human genome encodes for 1500 mitochondrial proteins which must be transported to the right mitochondrial compartment through translocase complexes (18). Thus, it is safe to assume that the genome of a cat encodes for at least a considerable number of similar proteins. Whether the synthesis of these proteins or of the ones mentioned above is affected by the viral infection would make a plausible object for future study.

While signs of secondary necrosis occur, mastocytes sense the presence of cellular fragments and get activated releasing factors like histamine and serotonin. This leads to capillary vasodilation needed for the inflammatory response. The phagocytosis of externalized cellular fragments is done through the use of polymorphonuclear leukocytes (predominantly macrophages). Macrophages are also releasing cytokines (Interleukin 1, Interleukin 6, Interleukin 8 and TNF) which are involved in the apparition of fever (7).

As mentioned above, other structures within the organism are affected in the same manner because the small virions are able to enter the bloodstream directly through the endothelial membrane which leads to infection in other organs besides the lymphoid ones.

4.1.1. Small intestine

At this level, the most favorable cells for the virus replication are the epithelial ones. These cells form a barrier between the intestinal structures and adjacent structures. Because of constant exposure of the epithelium to the to the enzyme and chemical filled intestinal juice, these cells divide frequently. In order to maintain the intestinal mucosa integrity and to fulfill their absorption role accordingly, mitosis and implicitly replication must occur very often. The moment the virus gets here, it takes

control over the cells' replicative machinery while also continuing to spread (2,14).

During a study done on 30 deceased cats due to FPV infection, diverse microscopical lesions were found. These were dependent on diverse factors like the infection gravity, resistance of the affected organism, etc.. The most predominant lesions were registered at the epithelial cells within the Lieberkuhn crypts, which in most cases resulted in the volume reduction of the crypts lumen and necrobiosis of the villi followed by a considerable shortage in size. (Fig. 10) Degeneration or hyperplasia of the epithelium which led to structural alteration and functional modification of the enterocytes was also present. At the nucleus there were either morphological changes like swelling, nuclei had a vesicular aspect with proeminent nucleoles or they had a picnotic aspect characteristic to apoptosis or fragmented which is characteristic to necrobiosis. Hyperplasia of caliciform cells could also be observed alongside the presence of intracellular inclusion bodies like viral fragments including viral DNA which is considered a specific marker. Another modification was also noticed in the lamina propria; to be more specific, the complete disparition of nuclei and the thinning of this specific layer. Smooth muscle fibers contained very low amounts of the viral antigen. However, in the case of secondary infections with fungi, bacteria or even viruses, massive neutrophilia was also observed (25).

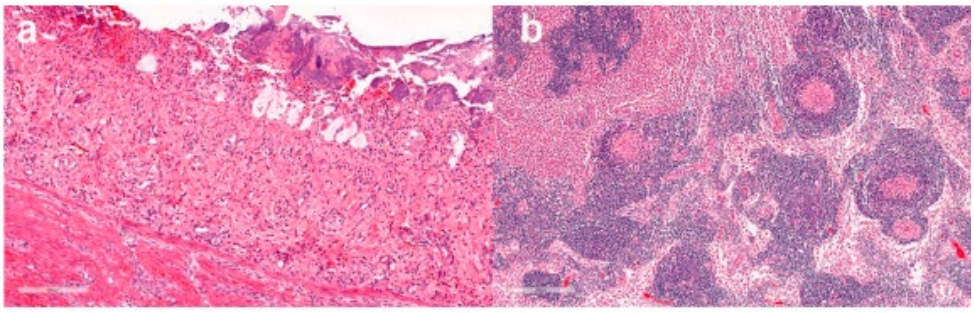


Fig. 10. A – Loss of villi and intestinal crypts of Lieberkuhn (intestinal glands), B – Lymphoid depletion within the mesenteric lymph node (4)

4.1.2. Bone marrow

At this lymphoid organ's level, most of the encountered cells are stem or precursory stages of the blood ones. The massive differentiation at this level favorizes the replication of FPV. Infection of these cellular lines leads to a drastic reduction of their numbers in the bloodstream. Furthermore, this can also be seen in the clinical signs (24).

4.1.3. Nervous system

When queens contact the FPV, usually they do not suffer big consequences compared to the future conception product. This is due to the fact that during

intrauterine life, the virus replicates in a multitude of tissues but the most obvious effects can be seen on the nervous system. Because of raised mitotic activity in the Purkinje cells and in the granular precursors situated in the external granular cerebellar layer (the germinal epithelium cells), virus activity leads to the apparition of cerebellar hypoplasia. However, the severity of infection varies in different individuals which means that some manage to survive. This affection can develop in kitten younger than a week. Other lesions in the same situation are of ocular nature: folding of the retina, dysplasia and hypoplasia or degeneration of the optic nerve (24,30).

Based on a study made on a cat lot infected with FPV, scientists demonstrated the presence of viral antigens at the cerebral neurons level (around the diencephalon) following PCR and immunohistochemical tests. An interesting finding also made available by these tests could be deduced from the negative coloration of the p27^kip (CDKN1B) protein in some neurons. This protein regulates cellular cycle by inhibiting the cyclin dependent kinase, preventing uncontrolled replication of cells and implicitly of tumoral processes (Fig. 11). This was true for cats 6-12 weeks of age but also for a cat as old as 4 or 5 years approximately. If this protein is absent or inactivated then the cellular cycle is reset. Feline panleukopenia virus, by a unique substitution suffered by the NS1 protein, manages to create for itself the proper environment for viral DNA replication by inhibition of the p27^kip1 protein. This can presumably even enable the nervous cell to enter mitosis because of the active CDK (11).

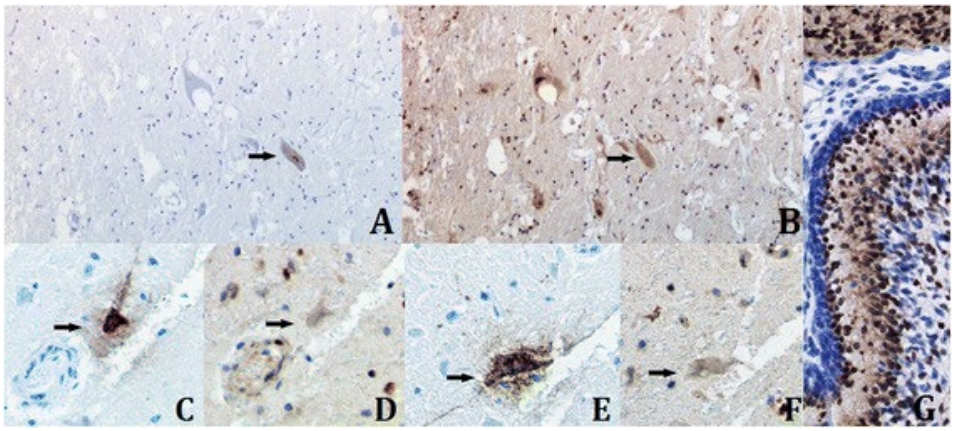


Fig. 11. Immunohistochemical staining for FPV and p27^kip1 antigens. The absence of nuclear staining for p27^kip1 is seen in FPV-infected neurons (C-F). Uninfected neurons still express it as seen in figure A, B respectively (11)

A pertinent conclusion is that by this change in the NS1 protein, FPV might be able to replicate in other cells besides the already mitotically active. Furthermore,

these changes occur in older cats as well as young ones. Future studies can also focus on the speed by which the p27^kip1 resumes it's activity post-infection. In the case that the p27^kip1 protein stays inactivated for longer times, formation of tumors following infection could be plausible.

4.2. Macroscopical changes

The best observation method for the macroscopical changes while the cat is still alive is ultrasound examination while in the case of dead cats it is the necropsy (26).

In the small intestine at the jejunum/ileum level, the intestinal lumen is thinned because of the diffuse catarrhal-hemorrhagic inflammation characterized by the presence of abundant mucus and hemorrhagic zones. The mucosa appears colored as dark red, with necrosis zones, congestion with fibrinous deposits can also be observed sometimes alongside ulcerations (Fig. 12) (26).

At the lymphoid tissue level, the aspect is edematous with hemorrhagic/ necrotic zones. The bone marrow appears dark red in color with a semifluid consistency. The tonsils suffer inflammation just like the pharynx mucosa and in some cases, there are small ulcerations on the edges and tip of the tongue (26).

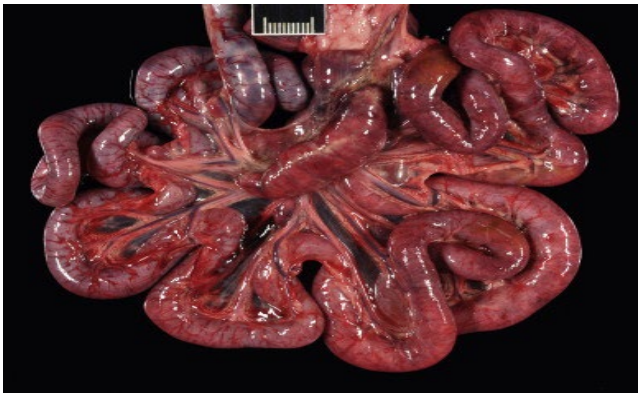


Fig. 12. Intestinal tract of a cat infected with FPV (34)

4.3. Clinical signs

Clinical signs appear after a 2-12 days period. Their gravity depends on the age and immunocompetency of the animal. There are distinct forms of the disease produced by the virus, one of which is supraacute (this results in the animal's death before the apparition of clinical signs). The debut of the disease is marked by hyperthermia (41 Celsius degrees or even more) followed by prostration, anorexia and depression. Shortly after, intestinal disturbances are also noticed. These can include: vomiting, diarrhea, yellow melena (sometimes in liquid form) with a characteristic smell. The affected individuals also suffer from severe dehydration and

polydipsia because of the severe loss of liquids. In the abdominal region, during palpation, high sensitivity might lead to vocalization from the cat indicating accentuated pain (26).

Blood tests highlight leukopenia, erythropenia, thrombopenia following unavoidable anemia. This is especially due to the destruction of hematopoietic cells in the bone marrow. In the rare case in which the leukopenia does not drop below 1500/mm3, recovery is possible (11, 26).

The nervous form of the diseases usually expresses itself in kitten younger than a week and it is obvious only after 2-3 weeks when they can walk. Their position is abnormal, some of them not being able to keep a characteristic posture. Slight tremors are noticed at the head level and in some case there is also complete lack of eyesight (11, 26).

4.4. Prognosis

Feline panleukopenia gets a worse prognosis than canine parvovirus (CPV) enteritis. The negative prognostic factors are the following: leukopenia, thrombocytopenia, neutropenia and cerebellar ataxia (in young kitten). Immunosuppression is by far the worst factor caused by this disease. Mortality rates can go as big as 90% in the case of young kitten even though this number can decrease if the cat receives supportive care. However, there is no effective treatment to date (32).

Conclusions

The current work was meant to raise awareness and make scholars further understand the gravity of this infection as well as provide insight regarding possible antiviral strategies. Vaccination is still the best weapon medicine has against this virus to date but through research we found out multiple hypothetically promising pathways that could be explored regarding treatment/prevention. Whether the solutions lie through genetical engineering in order to change the hosts' genome; synthetic immunology/interferon therapy; stricter laws in order to have better statistics during outbreaks or better molecular analysis of the processes happening during infection in order to provide efficient medication is not yet known. But the present work at least justifies the beginning of such studies or experiments.

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STUDY ON THE EFFICACY OF SUPPLEMENTING BROILER CHICKS' DIET WITH PARSLEY, CINNAMON, AND TURMERIC

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Summary

This study aimed to investigate the effects of dietary supplementation with parsley (*Petroselinum crispu*m), cinnamon (*Cinnamomum veru*m), and turmeric (*Curcuma longa*) on the growth and health of Ross 308 broiler chickens. A total of 51 one-day-old chicks were divided into four lots: Lot A (supplemented with parsley), Lot B (supplemented with cinnamon), Lot C (supplemented with turmeric), and a control lot (no supplementation). Various growth parameters, including body mass and feed conversion rate, were monitored over six weeks. Significant differences were observed in the third week between the control group and the groups supplemented with parsley and turmeric in terms of carcass mass. However, these differences were not sustained in the sixth week. In the fourth week, only the turmeric-supplemented group (Lot C) showed statistically significant improvements in body mass distribution. No significant differences were found in other zootechnical parameters or in biochemical analyses across the lots. The study suggests that while turmeric had a notable effect on body mass distribution in the fourth week, overall, the dietary supplements did not significantly improve the evaluated zootechnical parameters. The findings align with existing literature and indicate the need for further research to fully understand the potential applications of these supplements in the poultry industry. Limitations of the study include the lack of individual weighing in the first three weeks and the absence of successive weighing, which could have provided more nuanced insights into weight fluctuations.

Keywords: dietary supplementation, Ross 308 broiler chickens, zootechnical parameters, growth parameters.

The broiler chicken industry represents a vital segment of global food production, focusing on the efficient production of poultry meat for human consumption. The process of raising broiler chickens is based on solid scientific principles that have evolved over the years to maximize yield and the quality of the final product (18, 19).

In a concise synthesis of scientific literature, studies conducted by Ayşin Akıncı et al. (3), Zarei et al. (21), and Hui Zhang (13), Dorman et al. (10), have highlighted the antioxidant, digestive, and anti-inflammatory properties of parsley (*Petroselinum crispum*) in animals, particularly in mice and chickens. These research findings are supported by studies from Al-Musawi (6) and Mokalad Oraibi Hasan et al. (11), which have shown that parsley can be an effective feed additive for improving growth performance and gut health in chickens.

Research on cinnamon (*Cinnamomum verum*) has also shown multiple health benefits in animals. Studies conducted by Dhuley et al. (9), Symeon et al. (20)

have emphasized the antioxidant, digestive, and anti-inflammatory properties of cinnamon in mice and chickens. These findings are corroborated by studies from Al-Kassie, (5) and Krauzeet et al. (15), which have shown that cinnamon not only has antioxidant and antibacterial effects in broiler chickens but also improves their performance and immune function. Hussein et al. (14), evaluated the effect of cinnamon on growth performance, blood biochemical parameters, and antioxidant activities. The results indicated a significant improvement in growth performance and feed efficiency in broiler chickens.

Turmeric (*Curcuma longa*) has recently been the subject of research investigating its effects on the health of broiler chickens. The study conducted by 2. Abubakar et al. (2) highlighted the antioxidant properties of turmeric, particularly in reducing oxidative stress in the heart, liver, kidneys, and spleen of broiler chickens. These results are consistent with the study by Atay, also from 2023, which showed that adding turmeric to the diet of broiler chickens has beneficial effects on performance, carcass characteristics, and meat quality (7).

Additionally, research by Oluwafemi Ra (17), highlighted the positive effects of turmeric on some hematological and serum biochemical indices in broiler chickens. These findings are supported by the study by Hassan Reda M.A.(12) which investigated the efficacy of turmeric in alleviating the adverse effects of aflatoxin in broiler chickens. The study concluded that turmeric, in combination with other supplements, may have beneficial effects in managing aflatoxicosis in broiler chickens.

Thus, turmeric joins parsley and cinnamon as another agent with multiple beneficial properties in the field of animal health, with the potential to improve growth performance, feed efficiency, and combat oxidative stress and inflammation.

The aim of this paper is to analyze and demonstrate the effects that the active substances contained in three plants, namely parsley (*Petroselinum crispum*), turmeric (*Curcuma longa*), and cinnamon (*Cinnamomum verum*), have on the growth and health of broiler chickens. The motivation for this research stems from the potential of these plants in improving productive performance and the health status of broiler chickens.

Materials and methods

The experimental organization, rearing system, biological material, and equipment required for the research have the following characteristics:

The biological material consisted of 51 one-day-old Ross 308 broiler chicken hybrids, divided into four experimental lots.

The loting involved random selection and the use of four enclosures for four lots as follows: Lot A (13 chicks - feed with added parsley, marked with green, non-toxic dye applied to the head), B (13 chicks - feed with added cinnamon, marked with blue), C (13 chicks - feed with added turmeric, marked with purple), and the control lot D (12 chicks - feed without additives marked with yellow) arranged in a common

space to ensure identical growing conditions. Each enclosure was populated with 13 or 12 chicks. The stocking density was 17.33 and 16 chicks/m^2 in the first three weeks (pens of 0.5m/1) and 2.67 and 2.89 chicks/m^2 (pens of 1.5/3 m) (Directive 2007/43/EC).

Feeding was *ad libitum*, with periodic refilling of the feeders. The chicks had continuous access to waterers and feeders filled with fresh water and feed. The type and amount of feed were appropriate for each study lot and had the compositions in Tables 1 and 2.

Feeding in the first three weeks was done with starter feed (Table 1), and in the following three weeks with finisher feed (Table 2). For experimental lots A, B, and C, parsley, cinnamon, and turmeric were added to the basic starter or finisher feed at a rate of 5g/kg of feed (50 ppm), followed by homogenization. The chicks' food consumption was monitored and recorded daily. This included the total amount of food offered and the amount left uneaten. These data helped in estimating and calculating the amount ingested by the chicks and changes in intake with the addition of supplements.

Heating and light schedule during the startup period: In the first three weeks, infrared heat sources were used to ensure the heating of the growing enclosure. Subsequently, to ensure a 23-hour light schedule, LED bulbs with a power of 180 lumens/enclosure were used.

Table 1

Nutritional composition of starter feed

Substance	Quantity
Crude Protein	20.45%
Calcium	1.00%
ME for birds	11.72 Mj
Phosphorus	0.60%
Fat	2.57%
Sodium	0.14%
Cellulose	3.29%
Vitamin A	10045 IU
Methionine	0.45%
Vitamin D	5022 IU
Methionine + Cystine	0.80%
Vitamin E	75 IU
Lysine	1.03%

Based on (22)

Table 2

Nutritional composition of finisher feed

Composition	UM/kg	Composition	UM/kg
Crude Protein	15.00%	Calcium	0.52%
ME for birds	11.38 Mj	Phosphorus	0.25%
Fat	2.80%	Sodium	0.18%
Cellulose	4.51%	Vitamin A	9000 IU
Methionine	0.27%	Vitamin D	4000 IU
Methionine + Cystine	0.57%	Vitamin E	50 IU
Lysine	0.65%	Coccidiostatic	Absent

Based on (23)

The chicks' health status was monitored daily, and growth parameters (body mass and specific consumption) were calculated weekly, by weighing all the chicks together in the first three weeks and individually in the last three weeks. The chicks were closely monitored to record any changes in growth rate, development, behavior, or health status. Special attention was also given to any signs of illness or discomfort.

The birds were slaughtered in stages; the first slaughter was carried out in the third week and the second in the sixth week of the experiment, sampling one chick from each lot. After slaughter, the evisceration, cutting, and weighing of the carcass and its viscera followed, aiming to obtain data for evaluating the evolution of body mass and anatomical components of the chicks at different stages of the experiment.

Biochemical analyses were performed in the Animal Nutrition and Agronomy laboratory using an Automatic Biochemistry Analyzer FUJI DRI-CHEM 4000I for the following parameters: TP (Total Proteins, g/dL), ALB (Albumin, g/dL), ALT (Alanine Aminotransferase, U/L), GGT (Gamma-Glutamyl Transferase, U/L), GLU (Glucose, mg/dL), TG (Triglycerides, mg/dL), TCHO (Cholesterol, mg/dL), HDLC (High-Density Lipoprotein Cholesterol, mg/dL), CREA (Creatinine, mg/dL), and UA (Uric Acid, mg/dL).

Statistical analysis was performed using SPSS Statistics for Windows, Version 17.0 (Chicago: SPSS Inc. USA), using the Chi-Square Test and Kolmogorov-Smirnov Test to examine the significance and relevance of the obtained data. Differences were considered significant for threshold values of $p \leq 0.05$.

Results and discussions

The edible mass was determined after slaughter, cutting, and evisceration in the third and sixth weeks. The results revealed significant differences in the third week between the carcass of the control group and the group exposed to parsley, as well as between the control group and the group exposed to turmeric ($p < 0.05$).

However, in the sixth week, the study did not reveal any significant differences between the lots whose feeding was supplemented with cinnamon, parsley, or turmeric and the control lot.

Regarding the viscera such as the heart, liver, and muscular stomach, the study could not capture any significant differences ($p > 0.05$) between the experimental lots and the control lot in both the third and sixth weeks.

The live body mass of the chicks in the fourth week of the experiment had the following values: for Lot A, with parsley, the average was 1293.69 ±50.32g; for Lot B, with cinnamon, the average was 1267.23 ±27.52g; for Lot C, with turmeric, the average was 1326.46 ±37.88g; and for the control lot, the average was 1225.83 ±40.96g. The results of the Kolmogorov-Smirnov test showed that, in week 4, the distributions of the body mass values of the chicks in lots A, with parsley, and B, with cinnamon, were not significantly different from that of the control lot ($p > 0.05$). The only lot that had statistically significant performances in week 4 was Lot C, with turmeric ($p = 0.026$).

In the fifth week of the experiment, the body mass of the chicks was: for Lot A, with parsley, an average body mass of 1586 ±69.49g was observed. Lot B, with cinnamon, recorded an average body mass of 1609 ±48.80g. Regarding Lot C, with turmeric, the average body mass of the chicks was 1619 ±43.63g. The control lot recorded an average body mass of 1594 ±58.75g.

In the sixth week of the experiment, continuing the monitoring of the chicks' body mass, the following values were recorded: for Lot A, with parsley, the average body mass reached 1854 ±88.26g; Lot B, with cinnamon, recorded an average body mass of 1890 ±72.89g; Regarding Lot C, with turmeric, the average body mass of the chicks was 1880 ±51.81g. The control lot recorded an average body mass of 1879 ±63.50g.

For weeks 5 and 6 of the experiment, the study did not identify any statistically significant differences ($p > 0.05$). This indicates that, in the last two weeks of the experiment, the distributions of the body masses of the chicks subjected to treatments did not show significant differences compared to the control lot.

Regarding the average daily gain, average daily consumption, and feed conversion rate, the study did not reveal the existence of any statistically significant differences ($p > 0.05$).

Biochemical analyses did not reveal any significant difference between the experimental lots and the control lot.

By analyzing the obtained results, it can be observed that the distribution of body mass was much more uniform in the fourth week in the lot supplemented with turmeric (Lot C) compared to the control lot. However, the study did not identify significant differences in the distribution of body mass between the lots supplemented with parsley and cinnamon compared to the control lot in the same period. In weeks 4-6, the study did not record significant differences in the distribution of body mass between the experimental lots and the control lot.

In the preliminary phase of the experiment, more precisely in the third week,

statistically significant differences were recorded regarding the carcass mass between the lots of chicks supplemented with parsley (Lot A) and turmeric (Lot C), compared to the control group. However, in the sixth week of the experiment, these differences lost their significance.

Regarding the commercial carcass mass, the results obtained in the sixth week are consistent with the pre-existing specialized literature. The study by Abbas Rabia (1) which investigated the effect of parsley on carcass mass in chicks, reported similar results. Also, the research by Chowlu et al. (8) on the impact of cinnamon on broiler-type chicks, and the work by Aldiyanti et al. (4) which examined the effect of turmeric, confirmed this trend.

As for the zootechnical performance parameters, such as average daily gain, average daily consumption, and feed conversion rate, statistical analysis revealed that the experimental lots did not show statistically significantly higher values compared to the control lot. These results are consistent with the results obtained in the study by Majeed et al. (16) which examined the effects of parsley on similar parameters, as well as with the research conducted by Chowlu et al. (8) which investigated the impact of cinnamon. In both studies, supplementation with the respective additives did not have a significant impact on specific consumption or weight gain of the subjects. Therefore, it can be concluded that, under the conditions of the present experiment, dietary supplementation with parsley, cinnamon, and turmeric did not exert a significantly positive effect on the evaluated zootechnical parameters.

In the context of this experiment, it is important to note that, although the obtained data are considered valid, there are certain limitations that deserve to be mentioned for a complete understanding of the results. One of these limitations was that the chicks were not weighed individually in the first three weeks of life. In addition, successive weightings were not performed for each individual. These aspects do not invalidate the results, but the lack of individual weightings in the first three weeks and successive weightings limited the ability to monitor fluctuations in weight.

However, the results underline the subsequent importance of investigating the effects of these supplements on zootechnical parameters and suggest that they may have valuable applications in the poultry industry.

Conclusions

In conclusion, following the collection and analysis of experimental data, it can be stated that the administration of turmeric had a notable effect on the distribution of body mass weight in week four, leading to more uniform growth compared to the control lot. This result suggests a potential efficacy of turmeric in optimizing the growth of broiler chicks. In addition, both turmeric and parsley caused significant differences compared to the control lot in the third week regarding the weight of the chicks' carcasses.

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HISTOLOGICAL AND PHYSICOCHEMICAL CHARACTERISTICS OF TRADITIONALLY AND INDUSTRIALLY PREPARED FILLET MUSCLE – A SYSTEMATIC REVIEW

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Summary

The most powerful method of meeting the nutritional needs of humans remains the consumption of food products of animal origin. The rate of animal consumption per capita is constantly increasing, but for the 8 billion people consumption is becoming highly selective. The FAO of the United Nations predicts that dietary preferences will shift towards alternative sources of protein which will lead to a decrease in meat consumption. However, over the next decade, meat consumption will not be affected, consumer choice will continue to be influenced by the nutritional content of meat compared to protein substitutes. Due to the accelerated deterioration dynamics of meat products, preservation technologies are increasingly attractive and suitable for preserving quality, organoleptic and physico-chemical properties. In our study we aim to identify the impact of preservation methods on the histological and physicochemical properties of fillet muscle and also the benefits for human health. A search of the PUBMED/MEDLINE, EMBASE, Google Scholar, Web of Science and SCIENCEDIRECT databases was carried out for the period from 2000 to 2023. A total of 908 articles publications were initially identified. After exclusion of duplicates and application of inclusion criteria, 50 studies were selected for analysis. The development in processing strategies such as freezing, smoking, dehydration, salting, hedging, preservation with oils, influences the histological aspect and the physico-chemical properties and also showed the differences. The sustainable safety of meat is maintained by methods based on the control of temperature, available oxygen, water activity, which stop microbial growth, oxidation and enzymatic autolysis. Traditional meat preparation methods preserve the histological architecture as well as the physico-chemical properties compared to the industrial ones.

Keywords: fillet muscles, histological characteritics, physico-chemical characteritics, preservation.

Food is key to achieving and maintaining good health and to promote healthy behaviors, food must be safe and accessible. A high level of food safety and security impacts people of every age, race, gender and income level. The challenges to food safety and security cover a broad scope, and this means they can impact communities and individuals differently (34).

Causes of damage to human health are chemical contaminants - such as food additives and food preservatives, but also damage of ecosystems and biodiversity caused by climate change, soil loss, water and nutrient pollution, and decline in wild predators and herbivores (Fig. 1).

"Chemicals" represented a host of sub-categories, including cancer-causing chemicals, pesticides, food additives and ingredients and heavy metals (34).

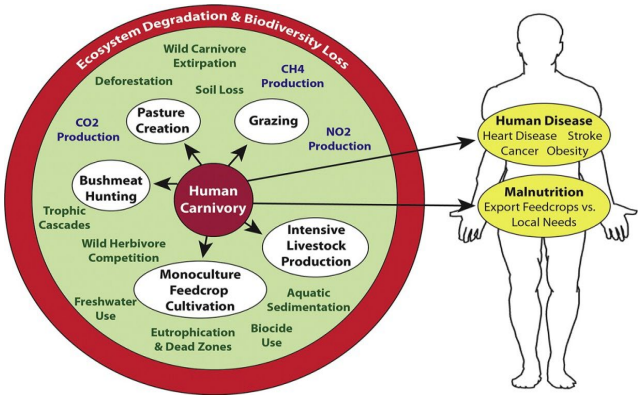

Fig. 1. The impact of consumption of animal products on human health (35)

In the human health risk assessment process, health professionals estimate the likelihood of adverse health effects in people who may be exposed to chemicals from contaminated environments, now or in the future. Thus, in November 2023, EPA (United States Environmental Protection Agency) released the Protocol for nitrates and nitrites (oral) IRIS assessment, which are used as preservatives for meat and fish curing and as color fixatives. At the same time there is an assessment protocol for perfluorohexanesulfonic acid (PFHxS) which is used in paper (including food contact), packaging (including food contact) (36, 39).

The satisfaction of the nutritional needs of people is mainly ensured through the consumption of food products of animal origin. The rate of animal consumption per capita is constantly increasing, but for the 8 billion people consumption is becoming highly selective. The potential solutions include more production through intensification and increasing grasslands, recycling food waste to feed animals and using alternative sources of animal fodder, such as insects. Laboratory-grown meat has also made an appearance, albeit only as a prototype, but may be expected to have an influence in the future, as will plant-origin meat substitutes, but meat will continue to occupy an important place in the planet's food ecosystem (6).

According to the Organization for Economic Cooperation and Development (OECD) and Food and Agriculture Organization (FAO) of the United Nations, the Agricultural perspective 2023-2032 for the next 10 years predicts an increase in meat consumption, due to the intensification of animal husbandry practices but also of growing demand, despite the fact that costs are expected to rise (Fig. 2).

The meat consumption patterns of consumers in most countries are found to focus on poultry (41% of protein consumed from all meat sources), followed by pork, beef and sheep. By 2032, it is estimated that worldwide meat consumption will

increase by 15% to that of poultry, with 11% of pork, 10% of beef and 15% of sheep (38). Per capita global meat consumption will increase by 2%, representing an increase of 0.7 kg/year/person based on retail edible weight equivalent by 2032, similar to that of the previous decade due mainly to the type and quality of the meat consumed.

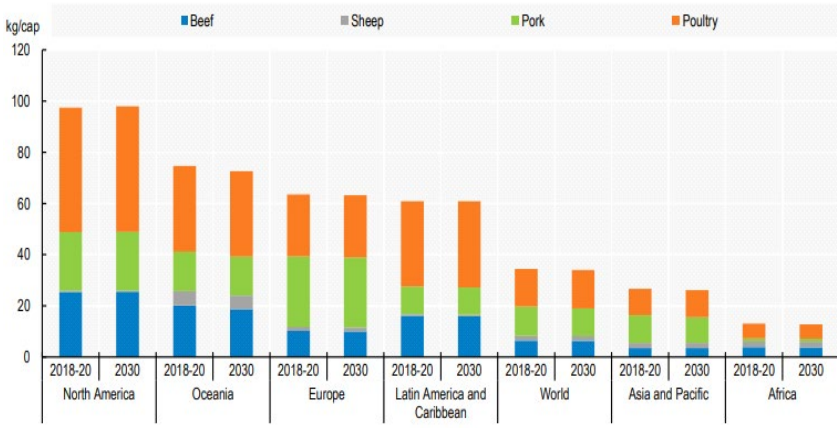

Fig. 2. Evolution of global meat consumption comparative 2018-20/2030 (37)

Russia's war against Ukraine and Israeli-Palestinian war adds uncertainties in food prices, energy, environmental, social, geopolitical and economic developments, with a negative impact on global food security, and undermining long-term supply capacity. Despite these facts, OECD predictions confirm an increase of meat demand per capita by 2% in 2032 compared to 2020-2022, depending also on consumer preferences and production technologies.

Objectives and methods

Preservation methods cause changes in the physico-chemical and also histological properties, important landmarks for the consumption pattern of the population but which influence the state of health.

In our study, we identify the changes that the preservation methods caused on the sensory characteristics (texture, aroma and color) of the food (moisture and fat content, structural carbohydrates and proteins) as well as the changes in the intramuscular connective tissue (collagen and elastin, the proteoglycan matrix fibers).

A computerized search of PUBMED/MEDLNE, EMBASE, Google Scholar, Web of Science and SCIENCEDIRECT databases resources was performed to identify all registered articles about characteritics of fillet muscles, published between 2000 and 2023, using the words ”fillet muscles”, ”histological”, ” physico-chemical” and ”preservation”. Publications were selected if they reported on

histological and physico-chemical characteritics of fillet muscles. A quantitative and a qualitative analysis of each method of preservation was performed in order to establish the benefits for human health.

Description of the preservation methods

Over time there have been transformations in food culture and culinary style, as people's history, culture, politics, sociology and religion have periodically influenced how and what people ate.

Previous studies have shown that the sustainable safety of meat products is maintained by methods based on the control of temperature, available oxygen, water activity to stop microbial growth, oxidation and enzymatic autolysis (11, 15, 32).

Various preservation methods have been used to ensure safety and extend the shelf life of the meat. The conservation principles are applied both on an industrial scale, with products with an extended shelf life in the trade, but also at the household level, respecting the ancestral recipes, inherited and passed down from one generation to another.

Industrial preservation methods use preservatives to extend the shelf life of meat. However, due to the potential health risks and toxicological factors associated with synthetic additives, the demand for healthier additive-free foods has increased. Therefore, there is a growing demand for natural preservatives to replace synthetic ones. Over the years, humans have consumed animal meat prepared/preserved in such a way that biochemical and microbial actions help both to increase the shelf life, but especially to improve the flavor and nutritional quality of the products (14).

Millennial methods of preservation, such as smoking, salting, sunlight drying, refrigeration at low temperature, freezing, have been supplemented with modern techniques such as: vacuum packaging (17), modified atmosphere packaging (8), radiation preservation (Thomas (28)), addition of preservatives (16) and active packaging – with natural materials containing starch-curcumin-gelatin dialdehyde particles (13, 21), high-intensity ultrasound (4, 25) (Fig. 3, 4).

Characteristics, physico-chemical characteristics, preservation allowed identification of 908 articles. Screening of abstracts revealed that 627 did not address issues relating to preservation or conform to inclusion criteria, with inadequate descriptions of properties of fillet muscles. Following examination of the full-length text, 231 articles were excluded as they failed to specify the physico-chemical characteritics. After exclusion of duplicates and application of inclusion criteria, 50 studies were selected for analysis

An initial search using the key words: fillet muscles, histological.

We identify a lot of studies which investigate effect of all preservative methods on histological and physicochemical characteristics with benefit on human health:

Wang et al. (33) studied the effect of salt on meat preservation, highlighting its role in the safety, quality and flavor of meat products, but also its effects on human health. High doses of salt can cause kidney failure, heart failure, even cancer (33).

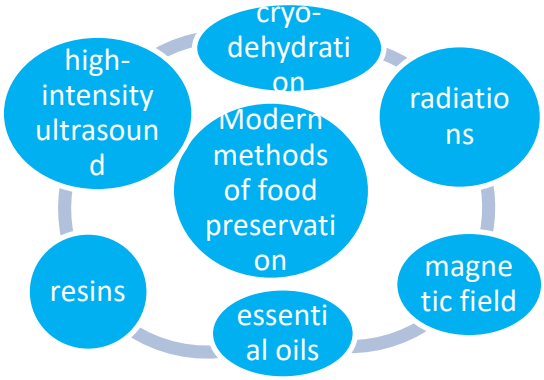

Fig. 3. Modern methods of food preservation

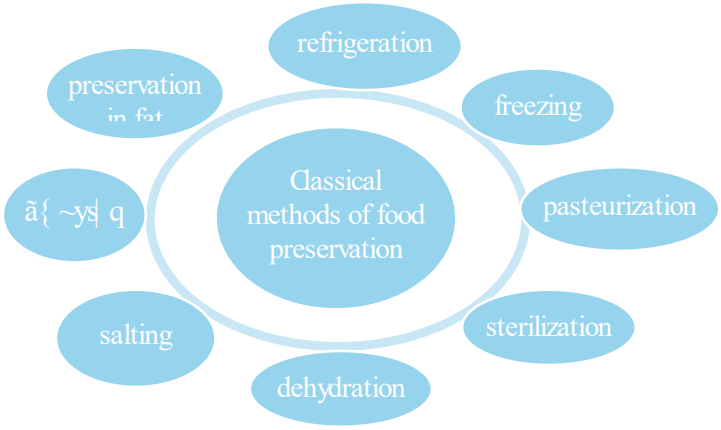

Fig. 4. Classical methods of food preservation

Aktaș et al. (2) investigated the role of salts on muscle fiber denaturation. Intramuscular connective tissue obtained from the *Longissimus dorsi* muscle of a 4-year-old beef carcass was treated with 2, 4 and 6% (w/v) NaCl solutions and 50, 100 and 150 mM $CaCl_2$ solutions and citric acid solutions and 0.5, 1.0 and 1.5% lactic for three different marinating periods (24, 46 and 72 hours). The changes in denaturation characteristics were investigated using differential scanning calorimetry and it was found that denaturation onset temperature (To) and denaturation peak temperature (Tp) increased as the NaCl concentration increased but decreased as the concentration of $CaCl_2$ increases regardless of the marinating time. Lactic and

citric acid lowered the To to about 39°C, from over 60°C breaking the fibril structure (2).

Zhang et al. (31) highlighted the role of freezing on meat quality. Freezing is an effective technique to extend the shelf life of meat. However, meat quality can gradually deteriorate following freezing and defrosting processes and lead to reduced water holding capacity (WHC), discoloration and alteration.

Sun et al. (27) in their study promote radiofrequency thawing of frozen meat for preservation of muscle fiber architecture and meat physicochemical properties compared to air flow. The study was conducted on frozen mutton at -18 -4°C, which was subjected to a 6 kW, 27, 12 MHz radio frequency system. The results demonstrated that radiofrequency defrosting has a higher value compared to air: shorter defrosting time of 30 min compared to air of 8.1 hours, radiofrequency defrosted samples maintained their color and texture better than air-defrosting samples, loss of total basic nitrogen content was less than in air-defrosting samples, reduction in myofibrillar protein content was less compared to air-defrosting, microstructure was less damaged with defrosting by radio frequency compared to air defrosting (9, 27).

Hernández et al. (10) focused on the analysis of the effects on the physico-chemical and histological properties of meat by adding waxy starch cryogels to pork muscles, using high-intensity ultrasound, which would allow minimizing the harmful effects caused by conventional freezing. The use of waxy starch cryogel in 2 cycles applied with high-intensity ultrasound generated a cryoprotective effect on the meat, covering the meat fibers, reducing the structural damage observed by (modulated differential scanning calorimetry (MDSC), total reflection Fourier transform infrared spectroscopy attenuated (FTIR-ATR), scanning electron microscopy (SEM), SEM, MDSC and FTIR-ATR, favoring the decrease in the size of the myofibrillar spaces about 75%, avoiding changes in the thermodynamics and chemistry of proteins, maintaining some aspects of quality in better conditions after conventional freezing in a chamber. The cryogel was found to generate significant ($P < 0.05$) changes due to its interaction with water and meat proteins, decreasing the size of spaces for ice crystal growth by about 75% compared to the control (meat without cryoprotectant). There were thermal, molecular and structural changes ($P < 0.05$) that influenced some quality aspects such as pH, water activity, shear force and color, avoiding important changes due to the freeze-defrosting process (10).

Elif Aykin-Dinçer et al. (4) investigated the effect of salt impregnation (4 g NaCl / 100 g solution) of beef cubes at different vacuum levels (250, 500 and 750 mbar), with and without ultrasound. Moisture content and salt content were monitored. The results showed that ultrasound exposure shortened the salting time and vacuum impregnation improved the salt and water gain during the salting process compared to the control (4).

Millar et al. (20) demonstrated the change in the color of poultry meat under the influence of ionizing radiation. The common effect in the muscles of all species was the appearance on the freshly cut surface of a deep red color. It has been

assumed that this color is the result of the formation of a carboxyheme pigment; carboxymyoglobin and/or carboxy hemoglobin (20).

Li et al. (18) used starch-curcumin-gelatin dialdehyde particles, prepared by encapsulating curcumin in the cross-linked body formed from starch dialdehyde and gelatin. Curcumin destroyed the hydrogen bonding system of the composite membrane, but also played a role in fixing the molecular chains in the film. The composite film not only had excellent resistance to ultraviolet radiation to protect 94.48% of ultraviolet radiation, and the elimination rate of free radicals reached 99%, but also showed significant antibacterial effects, which inhibited bacteria (18).

Zhang et al. (30) concluded that polycyclic hydrocarbons have toxic, mutagenic and carcinogenic properties and it is important that foods contain as little as possible to protect public health (30).

Liu et al. (19) reported the effects of cinnamaldehyde release from cinnamaldehyde nanoemulsion (CNE) on the shelf life of chilled pork. Cinnamaldehyde (Cin) was encapsulated in an oil-in-water emulsion using Tween 80 (Polysorbate 80) as an emulsifier. Ultrasound was used for incorporation/emulsification. Unlike previous studies when emulsions were used in direct contact with food as coatings or as immersion solutions (1, 22), in this study a non-contact way was adopted in which the nanoemulsion and the chilled meats are next to each other but not touching each other. The results showed that Cinnamaldehyde is continuously and uniformly released from the nanoemulsion, in a sealed package, having a natural bacteriostatic effect (5, 23, 26) while reducing exudate losses, pH, total volatile basic nitrogen and the total number of microbial colonies (19).

Noori et al. (22) demonstrated the antimicrobial and antioxidant effect of nanoemulsion-based edible sodium caseinate coating containing ginger essential oil (GEO) (3 and 6% in weight) that was applied to chicken breast fillet to extend shelf life his. Edible nanoemulsion-based coatings with 6% nanoemulsion containing ginger essential oil caused a significant decrease in total aerobic psychrophilic bacteria in chicken fillets refrigerated for 12 days. Overall, the nanoemulsion containing ginger essential oil was more effective than the conventional emulsion in extending the shelf life of chicken breast fillets (22).

The present authors found that the methods used most often in preservation are industrial ones in accordance with the litterature. The methods that preserve the histological and physicochemical properties of the fillet muscle remain the traditional ones or those that use natural additives that, in addition to improving the organoleptic properties, produce an improvement in the quality of the products.

All preservation methods cause changes in the physical-chemical and histological properties, important marks for the consumption pattern of the population but also for influencing its state of health (12). The sensory characteristics (texture, flavor, aroma, and color) of a food are determined primarily by its moisture and fat content, as well as the types and amounts of structural carbohydrates and proteins. The texture of meat can be defined as the composite of its structural elements (2,

29). Intramuscular connective tissue consists mainly of collagen and elastin fibers as a composite network encased in a matrix of proteoglycans. The morphology, composition and amount of connective tissue present depends on the muscle type, species, breed and age of the animal (24), and its impact on meat hardness depends on its composition, distribution and mechanical and thermal stability properties (7). The hardness of connective tissue can be reduced by long cooking times, which convert the collagen to gelatin with small changes in the thermal stability of the connective tissue having a considerable impact on cooking times (3, 24).

The development in processing strategies such as freezing, smoking, dehydration, salting, hedging, preservation with oils, influences the histological aspect and the physico-chemical properties and also showed the differences. This review shows that preservation methods have many advantages but also disadvantages.

The processing of preservation and preparation of meat causes denaturation of some of the meat proteins, resulting in changes in appearance, texture and water-holding capacity of the meat proteins, leading to different consumer perceptions and likings of the prepared meat dish. Muscle protein is a high-quality source of protein nutrition, with a good balance of dietary essential amino acids and high digestibility (6).

The changes that conservation methods produce on the structure of the fille muscle are presented in the table below (Table 1):

Table 1

Comparative changes induced by preservation methods on fillet muscle

Meat preservation method	**Advantages**	**Disadvantages**	**Changes in physicochemical properties of meat**	**Histological appearance of meat**
Smoking	The smoke contains antiseptic substances – various acids and phenols that act in the food preservation process, based on the principle of anabiosis. It can be done cold, hot or by exposure to hot smoke	Oxygenated polycyclic aromatic hydrocarbons appear with a carcinogenic.	Smoke can have chemical compounds that interact with meat proteins, changing its appearance and texture. Offers a rich flavor to meats but demands careful monitoring.	Changes in cellular structure occur depending on the duration and intensity of smoking.

	(maximum 170°C).			
Salted	It increases the osmotic pressure in their molecular structure, which gradually leads to slow dehydration. Destroys bacteria (botulinum toxin). Protects lipid content.	Foods preserved in brine have a low nutrient value because they pass into the salt water solution. The use of nitrate can determine in combination with hydrochloric acid nitrosamine with a carcinogenic effect.	It helps preservation by reducing the water available to microorganisms, but it can affect the texture and taste of the meat.	Leads to changes in salt concentration in muscle cells. Increasing concentrations of NaCl ions increase the thermal contraction temperature of collagen.
Dehydration	It decrease the level of humidity that favors microbiological and enzymatic processes. The volume and weight of the meat is reduced, which leads to a decrease in economic costs. A very gentle process that preserves more of the meat's natural flavor.	Dehydrated products lose their vitamin content and some sensory characteristics. Another disadvantage could be that dehydrated products become hygroscopic (absorb moisture from the air, absorb water) and therefore require special packaging.	The physico-chemical properties are preserved. If drying occurs at over 60°C, the starch gelatinizes.	Cause changes in cellular structure and changes in the texture and density of muscle tissue.
Pasteurization	Destruction of enzymes by prolonged and moderate heating.	The resistance flora is not destroyed.	Partially inactivates enzymes and kills microorganisms that cause spoilage.	Very good to retain (keep) the meat tissue microstructure.

Sterilization	It destroy all microorganisms by exposure to temperatures above the boiling point of 100°C.	Modify the taste, aroma and structure of the product.	Enzymes, vitamins are destroyed, proteins coagulate.	Change the structure of muscle fibers.
Refrigeration	Keep food at positive low temperatures of no more than 4°C and with a humidity level of up to 90%. Refrigeration is effective in the short term and is used more for transporting and storing meat.	Enzyme activity is slowed but not stopped. It applies for a short period of time.	Avoids the development of microorganisms, the oxidation of fats, the loss of specific flavors.	The structure of the products is not influenced.
Freezing	Consists in keeping food at temperatures below the freezing point, usually between -18 and -45°C. Freezers must ensure good ventilation and maintain the relative humidity of the air.	It produces a series of physical, biochemical and structural changes to the food product, which means that in order to be consumed or processed, it must be defrosting. This process can take place either at room temperature, letting warm room air work slowly to break the ice crystals, or by using steam and water or a microwave oven.	Protein denaturation and lipid oxidation in meat, leading to loss of quality.	Keeping the cell structure almost unchanged, with formation of ice crystals. When the meat is defrosting, a leakage of water from the cells can occur, which can lead to changes in the texture and quality of the meat.

High intensity ultrasound	Used to facilitate the diffusion of waxy starch cryogels in pork muscle.	It creates high-pressure bubbles in the liquid or semi-solid medium that burst quickly, generating intense heat and pressure.	Allows to minimize the harmful effects caused by conventional freezing.	It destroys the cell membrane. Changing the freeze-defrosting rate.
Ionization	It destroys the bacteria responsible for spoiling the meat. No side effects if used on frozen meat.	It changes the taste. Radiolytic products appear (hydrogen, hydrogen peroxide). Aldehydes appear. Alkylcyclobutanones (2-ACB) are formed during food irradiation and have been adopted by the European Committee for Standardization as biomarkers.	Increase ph. Proteins are split into amino acids, carbohydrates become more susceptible to depolymerization and formation of oxidative products, lipids are transformed into 1 alkylcyclobutanones, vitamins (K) are destroyed. Carboxyhemoglobin is formed.	It does not change.
Essential oils	Essential oils (EOs) are aromatic and volatile oily extracts, commonly used as a flavoring in food products. They are also known as a class of natural preservatives because their strong antimicrobial	They are very volatile.	It changes pH, exudate loss, total volatile basic nitrogen and total microbial colony count.	The histological appearance is preserved and durability is increased.

	and antioxidant. They can delay the oxidation reaction, the formation of free radicals and the growth of microorganisms *in vitro*.			

All preservation methods cause changes in the physical-chemical and histological properties of fillet muscle and influence public health.

Salting meat products increases their quality and flavor, but in large doses salt can cause kidney failure, heart failure, even cancer. In order to shorten the salting time, exposure to ultrasound has been proven to have a decisive role.

Freezing extends the shelf life of the meat, but the quality of the meat can gradually deteriorate following the air flow freezing and thawing processes. The architecture of the muscle fibers and the physicochemical properties of the meat is preserved by promoting the thawing of frozen meat by radiofrequency. Minimizing the harmful effects of conventional freezing can be achieved by adding waxy starch cryogels to pork muscles using high-intensity ultrasound.

The consumption of meat containing polycyclic hydrocarbons, with toxic and mutagenic properties, increases the risk of colorectal cancer.

Curcumin and essential oils protect against ultraviolet radiation but also showed significant antibacterial effects, inhibiting the development of bacteria.

In order to preserve the architecture of muscle fibers with beneficial effects on human health, traditional preservation methods must be refined with modern methods.

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USE OF CANNABIDIOL IN THE MANAGEMENT OF STRESS-RELATED ACRAL LICK DERMATITIS - CASE REPORT

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Summary

Acral lick dermatitis (ALD) is a chronic skin disease of canine patients that present behavioral issues such as excessive licking of specified skin areas, mainly the legs. The licking behavior leads to a localized alopecic, hyperpigmented, hyperplastic or even ulcerated single or multiple lesions, predominantly located in the carpal or hocks area. Repetitive licking is viewed as compulsive behavior suggesting that ALD is the canine equivalent of the human obsessive-compulsive disorder (OCD). The interest in using phytocannabinoids in veterinary medicine has seen an increase in the past 5 years especially in terms of treatment of various pathologies such as pain, epilepsy, anxiety and skin lesions. Nevertheless, studies are scarce and there is an increased need for research on the utility of these products. The present case report is focused on the use of a CBD (cannabidiol) and L-tryptophan commercially available oil (CroniCare®) for relieving stress and anxiety in a 6 year-old American Staffordshire terrier mix and to improve skin lesions caused by the compulsive licking behavior subsequent to stress. The dosage employed for the case was according to the product label, namely 2 drops/kg given twice daily *per os*. The clinical signs improved within three weeks of administration due to a decrease in intensity of the obsessive-compulsive licking behavior. A low maintenance dose of 1 drop/kg given once daily has been maintained in order to avoid recurrence of signs until the cause that has led to this behavior can be properly managed.
Keywords: dog, veterinary medical use, cannabidiol, acral lick dermatitis.

Cannabis (*C.sativa* L.) is a plant originating from Central Asia, documented in numerous cultures, such as ancient China, India, Europe, etc. as having multiple purposes: source of grain, fibre, textile, narcotics and medicine (1, 19). The plant has a wide range of therapeutic effects, being used in the treatment of diseases such as epilepsy (8, 16), anxiety (2, 17), inflammation (7), pain (6, 16).

Dogs that develop a self-initiated skin disease called acral lick dermatitis (ALD), also referred to as lick granuloma, frequently develop a localized alopecic, hyperplastic, fibrotic lesion near their carpus or hock areas as a result of repeated biting, licking, or scratching, which leads to ulceration and plaque formation (13). ALD is thought to be an animal model of obsessive-compulsive disorder (OCD) in humans since the condition's repeated licking is typically seen as a compulsive behaviour. The licking is caused by the primary factors associated with this disease, which are organic and psychogenic. On the other hand, the licking is perpetuated by factors such as secondary bacterial infections, keratin foreign bodies, furunculosis, bony changes, and the development of a secondary compulsive disorder/learned behaviour (13).

Endorphins are released into the brain when anything painful or exciting happens. For this reason, dogs who lick their paws as a coping method are 'self medicating' since the endorphins cover the gap between their brain chemistry and lifestyle.

Recently, research has been conducted on the possible impacts of CBD oil on the hypothalamus and other areas of health. An essential component of the brain, the hypothalamus, controls a wide range of physiological processes, including body temperature, appetite, thirst, and hormone production. The endocannabinoid system, which regulates a variety of physiological processes, including those regulated by the hypothalamus, has been demonstrated to interact with CBD oil, despite the paucity of study on the precise effects of CBD oil on the hypothalamus.

Anxiety is an emotional response towards the anticipation of a potential threat or and impending danger (8). Dogs can have a wide range of anxiety disorders, but the most prevalent ones are separation anxiety, generalized fear of strangers, other dogs, the environment, and noise aversions (such as thunderstorms and fireworks). Benzodiazepines, tricyclic antidepressants, selective serotonin reuptake inhibitors, and other veterinary medications are being utilized to treat various forms of anxiety in companion animals. However, because of the possible negative side effects, companion animal owners would rather see a decrease in drug use and a rise in interest in natural product-based anxiety therapy.

The control of stress reactions is closely related to the endocannabinoid system. Long-term exposure to environmental stress downregulates CB1 receptors, which lowers AEA levels and raises 2-AG levels. Reduced AEA levels are linked to the development of the stress response and an increase in anxiety-related behavior. In addition to cannabinoid receptors, newer research indicates that CBD affect a number of additional receptors, such as transient receptor potential vanilloid type 1 receptors and the serotonin 5-HT1A receptor (14).

Materials and methods

A 6-year-old American Staffordshire terrier mix with a body weight of 20 kg was presented at the Dermatology clinic from the Faculty of Veterinary Medicine in Timisoara, with skin lesions suggestive of self-trauma such as: hyperpigmentation, localized alopecia, erythema and mild erosions on the metatarsal region of the right hind limb and in the carpal region of the left front leg (Fig. 1, Fig. 2). In addition to the skin-related lesions, the animal was showing clear stress signals such as lip licking, hypervigilance, constant licking of itself or others, yawning, shaking off. Differential diagnosis was made for atopic dermatitis, bacterial pododermatitis and fungal infections. In this respect, we carried out diagnostic methods such as cytology and skin scrapes and they both came back negative. Thus, based on the licking behaviour and on the detailed history that we got from the owner which confirmed that the animal had gone through abrupt and significant lifestyle changes such as moving house and changing owners, the skin lesions were diagnosed as symptoms

of acral lick dermatitis. The patient received local treatment based on chlorhexidine digluconate spray in order to help the healing process and to prevent the occurrence of secondary bacterial or fungal infections. The treatment was applied by spraying on the affected areas once a day for 7 days in a row.

The cause of ALD in our patient was established to be of psychological nature so in order to manage and reduce the stress related self-traumatising behaviour we resorted to psychotherapy with a natural product that contained a mixture of cannabis oil and fish oil (Table 1, Fig. 6). The oil (Fig. 3) was administered on a daily basis according to the manufacturer's recommendations in a dose of 1 ml.

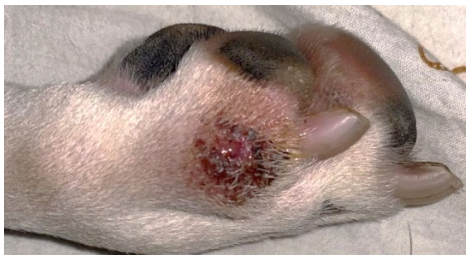

Fig. 1. Erosion on front leg due to excessive licking (original)

Fig. 2. Hyperpigmentation on hind leg due to excessive licking (original)

Table 1

Description of product label

Ingredients	Amount
Fish oil	750 mg
Cannabis extract	250 mg
Omega 3	285 mg
Phytocannabinoids	10 mg
THC	<0,05mg

Fig. 3. Cannabidiol based oil used for the treatment of ALD (22)

Results and discussions

In order to observe the reaction of the animal to the medication and to avoid any severe adverse effects, the product was first administered in half the recommended dose. Hypersalivation could be observed but no behavioural changes or adverse effects were noticed so the dose was increased to 1 drop per kg. During the treatment we noticed an increase of water intake and increased appetite. The indicators of stress and compulsive licking began to lessen as the dosage was gradually increased to 2 drops per kilogram. Her mood improved, she did not show any signs of stress around other people and the compulsive licking habit diminished. The aspect of the skin lesion improved within 1 week of treatment with visible epidermal repair (Fig. 4). Following three weeks of treatment (Fig. 5, Fig. 6) the aspect of the skin improved, the size of the lesion reduced considerably and the erosion started to heal so we proceeded to progressively reduce the psychotherapeutic treatment to observe whether a potential recurrence of the symptoms would take place. The patient did not pick up on her previous self-traumatizing habits. During the treatment period and after it, no changes to her lifestyle were made and the patient was kept in the same conditions as the ones that led to the compulsive behaviour in the first place. Following the complete withdrawal of the treatment, her condition stayed the same, her compulsive licking did not reoccur and the behavioural indicators of stress disappeared. No withdrawal-associated symptoms were observed.

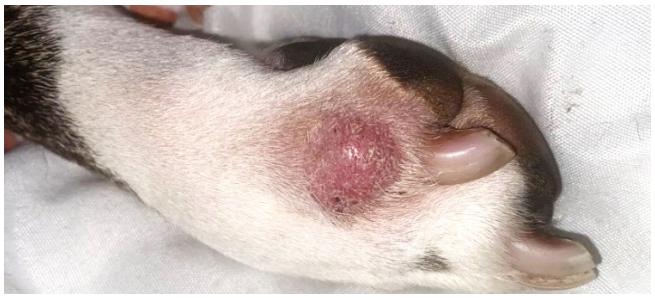

Fig. 4. Aspect of lesion following 1 week of treatment (original)

CBD oil is being used more and more frequently in various situations that imply anxiety control. One study reported significant reduction of canine stress compared to the placebo group in patients suffering from stress related to travelling in vehicles (21). Another study conducted on 98 dogs and based on the answers given by owners to tailored questionnaires revealed significant changes in pre- versus post- treatment behaviour of pet dogs. Thus, after only 14 days of treatment, owners reported reduced levels of stress-related behaviour in their pet dogs, including in terms of self-traumatizing behaviour such as excessive scratching,

licking or foot chewing, results that are similar to our case report, with visible improvement of licking-related lesions within seven days of treatment. The same study reports increased playfulness and calmness, also similar to what we noticed in the studied patient (10, 11).

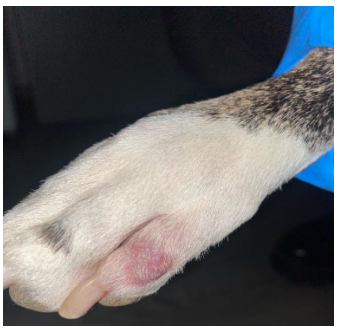

Fig. 5. Aspect of lesion after three weeks of treatment (original)

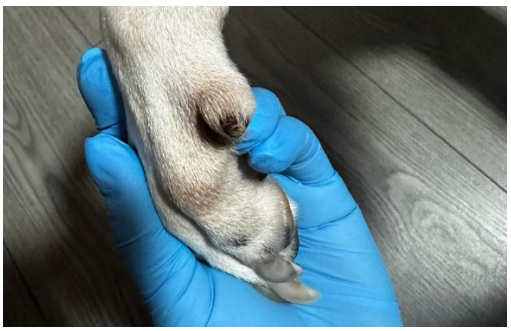

Fig. 6. Aspect of feet at the end of the treatment (original)

A neurotransmitter linked to pain, depression, and anxiety is serotonin (5-HT) 5-HT1A receptor found throughout the brain, particularly in areas that are frequently associated with stress and anxiety, such as the hippocampus, raphe nuclei, prefrontal cortex, amygdala, and hypothalamus. While the single study on CBD and anxiety in dogs found that CBD at 1.4 mg/kg BW/day did not have anxiolytic effects, there are presently no data on CBD use for anxiety in cats (5).

While administering CBD oil to shelter dogs has been demonstrated to lessen their hostile behavior toward people, neither CBD oil supplements nor CBD-infused snacks have been linked to anxiolytic effects in dogs. However, there were conflicting findings in investigations on humans and rodents about the ability of CBD to reduce anxiety (12). Recent randomised, double-blind, and controlled trials have shown clinical evidence that short-term oral CBD treatment can reduce anxiety related to public speaking and social anxiety. Additionally, anxiety-related comorbidities in a range of illnesses and diseases, such as mental disorders, neurofibromatosis type 1, Dravet syndrome, fragile X syndrome, Crohn's disease, etc., were also shown to be positively impacted by CBD (21). Conversely, a study by Wildes et al. (16) revealed conflicting results, showing that people who received opioids for chronic pain management experienced worsening anxiety or depression as the frequency and/or percentage of CBD oil consumption rose. Additionally, a small number of mouse studies discovered that intraperitoneal injections of CBD at doses ranging from 5 to 20 mg/kg BW produce negligible to no effects on anxiety; a dose of 20 mg/kg BW was even discovered to be anxiogenic.

Sacchettino et al. (5) described the case of a dog suffering from compulsive disorder which was managed using a combination between CBD and melatonin therapy with promising results compared to the previously administered conventional antidepressant therapy, further confirming the potentially beneficial effect of CBD in managing compulsive behaviours in dogs.

Conclusions

CBD oil appears to be promising in the treatment of anxiety-related skin disorders. The present case study had a positive outcome, with resolution of clinical signs following a month long treatment with CBD oil that aimed to reduce stress in a 6 year old Amstaff that came in presenting clinical signs specific for acral lick dermatitis. The ulcerative lesions reduced significantly and the stress-related behaviour improved as well, suggesting a beneficial effect of the CBD oil in managing the dermatological condition.

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COMPARATIVE STUDY ON THE ANTIHELMINTIC EFFICACY OF SOME MEDICATIONS IN GASTROINTESTINAL NEMATODE PARASITISM IN SHEEP

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Summary

One of the most common problems in ovine farm management encountered by farmers is represented by parasitological control. The effects of parasitological infestation in sheep are animal productivity losses and even animal deaths. In this study, the efficacy of some commonly used antiparasitic drugs against gastrointestinal nematodes infestation (*Vermitan 10%, Levaverm 10%,* and *Evomec Plus*), as well as the incidence of parasites' chemoresistance to the active substances, were noted. For this study, 3 sheep farms were taken into account. The sheep were raised in a free-range environment in Timiș County. A different type of anthelmintic drug was administered in each farm. For the efficacy determination, the initial infestation level was evaluated on day 0, followed by the infestation level evaluation on days 7, 14, and 21 post-treatments. The infestation level was rated via the quantitative method McMaster by assessing the number of parasitic eggs/gram of fecal matter (EPG). The efficacy was calculated with the FECRT formula. The efficacy of antiparasitic drugs against gastrointestinal nematodes determined during this study was 97,16% for *Levaverm 10%*, 96,51% for *Evomec Plus,* and 95,52% for *Vermitan 10%*. No chemoresistance phenomena were noted for the antiparasitic drugs evaluated in this study.
Keywords: sheep, nematodes, treatment efficacy.

Effective prevention and control of parasitic diseases require that all epidemiological features regarding parasite life cycle, mode of transmission, sources of infestation, etc. are well understood by both veterinarians and animal owners (3, 14, 44). The correct diagnosis and administration of the appropriate treatment promote therapeutic success and also decreases the possibility of chemoresistance. Worth mentioning is the fact that depending on the particular situation of each area, sustainable parasitemia management is performed differently (33, 39, 43).

The strategies applied for the parasitological control are evolving worldwide. In many countries the use of broad-spectrum anthelmintics in combination with pasture hygiene are still the predominant control strategies. However, in recent years significant production losses and high mortality rates due to helminth infestations have been reported. Those cases are registered especially in regions with intensive sheep farming (31, 36). This situation comes as no surpise since some common anthelmintics have relatively lost their efficacy in several countries after only a few years on the market (24). The way these drugs are used

needs to be considered in the current context in order for them to remain effective in the long run (16). Taken into account should be the fact that some studies suggest the fact that new antiparasitic substances are unlikely to emerge and be introduced on the market in the near future (22).

In Euope the biodiversity of gastrointestinal strongyles is very high. The most common species are: *H. contortus* and *T. circumcinta* (18). *H. contortus* is a haematophagous parasite and the most pathogenic gastrointestinal nematode (6). Moderately mixed parasitosis should not be neglected, as this usually comes associated with poor animal welfare and can generate important economic losses through decreased production and even death of animals (41). Therefore, a well thought out and efficient control strategy is needed.

In recent years, more and more examples have shown that the efficacy of anthelmintics has decreased considerably (32). The reason is the increase in parasites' chemoresistance to all major classes of drugs (28, 45) as well as to macrocyclic lactones (12, 26, 27, 29, 34, 35), benzimidazoles (4, 5, 7, 10, 19, 40) and imidazoles (13, 46, 47). This chemoresistance present especially in *H. contortus* is observed on all major continents, i.e. North America (15), South America (32), Asia (48), Africa (42) and Europe (20). On an international level this chemoresistance is classified as a threat to global food security (25).

In our country, the most commonly used antiparasitic drugs to control gastrointestinal parasites in sheep are benzimidazoles and ivermectin. It is noted that levamisole is only sporadically used (37).

In this study we had two objectives:

- to evaluate the degree of infestation of sheep from 3 farms in Timis County;
- to test the efficacy of levamisole (commercial product *Levaverm 10%*), ivermectin (commercial product *Evomec plus*) and albendazole (commercial product *Vermitan 10%*) in the treatment of gastrointestinal nematodes.

Materials and methods

The study was carried out in 3 sheep farms of the *Tsurcana* breed, in Timiș county for a period of 21 days. The villages where the farms are located are: Cadar (farm 1), Duboz (farm 2) and Nițchidorf (farm 3).

The experiment considered only adult animals aged between 2 and 8 years in each herd. All 3 herds were made up of adult animals only. The number of animals ranged between 200 and 250 heads per herd. All sheep were acclimatized to the conditions found in the farm locations. The conditions were similar in terms of climate and vegetation found on the pastures, since all the animals grazed in the Pogăniș meadow on adjacent pastures. The last internal deworming of the animals was done in the autumn of the year before the study by oral administration of albendazole (commercial product *Vermitan10%*). The last external deworming of

the animals was also done it the previous year, using diazinon bathing (commercial product *Diazinol*). Throughout the experiment the animals were kept in open range on communal pastures. They received additional salt pellets *ad libitum* and the only water source for the animals was the Pogăniș river.

The number of faecal samples collected from sheep throughout the experiment was 300, mentioning that there were 100 samples taken from each sheep flock. On day 0 of the experiment, the initial infestation degree was assessed according to the number of gastrointestinal nematodes eliminated through feces (EPG). After this assessment, until the end of the experiment, the evolution of EPG along with the effect of each antiparasitic drug against gastrointestinal nematodes were evaluated. The fecal samples were collected transrectally. Each sample was labeled and refrigerated for 48h, until examination.

The degree of sheep infestation with gastrointestinal nematodes was determined using the McMaster quantitative coproscopic method. Examination was performed using a 10x microscope objective (Bresser Researcher Bino 40-1000x microscope, Germany).

Treatment of sheep on day 0 in each farm was carried out as follows:

- Farm 1 with *Levaverm 10%* 1ml/10 kg body weight, orally;
- Farm 2 with *Evomec plus* 0.2 mg/kg, subcutaneous injection;
- Farm 3 with *Vermitan 10%* 7.5 mg/kg, orally.

Each drug was used separately in one farm, i.e. the animals in the Cadar farm were treated with levamisole, those in the Dubozv farm with ivermectin, and the ones in the Nițchidorf farm with albendazole. For the most correct dosing of the drugs during administration, automatic veterinary syringes were used, namely:

- for the administration of the injectable solution *Evomec plus*, the SAS Henke HSW ECO-MATIC 2 ml automatic syringe;
- for the administration of the oral solutions *Levaverm 10%* and *Vermitan 10%*, the HSW DRENCH-MATIC 25 ml automatic syringe.

Statistical interpretation of the data on differences between the 3 livestock farms was done using statistical functions implemented in Microsoft Excel 365 (version 2208, Redmond, WA, USA). This interpretation was done one at a time between each farm, taken into pairs.

Results and discussions

Following the McMaster method to identify the degree of infestation before and after treatment (days 7, 14, 21) the following results were identified in animals from all 3 farms (Tables 1, 2, 3 and Fig. 1, 2, 3).

The anthelmintic efficacy (E%) of products used in animal deworming was calculated according to the FECRT formula:

$$E\% = \frac{E.P.G.\ before\ treatment\ (day\ 0) - E.P.G.\ day\ 14}{E.P.G.\ day\ 0}\ x\ 100.$$

Table 1

Degree of infestation of sheep in farm 1 before and after treatment with *Levavermin* 10%

	Larval eggs of *Strongyloides spp.*				Morulated eggs from gastrointestinal strongyles			
Day	0	7	14	21	0	7	14	21
Total EPG /samples*	8100	500	750	900	116650	1900	2800	3700
EPG average /samples*	324	20	30	36	4666	76	112	148
Minimum	0	0	0	0	500	0	0	0
Maximum	750	100	200	150	12300	300	400	450
Mean standard error*	±229	±28	±50	±42	±2674	±80	±123	±135

*25 samples were collected for each day of determination, i.e. n = 25 samples;
*Statistical calculations were performed using statistical functions implemented in Microsoft Exel.

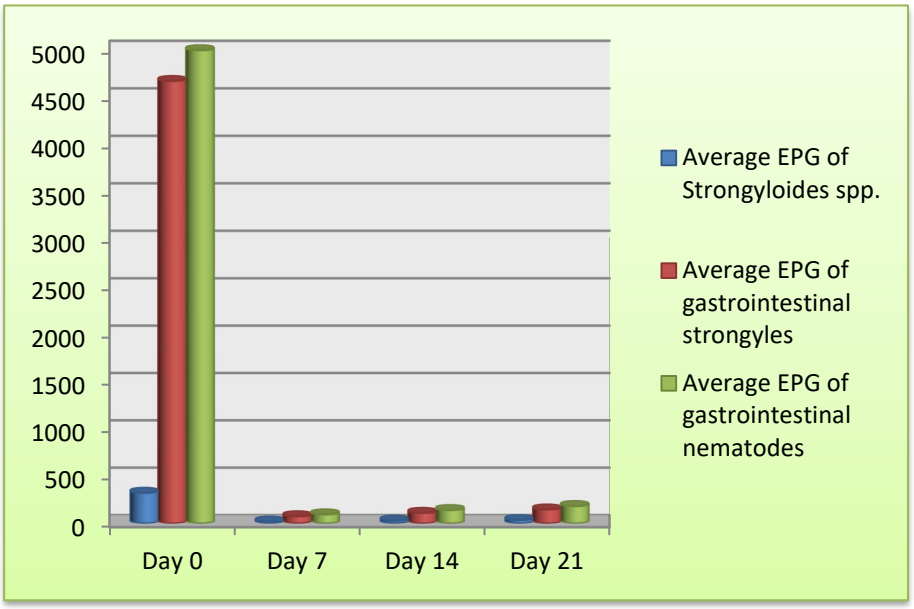

Fig. 1. The evolution of parasitism in sheep from farm 1, treated with *Levaverm* 10%

Table 2

Degree of infestation of sheep in farm 2 before and after treatment with *Evomec Plus*

	Larval eggs of *Strongyloides spp.*				**Morulated eggs from gastrointestinal strongyles**			
Day	0	7	14	21	0	7	14	21
Total EPG /samples*	7150	550	600	900	128850	2550	4150	4750
EPG average /samples*	286	22	24	36	5154	102	166	190
Minimum	0	0	0	0	550	0	0	0
Maximum	1000	150	150	150	12000	400	500	600
Mean standard error*	±284	±38	±43	±51	±3187	±100	±130	±162

*25 samples were collected for each day of determination, i.e. n = 25 samples;
*Statistical calculations were performed using statistical functions implemented in Microsoft Exel.

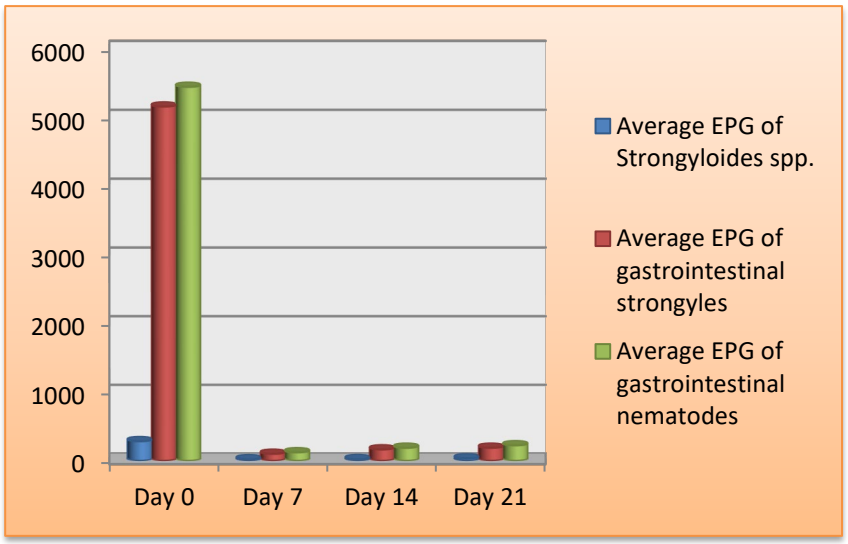

Fig 2. The evolution of parasitism in sheep treated with *Evomec Plus*

Table 3

Degree of infestation of sheep in farm 3 before and after treatment with *Vermitan* 10%

	Larval eggs of *Strongyloides spp.*				Morulated eggs from gastrointestinal strongyles			
Day	0	7	14	21	0	7	14	21
Total EPG /samples*	10050	650	800	950	128250	3850	5400	6050
EPG average /samples*	402	26	32	38	5130	154	216	242
Minimum	0	0	0	0	500	0	0	0
Maximum	1200	150	150	150	11000	450	550	650
Mean standard error*	±339	±45	±49	±50	±2833	±134	±175	±200

*25 samples were collected for each day of determination, i.e. n = 25 samples;
*Statistical calculations were performed using statistical functions implemented in Microsoft Exel.

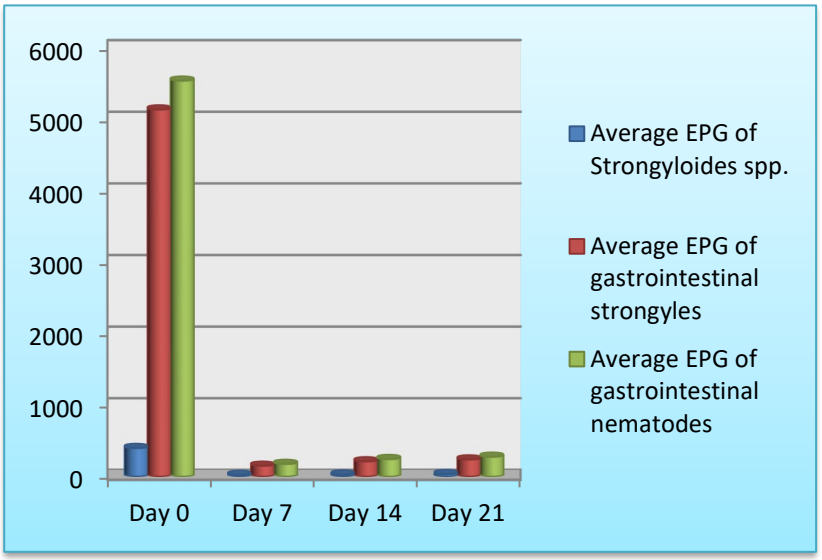

Fig 3. The evolution of parasitism in sheep treated with *Vermitan* 10%

In the case of *Levaverm 10%,* the efficacy of the antiparasitic treatment of

sheep, according to the FECRT formula, was: 90.74% for strongyloidosis and 97.60% for trichostrongylosis and 97.16% for general gastrointestinal nematodes.

In the case of *Evomec Plus*, the efficacy of the antiparasitic treatment of sheep, according to the FECRT formula, was: 91.61% for strongyloidosis and 96.78% for trichostrongylosis and 96.51% for general gastrointestinal nematodes.

In the case of *Vermitan 10%*, the efficacy of the antiparasitic treatment of sheep, according to the FECRT formula, was: 92.04% for strongyloidosis and 95.79% for trichostrongylosis and 95.52% for general gastrointestinal nematodes.

The comparison of the results obtained for each drug (Fig. 4) shows a lower efficacy for the anthelmintic effect of albendazole. This can be attributed to the fact that the animals were dewormed internally with the same substance last year (at the last deworming), therefore the parasite population that infested the sheep were descendants of the generations that survived that last deworming. This phenomenon demonstrates that repeated deworming with the same substance can accelerate the emergence of chemoresistance.

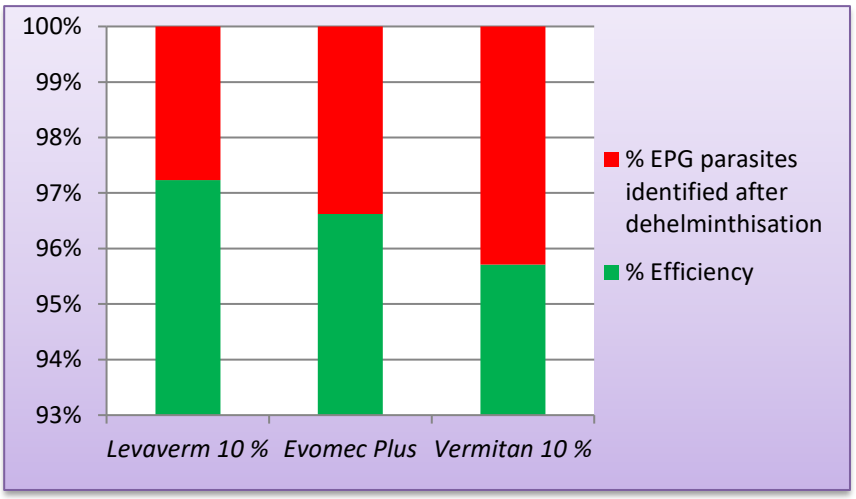

Fig. 4. Difference in the antiparasitic efficacy of the drugs used

The fact that the sheep flocks grazed on neighboring pastures and had a similar history of deworming, could have led to the identification of a relatively similar infestation degree between all 3 farms at the start of the experiment (on day 0 the total EPG load of gastrointestinal nematodes was between 124 750 - 138 300). According to the t test the differences are not significant ($p > 0.05$). This similarity in terms of parasitical infestation degree at the start of the experiment for all 3 farms allowed an objective assessment of the anthelmintic efficacy of the drugs used. Similarly, the statistical differences at the end of the experiment are

insignificant between the 3 farms evaluated ($p > 0.05$). This can only be observed by comparing the percentage differences in efficacy.

In our country, similar studies have been conducted showing the efficacy of albendazole against intestinal nematodes of sheep: 91% in Vâlcea county (30), 97.03% in Bihor county (21), 98.46% in Arad county (23) and 99.84% in Timiș county (11). Ivermectin efficacy was observed up to 99.73% in Timiș county (11) and 91.31% in Alba county (8). The efficacy of levamisole against gastrointestinal nematodes in sheep was demonstrated by Mitrea et al. (2006) in Vâlcea county. The study showed up to 97% reduction of parasitical infestation (30). Since levamisole is used sporadically in the treatment of gastrointestinal helminthiasis of sheep in our country, there is no recent data on the efficacy of this drug (37).

Worldwide, studies show variable data on the efficacy of anthelmintic drugs in the treatment of parasitosis depending on the frequency of their use. Therefore, worth mentioning are studies done on:

- benzimidazoles - 91% efficacy in Uganda (9), 23% (17), and 54.95-90.86% (38) in India, 83% in Mexico (32), 48-85% in Portugal (1) and 80.3-86.5% in Slovakia (2);
- ivermectin - 98% efficacy in Uganda (9), 98.11% in India (17), 57% in Mexico (32) and 60% in Slovakia (2);
- levamisole - 91% efficacy in Uganda (9), 63% in India (17) and 94% in Slovakia (2).

Although the animals in all 3 flocks were dewormed in the autumn of the year prior to the experiment, they showed a high degree of infestation on day 0. This phenomenon might’ve occured due to the fact that the parasite populations on the pasture benefited from the rainy weather, coupled with high temperatures, both before and during the experimental period. This favoured the development of parasitical larvae found on the pasture. The increasing number of parasitic elements observed at the end of the experiment on all 3 farms can be attributed to the fact that the animals were continuously infested with larvae from the contaminated pasture.

The differences in efficacy between the drugs used in the experiment demonstrate that the occurrence of chemoresistance is directly proportional to the rate of drug use. Albendazole, used the most (because it’s cheap and easy to administer to sheep) showed the lowest efficacy. A higher efficacy than albendazole was found for ivermectin which, because of its route of administration (injectable), requires more rigorous containment of the animals, therefore it is less preffered by the farmers. Finally, the best result was obtained by administering levamisole which is less known among sheep farmers.

Conclusions

The evolution of OPG from day 0 to day 21 post treatment in all sheep on the 3 farms shows that *Vermitan 10%*, *Evomec Plus* and *Levaverm 10%* are still in the "safe group" in our country.

Chemoresistance is favoured by increasing frequency of use of a drug in antiparasitic treatment.

To avoid the development of chemoresistance, it is mandatory to rotate the use of antiparasitic drugs.

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LESIONS OF THE BEAK AND CEROMA IN BUDGERIGARS (*MELOPSITTACUS UNDULATUS*)

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Summary

In avian pathology, external lesions or anomalies of the beak and the cere are one of the external causes for a veterinary consultation in budgerigars (*Melopsittacus undulatus*) most common pet birds among bird enthusiasts. The beak is derived from the maxillary and mandibular bones, covered by a thin layer of keratin that continues into the nasal area. Modified beak conformations can affect a parakeet's life, leading to difficulties in feeding and preening, often resulting in untidy feathers and reduced weight. Another structure, the cere or the ceroma is the outer segment of the respiratory system and has a role in sexual dimorphism in budgerigars. Different pathologies, both systemically or locally can affect a parakeet when the nostrils become partially obstructed (sinusitis, rhinoliths) making breathing difficult. The present study was conducted at the department of Pathological Anatomy from the Faculty of Veterinary Medicine of Bucharest and comprised eight cases of budgerigars. The birds were examined for suspected lesions in the ceroma and the beak region and the methods used included macroscopic evaluation, cytopathologic examinations of cutaneous raclates (native slides and M.G.G. stain), microscopic examination of facial regional feathers and also necropsy examinations for three cases to establish the potential lesional link between external lesions and internal pathologies.The results of anamnesis, clinical and macroscopic examination of exclusively indoor, adult budgerigars revealed five females and three males, aged between 2 and 8.One case of a female budgerigar presented overgrowth of cere with a crusty, brown aspect that indicated a brown cere hypertrophy. Two cases presented abundant white, crusts on the cere extending to the beak and lack of surrounding feathers. Cytologic feather examination revealed normal aspects. Cytological examination of raclates were negative for parasites, but the clinical appearance and the favorable therapeutic response indicated parasitic hyperkeratosis. Four budgerigars manifested acute and chronic rhinitis and rhino-sinusitis associated with external tissue loss and wide choanal orifices as manifestation of rhinoliths sequelae or long term cicatrisation, although one case presented acute rhinitis at necropsy and only tissue loss and fibrosis at the exterior. One conformational abnormality of the upper beak valve manifested as upwards curving was diagnosed in an adult bird associated with emaciation and serous atrophy at necropsy examination. In conclusion, beak and ceroma pathologies in budgerigars can include multiple causes and a careful examination associated with complementary tests helps to determine the underlying pathologies.

Keywords: avian pathology, budgerigar, *Melopsittacus undulatus*, beak, ceroma.

The avian respiratory system, particularly in parrots, is highly susceptible to avian pathologies due to its distinct structure compared to mammalian respiratory systems. Gas exchange occurs in two stages, involving specialized structures known as air sacs. This system comprises two inextensible, non-lobed lungs, a trachea

formed by complete tracheal rings, and four pairs of air sacs (cervical, clavicular, cranial thoracic, caudal thoracic and abdominal) (2, 5, 8, 20).

Respiratory disorders have a multifaceted etiology, including biological factors like bacterial, viral, or parasitic infections, along with mechanical influences regarding air quality, as well as hypovitaminosis and congenital anomalies (3, 4, 15, 16, 18).

In the context of infectious diseases, various microorganisms, such as viruses, bacteria, fungi, and nematodes, determine or contribute to the development of inflammatory processes in psittacine birds. Among avian external parasites, it is noteworthy that *Cnemidocoptes pilae* is frequently diagnosed in the case of parrots, often instigating a condition known as "scaly face" These microscopic arachnids affect regions such as the ceroma, beak, and legs of the afflicted birds, thereby necessitating a targeted therapeutic approach, specifically in the form of antiparasitic treatment modalities (1, 3, 4, 6, 11, 17, 18).

Noninfectious oral lesions encompass a spectrum of conditions, from trauma caused by foreign objects to chemical or thermal burns. Psittacine birds are at risk from ingesting overheated foods or exposure to corrosive substances like silver nitrate sticks and toys made of wood or plastic. Acute lesions manifest as lacerations or abrasions with variable bleeding, progressing over time to chronic conditions characterized by inflammatory responses, fibroplasia, and irregular thickenings in the affected oral region (17, 18).

Various neoplastic conditions may exhibit metastatic behavior or local infiltration into the beak, with fibrosarcomas, squamous cell carcinomas, and melanomas being the most prevalent. Notably, fibrosarcoma is recognized as the predominant primary tumor affecting the beak (10, 14, 17).

In addition to the inherently stable structures of the respiratory system, the beak is a structural formation originating from the maxillary and mandibular bones, encapsulated by a delicate keratin layer that seamlessly extends into the nasal region. Deviations in the typical conformation of the beak can significantly impact a parakeet's quality of life, introducing challenges in essential activities such as feeding and preening. These alterations often manifest in unkempt feathers and a notable decrease in body weight. Deformities of the beak can arise from congenital or acquired factors, including genetically induced congenital deformities in budgerigars. Additionally, beak trauma, often resulting from bite wounds by other birds, is a common occurrence in psittacines (2, 9, 12, 13, 17, 18).

Furthermore, the cere or ceroma, with role in sexual dimorphism in budgerigars, represents the outer segment of the respiratory system. Various pathologies, whether systemic or localized, can adversely affect a parakeet, especially when partial obstructions occur in the nostrils (such as sinusitis or rhinoliths), leading to compromised breathing functions (1, 3, 17, 18).

The aim of this study is to evaluate lesions in budgerigars, focusing on the break and ceroma regions in order to document the nature, prevalence, and potential implications of such lesions within this particular avian species seeking to contribute

to the broader understanding of pathologies encountered in this particular anatomic region.

Materials and methods

The present study was conducted in the Laboratory of Anatomical Pathology, from the Faculty of Veterinary Medicine, University of Agronomic Sciences and Veterinary Medicine of Bucharest.

The materials were represented by eight cases of budgerigars (*Melopsittacus undulatus*) submitted to antemortem and postmortem investigations by private owners that observed abnormalities in the beak and cere region.

The methods used for evaluation were represented by gross examination and anamnesis data collection, cytopathological examination of crusts, obtained by scraping and imprinting of lesions, using direct, non stained smears and May-Grunwald-Giemsa stained cytologic samples and direct microscopic examination of feathers collected from the perioral region. In addition, three cases of budgerigars were evaluated by complete necropsy examination followed by histopathological examinations of various tissues, using standard protocol, respectively, Hematoxylin-Eosin (H.E.) stain.

Results and discussions

The results regarding anamnesis of the budgerigars indicated eight adult *Melopsittacus undulatus*, aged between 2 and 8 - three males and five females. They were all kept exclusively indoor, either as solitary birds or in groups, and predominantly fed with standard commercial food.

One case involved a female budgerigar with overgrowth of the cere, presenting a crusty, brown aspect indicative of brown cere hypertrophy.(Fig. 1).

In female budgerigars, especially during their breeding cycle, brown cere hypertrophy is a common occurrence. This phenomenon is often linked to excessive estrogen levels, a primary hormone in female reproduction, causing the cere to develop a keratinized enlargement (21).

Fig. 1. Brown cere hypertrophy

The cytopathological examination of the dry and crumbly crusts with brownish hues revealed immature anucleated and some nucleated keratinocytes, confirming the hypothesis suggested during macroscopic examination. Physiologically, the ceres exhibit a white color in females and transition to shades of brown during the mating season. Microscopic examination of the feathers did not reveal the presence of any parasites or mycosis linked with the overgrowth of the cere (Fig. 2).

Fig. 2. Feather (direct examination on microscope) (Ob. 20)

Two cases presented abundant white, crusts on the cere, extending to the beak, and lack of surrounding feathers (Fig. 3).

Cytopathologic examination of the feather revealed normal aspects, and examination of raclates were negative for parasites. However, the clinical appearance and the favorable therapeutic response indicated parasitic hyperkeratosis.

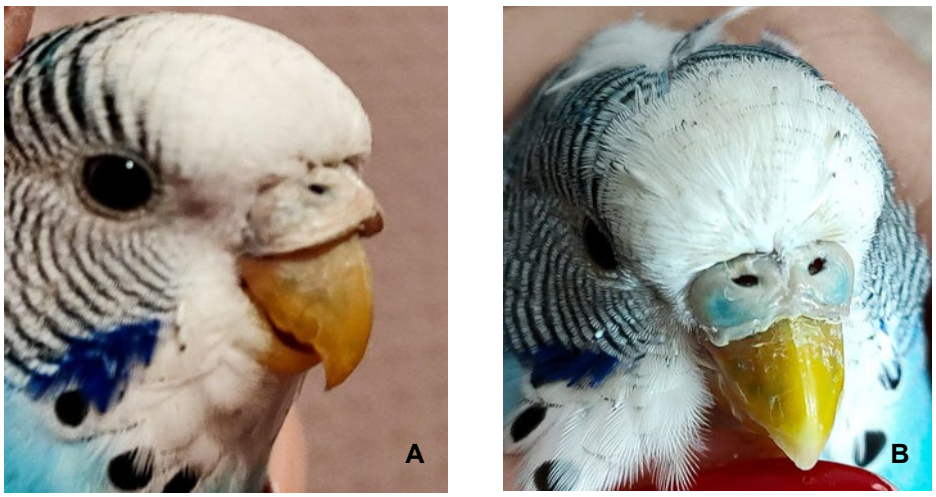

Fig. 3. A) Cere hyperkeratosis, B) Normal cere appearance after therapeutic trial diagnosis

Four budgerigars exhibited signs of both acute and chronic rhinitis, along with rhino-sinusitis, characterized by external tissue loss and widened choanal orifices - an indication of rhinolith sequelae or prolonged cicatrization.

In the acute form of rhinosinusitis, birds commonly exhibit symptoms like swollen eyes, or discharge from both the eyes and nostrils. The presence of nasal discharge, frequent sneezing, or swelling around the eyes are key indicators of sinus issues (20). One case of acute rhinitis was associated with cere discoloration, increased volume of the cere and surrounding skin and nasal orifices obstructed by exudate and only discrete palpebral oedema, which did not progress to conjunctivitis or lateral subcutaneous volume increase indicators for sinus infection (Fig. 4). Cytopathological examination of imprints indicated multiple coccoid bacteria (Fig. 5), but a further microbiological examination was not followed in this case.

Fig. 4. Acute purulent rhinitis with choanal orifice obstruction, ceroma discoloration and discrete palpebral oedema

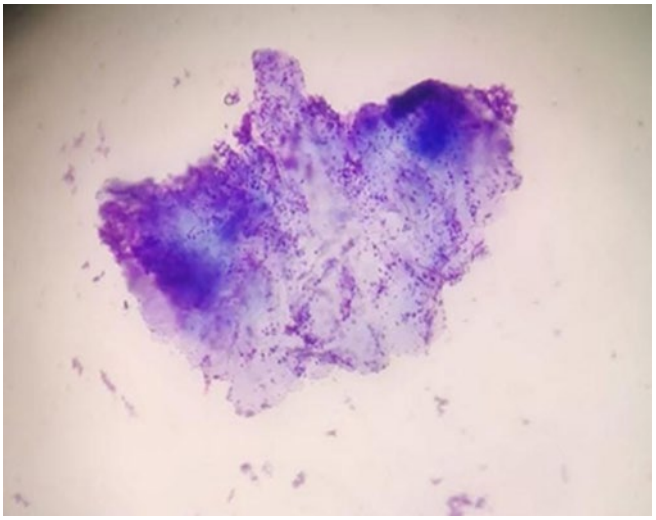

Fig. 5. Keratinocytes and coccoid bacteria in a smear obtained from an imprint (MGG stain, Ob. 20)

Chronic sinusitis can escalate, affecting adjacent jaw muscles. In these cases, birds suffer from necrotizing myositis, perineuritis, and osteomyelitis, which impact the jaw muscles and cranial bones. Macroscopic lesions include nasal discharge, emaciation, sneezing, swelling of the infraorbital sinus, refusal to feed, corneal oedema and conjunctivitis, and, in some cases, by extension airsacculitis and pneumonia. Post-mortem examinations often reveal caseous material in the sinus and airsacculitis. Traditionally, such conditions were thought to be linked to a Vitamin A deficiency in the bird's diet, indicating a nutritional factor in the onset and progression of the disease (1, 22, 23).

Notably, in cases of acute, but most frequently chronic inflammation of the nasal cavities, densified material, sometimes mineralized, is formed, known as choanaliths or rhinoliths. In the present study, 2 out of 4 cases presented tissue loss associated with mechanical or spontaneous opening of the nasal cavities and elimination of these structures (Fig. 6).

Particularly, one adult male, *Melopsittacus undulatus,* was diagnosed with acute rhinitis during necropsy, while previous external examination revealed only tissue loss and enlarged choanal orifice, proving the importance of careful macroscopic examination of the cere and the permeability of choanal orifices in this species.

Fig. 6. Tissue loss following necrosis in the left cere and the outer layers of the beak

One male exhibited chronic inflammation resulting in the loss of the cere substrate, replaced by a caseous material, and a female displayed acute necrotizing inflammation resulting in the loss of the cere and local congestion/hemorrhage.

One conformational abnormality of the upper beak valve manifested as

upwards curving was diagnosed in an adult *Melopsittacus undulatus* associated with emaciation and serous atrophy at necropsy examination. The bird had a congenital anomaly of the upper valve of the beak, by curving upwards. Macroscopically, the structure of the upper valve showed an irregular, continuously increasing shape, and elastic-case consistency of lower than normal hardness. This developmental disorder was compatible with life, with the bird reaching the age of 8 years, requiring only periodic care of the upper valve by clipping to avoid lesions in the skin area above the nostrils. The anamnesis, coming directly from the breeder, made it possible to know that the parrot showed the lesion from the moment of hatching. Such deformities are described in the literature as congenital or acquired, and these types are determined on the basis of anamnesis and species, with congenital being described in *Melopsittacus undulatus* (17). The level of curvature or size varies from individual to individual, as does their survivability (16). This lesion requires differential diagnosis with the protrusion on the upper valve of the bird chick immediately after hatching, which is a normal feature, and with changes in beak valve position that may occur as a result of force feeding by humans, especially in large parrots for which early taming is desired (16). In the case of the specimen diagnosed with this anomaly, repeated clinical and macroscopic examinations demonstrated the bird's ability to adapt, without requiring special care, except for periodic beak trimming The necropsy examination performed at the time of passing revealed a reduced body mass of only 20g (normal: 30-40g) resulting from obvious muscle wasting and moderate dehydration (dry, white, slightly wrinkled appearance of skin) (Fig. 7). Also, severe pulmonary edema was identified, with white-pink frothiness on the trachea and on evisceration and sectioning of the lung, which led to the diagnosis of death by acute respiratory failure. A very small amount of seeds was revealed in the digestive tube, signifying a lack of nutrition approximately 6-8 hours before passing. No other lesions or abnormalities were identified in this individual. In this context, the developmental disorder identified could only be correlated with muscle wasting, but taking into account the age reached of 8 years, which is considered the average age of survival for this species, it is considered not to have contributed decisively to the lesional evolution.

Fig. 7. Beak anomaly - upwards curvature of the beak valve with decreased consistency and elongated form and amyotrophy of pectoral muscle

Conclusions

Beak and ceroma pathologies in budgerigars can include multiple causes and a careful examination associated with complementary tests helps with determining the underlying pathologies.

Most frequent lesions in the cere and beak of budgerigars include inflammatory processes that result in tissue loss with chronicization.

Progressive hyperkeratosis in *Melopsittacus undulatus*, particularly in the perioral region such as the cere and lateral sides of the beak, is often parasitic, commonly attributed to *Cnemidocoptes pilae*, and a negative cytopathologic result does not rule out the need for a therapeutic response diagnosis based on the macroscopic appearance.

Beak anomalies are not necessarily life threatening conditions, but, over time, they can influence the quality of life of budgerigars and lead to emaciation due to feeding difficulties.

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ANATOMO-SURGICAL APPROACH OF TIBIOTARSAL BONE FRACTURE IN DOMESTIC PIGEON (*COLUMBA LIVIA*): A CASE REPORT OF A 5-MONTHS-OLD DOMESTIC PIGEON

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Summary

A domestic pigeon (*Columba livia*) weighing 350 grams with a history of a left pelvic limb stuck in the cage was presented at the clinic for consultation. On presentation, the bird was unable to bear weight on the affected limb. A crepitus was felt on palpation at the tibiotarsal region during physical examination. A radiographic examination confirmed a simple, transverse, distal-diaphyseal fracture of the left tibiotarsal bone. The bird was anesthetized with xylazine and ketamine. Surgery was performed by the introduction of a sterile hypodermic needle intramedullary from distal epiphysis to the proximal extremity of the tibiotarsus for cooptation of the fracture fragments. The needle has been fitted with an intravenous catheter plug to prevent blood leakage and ascending infection. To prevent rotation of the distal extremity of the pelvic limb, a plaster cast was used. The plaster cast serves in one hand for stabilizing the needle intramedullary and in another hand for external fixation of the fracture. The movements were restricted by placing the pigeon in a cage, for six weeks. The postoperative follow-up radiograph after six weeks shows a complete union of fractured ends. The bird recovered uneventfully with complete weight bearing on the affected limb and the intramedullary needle and plaster cast were removed at 6 weeks after surgery. To prevent infections and inflammation, the bird received antimicrobial and antiinflammatory medication for seven days. The intramedullary sterile hypodermic needle in association with a plaster cast under xylazine and ketamine anesthesia is a safe technique for the repair of tibiotarsal fractures in pigeons.
Keywords: avian, pigeon, anatomy, orthopedic.

The rock pigeon, or common pigeon (*Columba livia*) is a member of the bird family Columbidae (doves and pigeons). It is the only family in the order Columbiformes. Among the many representatives of the Columbiformes, the rock dove *Columba livia* is the most universally kept by humans on all continents. It has been domesticated and taken around the world, raised for food, trained for homing, racing, and carrying messages, and used in research (23, 24, 25).

Bone fractures are common in both wild and domestic birds being the cause of car accident, gunshots, fight with other birds, and animal attacks (24).

Materials and methods

A domestic pigeon (*Columba livia*) weighing 350 grams with a history of a left pelvic limb stuck in a cage was presented at the clinic for consultation. On presentation, the bird was unable to bear weight on the affected limb. A crepitus was

felt on palpation at the tibiotarsal region during physical examination. For certain diagnostics, a digital radiograph was used and as a result, the pigeon had a simple, transverse, distal-diaphyseal fracture of the left tibiotarsal bone (Fig. 1) (2, 7, 9, 11, 15, 17, 18, 21).

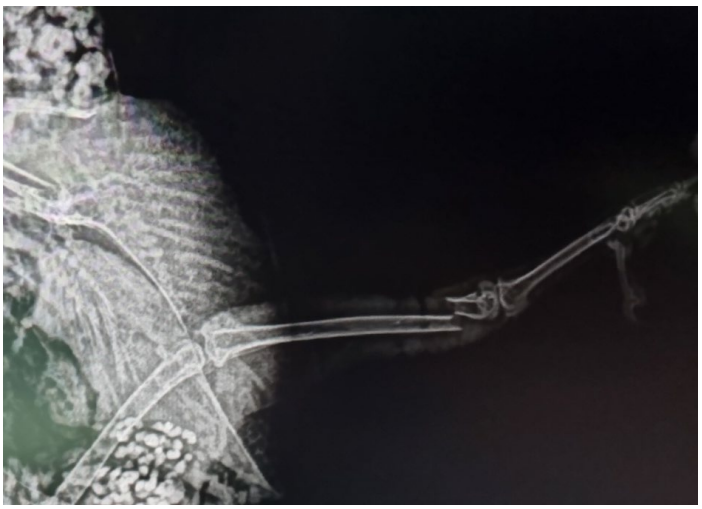

Fig. 1. Lateral radiograph of the left hindlimb of the patient that reveals the tibiotarsal fracture (original)

After examination, the bird received antiinflammatory and antimicrobial medication represented by meloxicam subcutaneously in a dose of 2 mg/kg and enrofloxacin orally in a dose of 15 mg/kg (4, 5, 6, 7, 8, 9, 20).

To perform the surgical procedure the bird was anesthetized with a combination of xylazine and ketamine. Xylazine was administrated intramuscularly in the dose of 5mg/kg, followed after ten minutes by the ketamine, also intramuscularly, in the dose of 40 mg/kg (4, 5, 6, 7, 8, 9).

Before performing the surgery, the affected limb was prepared for the standard orthopedic procedure, and consisted of deplumation and disinfection of the tegument. The deplumation is characterized by avulsion of the feathers at the level of the tibiotarsal region and then this region and distal extremity of the limb was antiseptized with a 10% iodine solution (1, 3, 5, 7, 9, 10, 12, 13, 14, 16, 17, 19, 24).

During the procedure, the intramedullary pin was replaced by a sterile hypodermic needle 18 gauge. Before the surgery, the distal epiphysis of the tibiotarsal bone was fixed with a bone fixator to favor the insertion of the needle intramedullary from the distal extremity to the proximal extremity of the bone (Fig. 2A). The needle was inserted gently with the needlepoint orientated proximally, the needle penetrated the skin of the distal extremity of the tibiotarsus and then, passed

the medullary canal from distal to proximal direction (Fig. 2B). The needle served for the cooptation of the fracture fragments (Fig. 2C). The needle has been fitted with an intravenous catheter plug to prevent blood leakage and ascending infection (1, 3, 5, 7, 9, 10, 12, 13, 14, 16, 17, 19, 24).

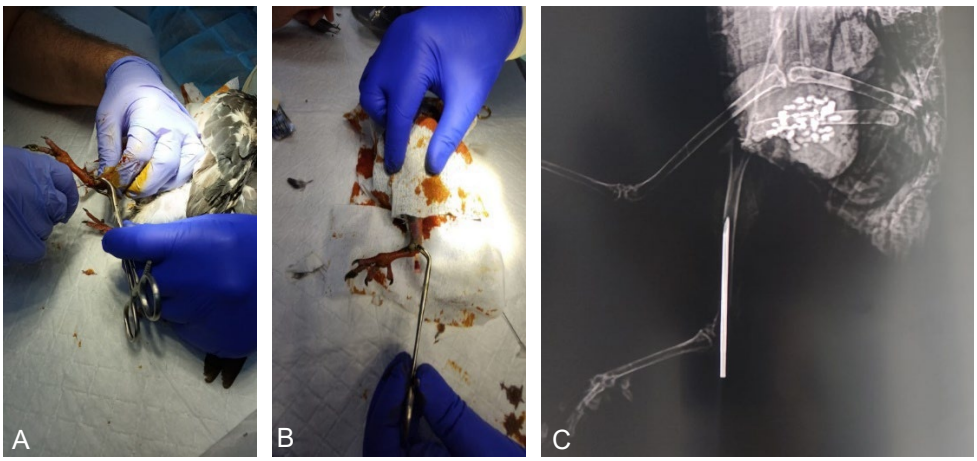

Fig. 2. Intramedullary needle insertion and radiographic imaging: A. Intramedullary needle insertion (original), B. Final aspect of the intramedullary needle insertion (original), C. Lateral radiograph of the affected pelvic limb after intramedullary needle insertion before applied plaster cast (original)

To prevent rotation of the distal extremity of the pelvic limb and the exteriorization of the needle within the medullary canal a plaster cast was used. After the insertion of the intramedullary needle, the limb was bandaged with cast padding (Fig. 3.). The plaster cast was applied latero-lateral on the tibiotarsus and metatarsus by two layers kept in tension with helping two hemostatic forceps one applied cranially and another one caudally to the bone. The hemostatic forceps were removed after the plaster had dried and then, the cranial and caudal margins of the plaster cast were wet and remodeled on the limb (Fig. 4.) (1, 3, 5, 7, 9, 10, 12, 13, 14, 16, 17, 19, 24).

Fig. 3. Cast padding bandage application on the limb (original)

Fig. 4. Final aspect of the limb with the plaster cast (original)

The plaster cast serves in one hand for stabilizing the needle intramedullary and in another hand for external fixation of the fracture. After surgery, the pigeon was placed in a cage for six weeks to restrict movements and to protect it from harmful environmental factors. After six weeks a control radiograph was performed to check up on callus formation, resulting in consecutive bone healing. Because the control radiograph showed the callus formation, the plaster cast and needle were removed under general anesthesia with the same substances in the same doses as in general anesthesia. After the external and internal fixators had been removed, the pigeon was placed in the same cage for another three weeks for protection (Fig. 5B) and then, the bird was externed (Fig. 5C).

Fig. 5. Postoperative evolution of the patient: A. The patient in the first day after surgery (original), B. At six weeks after surgery, the external and internal fixators were removed (original), C. The patient at nine weeks after surgery (original)

Nine weeks after surgery another control radiograph to check up on bone healing was done (Fig. 6.).

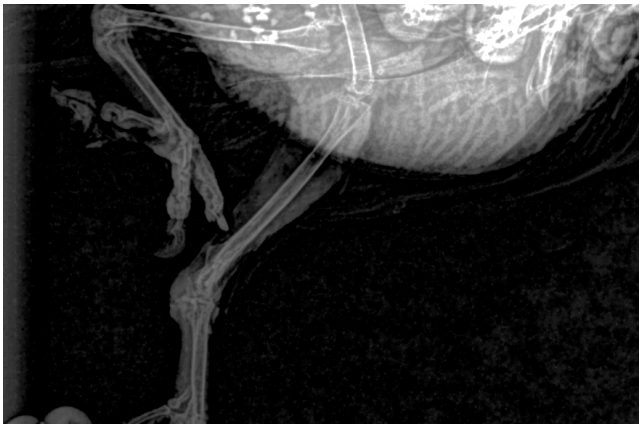

Fig. 6. Lateral radiograph of the left hindlimb of the pigeon that reveal the callus formation at three weeks after removed the internal and external fixators (original)

Results and discussions

This combination of internal and external fixation prevented rotation of the distal extremity of the hindlimb and ensured the stability of the bone fragment.

To prevent ascending infections, the pigeon received antimicrobial medication immediately after surgery for 7 days and another seven days after the plaster cast and needle were removed. The antibiotic medication was represented by the oral suspension of enrofloxacin in a dose of 15 mg/kg.

The pigeon steps on the affected limb from the first day after surgery.

The major part of the callus tissue during healing comes from the periosteal surface, and the blood supply to the periosteum from surrounding soft tissues is very important the intramedullary circulation appears to be of less significance in avian bone healing than in mammals explaining the healing in birds is faster than in mammals (21, 22, 24).

Internal fixation combined with external coaptation is also required to control rotation forces in birds (7, 9, 21, 24).

Conclusions

The combination of internal and external fixation of the tibiotarsus in the pigeon provided good stability, did not affect the movement of the bird, the peripheral blood circulation was not affected, and the callus formation was not excessive (*vicious callus*) but safe.

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THE NUTRITIONAL VALUES DERIVED FROM RED DEER MEAT ORIGINATING FROM THE WILD AND THAT FROM FARMS A REVIEW

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Summary

The consumption of red deer meat has been steadily increasing due to its perceived health benefits. In response to the high demand of red deer meat, farmers have adapted by initiating the farming of this species. This review aims to present the difference and nutritional value between wild-harvested red deer meat and farm-raised deer meat.Due to high market demand and the inability of game-sourced venison to meet it, this has led to the emergence of red deer meat farms.

Keywords: meat, red meat, red deer meat, nutritional value.

Due to the global population growth, the demand for meat has substantially increased (9, 19). In the last 50 years, meat consumption per capita has risen from 23 kilograms per year in 1961 to 42 kilograms in 2011. As a result, the demand for meat, particularly red deer meat, has significantly grown (6, 12, 13). With the increased consumption of red meat, there has also been a rise in the demand for red game meat, specifically red deer meat. When we think of red deer meat, we typically consider two sources of production: one from wild games and the other from farms. Despite evidence showing that the nutritional value of wild-harvested red deer meat is superior to farmed red deer meat, this has not deterred consumers from increasing demand in both areas (14, 15, 16). Game meat is clean and flavored and serves as a rich nutrient source, especially for obese or elderly individuals. It is high protein, low in fat, which positively impacts health (8, 12).

On primary example of red dear meat production is New Zealand, which leads the world in farm-raised red deer meat. Spain, ranking second, export approximately 11.250 tons of exclusively wild-harvested red deer meat (1, 3, 7, 17).

However, despite its nutritional richness, red deer meat often presents a significant deficiency in copper (Cu). Consequently, farmed red deer receive copper-rich supplements as it improves new born growth, leading to increased profits (4, 5). Due to this practice, the European Union has established a safety level for copper content in meat, specifically 5mg/kg of fresh meat (20).

Another critical factor influencing meat quality is tenderness, which is determined by the composition of connective tissue, muscle location and physical activity (10, 11, 13, 16).

According to a study conducted in two countries with distinct methods of

acquiring red deer meat and during different seasons, significant differences were observed (Table 1).

Table 1

Effects of country of origin (Spain vs. New Zealand) and hunting type most commonly used in Spain (stressful-winter vs. stalking summer) on quality traits and chemical composition of red deer meat (2, 5, 8, 17)

Effect	Country of origin (OR)				Hunting type (HT)			
Items	Spain	New Zealand	SEM	P. value	Stressful-winter	Stalking-summer	SEM	P. Value
pH	5.67	5.68	0.027	Ns	5.78	5.55	0.037	***
Colour traits								
Lightness	35.3	29.1	0.82	***	34.8	35.7	0.84	ns
Redness	15.4	14.6	0.42	ns	14.1	16.6	0.67	0.07
Yellowness	11.8	8.9	0,45	***	10.9	12.7	0.53	0.08
Chroma	19.5	17.1	0,54	*	17.9	21.0	0,78	0.05
Hue angle	0.66	0.55	0.017	***	0.66	0.65	0.019	ns
Chemical composition								
Moisture (%)	75.2	73.5	0.28	**	76.5	73.8	0,39	***
Protein (%)	22.7	24.1	0.20	***	21.9	23.5	0.26	***
Fat (%)	0.51	0.75	0.087	ns	0.11	0.90	0.137	***
Ash (%)	1.18	1.33	0.026	**	1.07	1.28	0.035	***
Cholesterol (mg/100g)	41.1	41.8	1.59	ns	40.5	41.6	2.18	ns
Cooking losses (%)	24.9	23.9	0.77	ns	20.5	27.4	1.19	***
Shear force	42.9	25.2	2.39	***	46.5	39.3	2.34	ns

Quality traits and chemical composition of red deer meat: Not significant(P>0.10); *P<0.05; **P<0.01; ***P<0.001

Another study on essential amino acids reveals that the most dominant amino acid is Lysine, followed by Arginine and Leucine, these amino acids collectively represent approximately 50% of the total essential amino acids. In contrast, Methionine has the lowest value, accounting for around 2,4% of the total essential amino acids (Table 2).

Regarding non-essential amino acids, Glutamic acid, Aspartic acid and Alanine were the primary non-essential amino acids observed, constituting approximately 71% of the total non-essential amino acids.

Table 2

Effects of country of origin (Spain vs. New Zealand) and hunting type most commonly used in Spain (stressful-winter vs. stalking summer) on amino acids (AA) profile of red deer meat (expressed as mg/100 g of sample) (11, 17)

Effect	**Country of origin (OR)**				**Hunting type (HT)**			
Item	Spain	New Zealand	Sem	P value	Stressful-winter	Stalking-summer	Sem	P value
Essential AA								
Histidine	883	427	54.9	***	773	993	34.8	***
Threonine	1.081	1.151	16.9	*	1.077	1.084	20.4	ns
Valine	1.278	1.159	29.1	*	1.184	1.371	39.2	*
Methionine	295	295	6.4	ns	278	312	8.9	0.05
Lysine	4.315	2.081	53.8	ns	2.023	2.292	74.4	0.07
Isoleucine	1.216	1.025	29.2	***	1.170	1.261	31.3	ns
Leucine	2.043	1.778	45.1	***	1.992	2.094	51.8	ns
Phenylalanine	1.086	936	20.9	***	1.048	1.123	21.0	0.08
Tyrosine	834	602	25.5	***	839	828	15.2	ns
Arginine	2.072	1.833	40.3	**	2.179	1.964	51.9	*
Non-essential AA								
Aspartic acid	2.084	1.414	79.0	***	2.061	2.107	57.2	ns
Serine	883	1.015	24.0	**	800	966	30.7	**
Glutamic acid	3.635	3.324	79.9	0.07	3.746	3.523	101.6	ns
Glycine	987	886	19.1	**	978	996	22.9	ns
Alanine	1.299	1.213	28.4	ns	1.375	1.223	39.6	0.05
Proline	906	810	19.0	*	954	857	25.7	0.06
Total AA	22.734	19.951	480.6	**	22.476	22.992	537.3	ns
Essential AA	12.941	11.287	276.6	***	12.562	13.320	304.1	ns
Non-essential AA	9.793	8.664	211.6	**	9.914	9.672	250.4	ns
Essential/non-essential AA	1.33	1.30	0.011	ns	1.27	1.38	0.017	***

Quality traits and chemical composition of red deer meat: Not significant(P>0.10); *P<0.05; **P<0.01; ***P<0.001

Furthermore, in terms of minerals, it has been identified that primary mineral is Potassium, followed by Phosphorus. Additionally, significant quantities of Zinc and Iron have also been discovered among the minerals (Table 3).

Table 3

Effects of country of origin (Spain vs. New Zealand) and hunting type most commonly used in Spain (stressful-winter vs. stalking summer) on mineral content of red deer meat (17, 19)

Effect	Country of origin (OR)				Hunting type (HT)			
Item	Spain	New Zealand	SEM	P. Value	Stresful -winter	Stalking-summer	SEM	P value
Macrominerals, g/kg								
Calcium	0.038	0.038	0.0011	ns	0.044	0.032	0.0018	***
Potassium	2.97	3.65	0.085	*	3.03	2.90	0.062	ns
Magnesium	0.22	0.37	0.017	***	0.20	0.24	0.0056	*
Sodium	1.25	1.20	0.043	ns	1.43	1.06	0.063	***
Phosphorus	2.14	2.29	0.040	ns	1.93	2.34	0.061	***
Trace-minerals, mg/kg								
Iron	29.5	34.1	1.06	*	31.9	27.0	1.18	*
Manganese	0.14	0.005	0.17	**	0.15	0.13	0.005	ns
Zinc	39.2	20.1	3.28	**	56.4	22.0	4.80	**
Copper	1.45	2.04	0.082	***	1.34	1.56	0.074	ns

Quality traits and chemical composition of red deer meat: Not significant(P>0.10); *P<0.05; **P<0.01; ***P<0.001

Another significant study was conducted in Poland, where the analysis of metals in red deer meat from the Northeast and Southwest regions of the country was undertaken. This study focused on red deer meat from farms, and the results are presented in Table 4 (18).

As evident from the analysis of metals in red deer meat, tha values area within normal limits and do not pose a danger to consumers.

The supplementation of Cu to farmed red deer meat does not alter the meat quality and does not significantly affect the Cu levels in the meat.

From the top 2 countries in the red meat market we can say that both have exceptional nutrition’s value but the wild-harvested deer meat consistently exhibits higher nutritional value compared to farm-raised deer meat, regardless of the season in which the meat was harvested.

Table 4

Correlation between the concentration of chosen metals in the muscles of red deer according to the geographical location (18)

Region South-Western (1)		Sr	Cs	Ba	Pb	Cu	Zn
	Cs	0.0673	-	-	-	-	-
	Ba	0.0156	0.4059	-	-	-	-
	Pb	-0.1405	0.4533	0.4531	-	-	-
	Cu	0.2198	0.0616	0.2430	0.0765	-	-
	Zn	-0.1315	-0.0869	-0.1740	-0.0138	0.0277	-
	Rb	0.2311	-0.0663	-0.0849	-0.1491	0.4210	0.0327
Region North-Estern (2)	Cs	-0.1383	-	-	-	-	-
	Ba	0.5549	-0.1371	-	-	-	-
	Pb	0.4675	0.5139	0.5301	-	-	-
	Cu	0,0871	0,2241	0,2010	0,4248	-	-
	Zn	-0.0975	0.1407	0.1200	0.1304	0.6579	-
	Rb	-0.1788	0.6091	-0.3591	0.1375	0.2761	0.3884

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DIAGNOSTIC METHODS IN THE MOST COMMON BACTERIAL AND PARASITIC TICK-BORNE DISEASES OF WILD BOAR IN EUROPE – MINI REVIEW

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Summary

Ticks are important ectoparasitic arthropods, responsible for substantial economic losses resulting from the direct or indirect effect exerted on their hosts. They are obligate parasites that feed on blood and they serve as vectors for pathogens of domestic and wild animals being second in this category only to mosquitoes. They transmit pathogens such as *Anaplasma* spp., *Babesia* spp., *Ehrlichia* spp. etc. Clinical signs are often non-specific and can easily be confused with other pathologies. Identification of the pathogen can be done by direct methods and by molecular and serological methods. The aim of this study was to assess the common methods of diagnosis of tick-borne diseases in wild boar in Europe listed in reference literature. Information on the occurrence and prevalence of tick-borne diseases is difficult to assess and evaluate due to limited information and difficulties in comparing results from studies with different designs and purposes and due to the employment of different diagnostic tools. The sensitivity of microscopic examination of blood smears is low, so the probability of false negative results is also present. In conclusion, commonly used methods to diagnose pathogens in wild boar blood are PCR and ELISA.

Keywords: Ticks, *Anaplasma* spp., *Babesia* spp., *Ehrlichia* spp., wild boar.

The number of tick-borne pathogens (TBPs) and the incidence of tick-borne diseases are rising globally as a result of multifaceted global changes. Ticks are the most significant vectors of disease transmission to livestock, pets, and humans (33). As a result, diseases spread by ticks to humans are becoming more common and pose a serious threat to public health (15, 41, 48). Environmental factors, such as host array and abundance, influence tick ecology and the epidemiology of TBPs (32; 54). The likelihood of ticks finding a suitable host, completing their life cycle, and multiplying increases with host density (18, 54). Because they can act as reservoirs for human pathogens and increase the range and abundance of ticks, wildlife can therefore play a significant role in the epidemiology of TBPs (12, 72). Furthermore, we face new epidemiological scenarios where zoonotic pathogens can spread due to the rise in human-wildlife interactions in densely populated areas (7, 10, 19).

This study assessed three diseases: ehrlichiosis, anaplasmosis, and babesiosis. The data used in the analysis came from academic publications that were published in international journals.

Anaplasmosis is a zoonotic disease spread by infected hard ticks (*Ixodidae*) (75). Rodents, equines, deer, and other mammals are among the food sources that hard ticks survive on. The white-tailed deer and the white-footed mouse are the two main reservoir species (3).

Anaplasma species are known as both human and veterinary pathogens; however, *A. phagocytophilum* is the primary species that infects humans. Thieler, 1910, first identified *Anaplasma marginale* as the etiological agent of a devastating pathogen of cattle in 1910 (75). *A. phagocytophilum* was first discovered in humans in 1994 and was thought to be a new *Ehrlichia* species found in neutrophils, which garnered it the name *Ehrlichia phagocytophilum*, the causative agent of human granulocytic ehrlichiosis (HGE) (4, 40). The bacteria resembled *Ehrlichia equi*, which also has tropism for neutrophils and is an equine pathogen. In 2001, *E. phagocytophilum* was officially renamed *Anaplasma phagocytophilum*.

Ticks belonging to the genera *Dermacentor, Haemaphysalis, Hyalomma*, and *Rhipicephalus* are the carriers of babesiosis, which is caused by *Babesia* species. Severe forms of the illness can be fatal and cause anaemia, jaundice, and hemoglobinuria (53).

Dr. Victor Babeș, a Romanian physician, noticed microorganisms in the erythrocytes of sheep and cattle that had hemoglobinuria towards the end of the 1800s. The genus name *Babesia* was given to these microorganisms in honour of their discoverer, and they were subsequently designated *Babesia bovis* and *Babesia ovis*, respectively (71). The first report of *Babesia* spp. infection in dogs was published in Italy in 1895, not long after these observations in ruminants (56). Today, these protozoan illnesses are spread worldwide (5, 63).

It was established that a wild boar population in the same region did not harbour the etiological agent responsible for swine babesiosis in domestic pigs, as reported in Sardinia, Italy (80). *B. trautmanni* and *B. perroncitoi*, the only two known *Babesia* species that infect pigs (including wild boar), were primarily identified in the 1990s based solely on morphology and without the use of molecular data (49).

According to epidemiological estimates, it is seasonal, zonal, and enzootic. The disease mostly affects pastures when animal tick attacks are at their peak. Ticks on pasture and the potential addition of new animals to the herd are additional epidemiological factors to consider.

A significant veterinary hazard and potential hazard to humans is posed by piroplasmids.

Obligate intracellular bacterial pathogens of the species *Ehrlichia* belonging to the *Anaplasmataceae* family in the order *Rickttesiales* parasitize a wide variety of mammalian reservoir hosts and are responsible for several emerging human infectious diseases (46, 70).

Ehrlichia and *Anaplasma* are related genera of the family *Anaplasmataceae*, which also includes *Neorickettsia* and *Wolbachia* (16). Among the related bacteria that appear to infect humans, *E. chaffeensis* is more pathogenic than *A. phagocytophilum*, *E. ewingii*, *E. muris*, and *E. canis* (8, 43, 51).

The ability of these bacteria to replicate within two hosts—a mammalian host and a tick vector—as well as their ability to plan extremely complex and sophisticated survival strategies, are characteristics that set them apart from other bacteria (55).

Certain species are linked to various host reservoirs; deer, for instance, are considered significant reservoir hosts for *E. chaffeensis* (67). The bite of an infected tick can spread these bacteria (55).

The European wild boar population is devoid of the *B. burgdorferi sensu lato* bacteria that cause Lyme borreliosis, as well as other tick-borne pathogens like *Francisella* spp., *Rickettsia* spp., and *Neoehrlichia* spp. (35, 50, 61, 62).

Materials and methods

A systematic multi-stage search of PubMed Central® (PMC) and Web of Science (WOS) databases was conducted to identify all eligible studies for the purpose of this paper.

The keywords, “*Babesia* wild boar”, “tick disease ELISA”, “wild boar ELISA”, “ehrlichiosis swine”, “wild boar *Rickettsia* Romania”, “wild boar *Anaplasma*”, “*Anaplasma* ELISA”, “review *Anaplasma*”, “*Babesia* in Europe”, "wild boar PCR", "molecular diagnosis", "anaplasmosis", "wild boar blood smear" were entered. Articles were selected from scientific papers that have the subject of diagnosis by molecular methods of Anaplasmosis, Ehrlichiosis, Babesiosis, and also identification of parasites by direct microscopy and serological methods in six countries of Europe (Fig. 1).

After selecting papers based on titles and abstracts, the full text of the studies was thoroughly analysed. Articles that were included in the study had to meet all of the following criteria:

- study conducted over the last decade;
- original research articles based on molecular diagnostic techniques, direct microscopy and serological methods;
- the diagnostic method must be specified.

The research resulted in 88 articles, which were subsequently checked to determine whether they met all the proposed criteria as well as to eliminate duplicates. Six publications were considered eligible after screening of the selected studies (Fig. 2).

Fig. 1. Countries where the studies were conducted

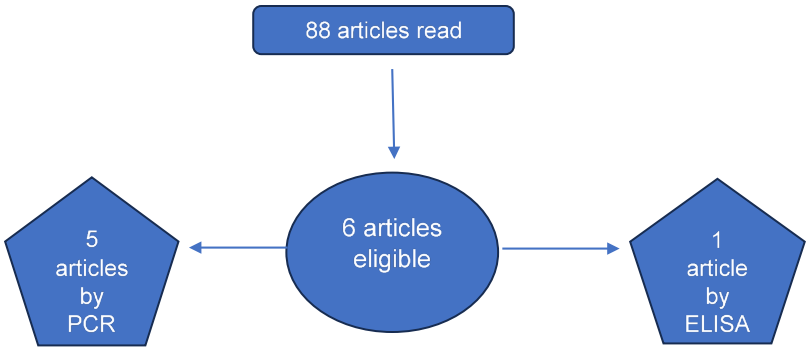

Fig. 2. Selection process of studies by methods of diagnostic

Results and discussions

Finding the most widely used diagnostic technique with the best specificity, sensitivity, and least amount of restrictions was one of the key goals of our analysis. Finding carrier animals is crucial to the diagnostic process because they pose a danger of transmission and serve as an illness reservoir for ticks and other animals (20).

The sensitivity of blood smear microscopic examination is low, so false negative results are regularly observed (74). This is especially encountered if the operator does not possess extensive experience to perform a correct diagnosis (6). The most important feature is that this method is only useful in detecting infected erythrocytes in the acute phase of the disease.

Microscopic examination of blood smears stained using the Giemsa method or with a fluorescent dye, such as acridine orange is a classical method of identifying the pathogen in infected animals. Smears are examined using immersion oil, at 100-x magnification. The detection sensitivity of the blood smear technique is below 1 parasite in 10^{-6} red blood cells (2).

It is efficient for identifying acute infections, but not for identifying carriers with very low parasiteamia. No evidence of piroplasmids was found in wild boar populations from Hungary, Slovakia, Germany, or Portugal, according to molecular studies conducted throughout Europe that focus on the 18S rRNA gene (Table 1) (26, 35, 50, 61). When considering piroplasmids, multiple reports from Italy or Portugal have failed to identify *Theileria* sp. in wild boar (50, 65, 77). There is however, an Italian report on *B. bigemina* (77).

Following the manufacturer's instructions, a commercial kit (QIAampDNA Blood & Tissue; Qiagen, Hilden, Germany) was used to extract nucleic acid from a 200 µL homogenized wild boar blood sample. *Theileria* spp. in Italy, was directly detected using a semi-nested PCR protocol that targets the V4 hyper-variable region of the 18S ribosomal RNA gene (60).

In the first round, Promega PCR Master Mix (Promega Corporation, WI, United States), 20pM of each primer, and approximately 100 ng of DNA template measured with the Biofhotometer plus (Eppendorf, Hamburg, Germany) were used in a final reaction volume of 25 µL. The primers were RLB-F2 (5'-GACACAGGGAGGTAGTGACAAG-3′) and RLB-R2 (5'-CTAAGAATTTCACCTCTGACAGT-3′). The manufacturer's instructions were followed (36, 77).

Most studies from the Czech Republic focus on viral tick-borne pathogens rather than parasitic ones (28, 29).

Anaplasma phagocytophilum was discovered using nested PCR amplification of 407 bp of the groEL gene. Using the technique for positive samples, the 1297 nt of the GroESL operon was amplified. By concentrating on the variable region of the 18S rRNA gene, piroplasmids were found utilizing the extremely sensitive nested PCR approach. To visualize the PCR results on a 1.5% agarose

gel, Nippon Genetics Europe, Germany's Midori Green Advance was employed. After utilizing the Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd., Taiwan) to purify products of the desired size, the products were sequenced using Macrogen capillary sequencing services (Macrogen Europe, the Netherlands) and amplification primers (21, 36).

Table 1

Specific primers for each method

No	Country	Gene	Primers Name	Primers (5' -> 3')	Ref.	Database
1	**ITALY**	18S rRNA	RLB-F2	**GACACAGGGAGGTAGTGACA AG**	(60)	**WOS**
			RLB-R2	**CTAAGAATTTCACCTCTGACA GT**		
2	**CZECH REP.**	groEL	ApNest-F	**GTGGAATTTGAAAATCCATAC**	(31)	**PUBMED**
			ApNest-R	**GTCCTGCTAGCTATGCTTTC**		
3	**HUNGARY**	groEL	EphplGro EL(569)F	**ATG GTA TGC AGT TTG ATC GC**	(25)	**PUBMED**
		groEL	EphGroEL (1142)R	**TTG AGT ACA GCA ACA CCA CCG GAA**		
4	**PORTUGAL**	16S rRNA	cytB1-F	**GGTACCYACAGAAGAAGTCC**	(22)	**PUBMED**
			cytB1-R	**TAGCACTCATCGTTTACAGC**		
5	**ROMANIA**	18S rRNA	BJ1	**GTCTTGTAATTGGAATGATGG**	(27)	**WOS**
			BN2	**TAGTTTATGGTTAGGACTACG**		
6	**SLOVENIA**	ELISA	ELISA kit	**CHEKIT-CSF-SERO**	(73)	**WOS**

Dermacentor and *Haemaphysalis* spp. adults, as well as nymphs and *Haemaphysalis concinna* and *I. ricinus* larvae (n = 253) were included in molecular analyses conducted in Hungary (11, 25).

To investigate the genetic diversity of *A. phagocytophilum*, amplification of an approx. 600-bp-long fragment of the heat shock chaperonin (GroEL) gene was also attempted from all real-time PCR positive samples (1). The primers EphplGroEL(569)F (5′-ATG GTA TGC AGT TTG ATC GC-3′) and EphGroEL(1142)R (5′-TTG AGT ACA GCA ACA CCA CCG GAA-3′) were used in a reaction volume of 25 µl, which included 5 µl of extracted DNA.

Samples were screened for the presence of piroplasmids using conventional PCR modified by Casati et al. (9). The primers BJ1 (forward: 5′-GTC TTG TAA TTG GAA TGA TGG-3′) and BN2 (reverse: 5′-TAG TTT ATG GTT AGG ACT ACG-3′) were used to amplify an approximately 500-bp-portion of the 18S rRNA gene of *Babesia*/*Theileria* spp. (37).

A total of 141 free-ranging ungulates, including 73 red deer (*Cervus elaphus*), 65 wild boars (*Sus scrofa*), and 3 fallow deer (*Dama dama*) from both sexes, were sampled in the districts of Castelo Branco (n = 31), Portalegre (n = 16), Lisboa (n = 19), Évora (n = 15), and Beja (n = 60), Portugal, during the hunting

seasons, from December 2013 to March 2015. Before DNA extraction, samples were collected and stored at -20 °C (50).

The acquired blood samples were subjected to DNA extraction using a commercial kit (PCR-template Preparation kit, Roche Diagnostics GmbH, Germany), according to the manufacturer's instructions.

For PCR extraction, in order to avoid false-negative results, cyt-b specific primers (cytB1-F and cytB2-R) were used to amplify a 350 bp segment of the host mitochondrial cytochrome b gene (cyt-b) (39).

Detection of *Anaplasma*/*Ehrlichia* spp., *A. marginale*/*A. ovis*, *A. phagocytophilum*, *Babesia*/*Theileria* spp., *B. burgdorferi* (s.l.) and *Rickettsia* spp. DNA in blood samples was assessed by PCR, according to previously described protocols. (34, 66).

Most Slovenian studies also focus on viral tick borne pathogens such as the classical swine fever virus (73).

In Romania, samples were collected throughout three hunting seasons in 2019 and 2022. From the carcasses of wild boars, ticks were removed following inspection and blood samples were collected. In a particular hunting area from Sălaj county, a total of 54 boar (*Sus scrofa attila*) blood samples were obtained throughout two hunting seasons (2019–2020 and 2020–2021). All of the animals that were hunted during the chosen hunting seasons were represented by these samples (42).

Genomic DNA was obtained independently from each sample using the ISOLATE II Genomic DNA Kit (Meridian Bioscience, Newtown, OH, USA).

The DNA was evaluated by conventional and nested PCR amplifying fragments of the 16S rRNA genes of *A. phagocytophilum* and *Ehrlichia* spp., 18S rRNA gene of *Babesia/Theileria* spp. *Anaplasma*-positive samples were further evaluated by semi-nested PCR amplifying fragments of the groEL gene of *A. phagocytophilum* (3% positivity) (42).

The tick-borne pathogen that has been investigated the most in wild boar throughout Europe is *Anaplasma phagocytophilum*. According to several studies (14, 52, 69), the prevalence of wild boar ranges from 0% in Spain and Italy to 28.0% in Slovakia (35).

The presence of *A. phagocytophilum* has also been documented in Belgium (47), Portugal (50), Slovenia (64), Romania (38), Poland (44), Germany (61), and other countries. These reports are arranged in decreasing order of prevalence, ranging from 1.0% to 12.5%. The comprehensive serological analysis on 224 wild boars in Slovenia revealed a 69.6% *A. phagocytophilum* seroprevalence (78), backed up the idea that wild boar may play a part in the endemic life cycle of this infection.

Due to its genetic polymorphism, *Anaplasma phagocytophilum* is a pathogen with a various range of hosts, clinical manifestations and zoonotic potential among its circulating genetic variations.

A. phagocytophilum is carried by *Ixodes ricinus* ticks, which are found throughout much of Europe and central Asia. *Ixodes persulcatus* ticks have a

distribution that extends further into Asia and coincides with that of *I. ricinus* ticks (17, 40, 57). Milder winters have been linked to the northward spread of *I. ricinus* ticks in Sweden, Russia, and other regions of northern Europe (Fig. 3).

Ticks may benefit from this climate variation in several ways, including increased host food supplies and improved host survival. Climate change has also been connected to the northern expansion of I. persulcatus (76).

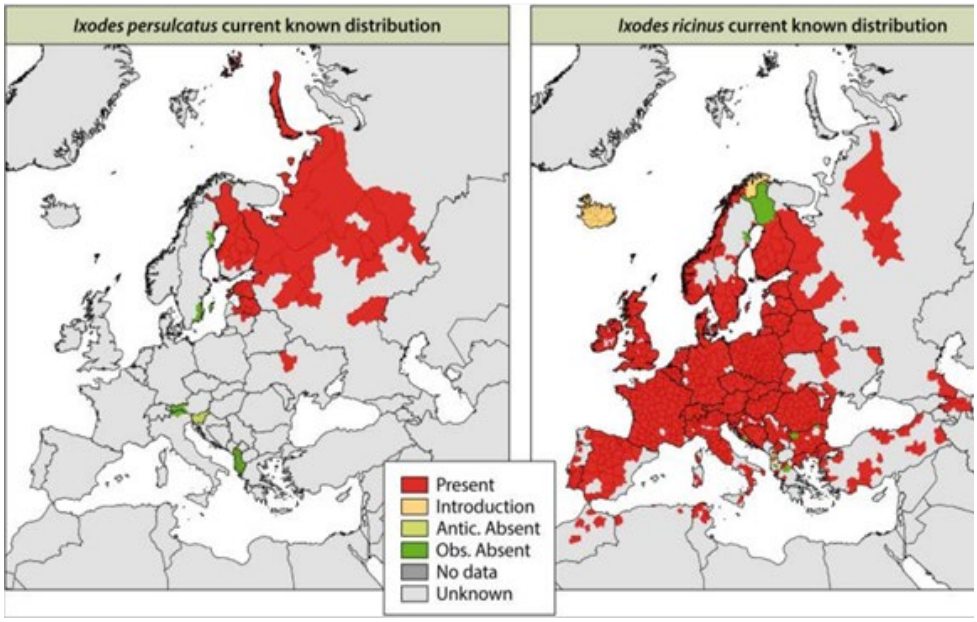

Fig. 3. Areas where *Ixodes* species are endemic are at risk for transmission of Lyme borreliosis, *A. phagocytophilum*, and *Babesia* spp. (81)

Worldwide, piroplasmid infections infecting free-range animals are becoming more and more common and are being recognized as a new tick-borne zoonosis (23, 24, 58).

The danger of acquiring zoonotic diseases has increased as a result of more frequent encounters between humans and wildlife brought on by socioeconomic changes (65). This is particularly true for vector-borne diseases, where the presence of the vector in the region has grown as a result of environmental, climatic, or host species changes (13, 45, 59). *Babesia* is well known for its significant economic effects on human health and on the livestock sector.

Ehrlichia, Anaplasma, Babesia, and *B. burgdorferi* s.l. species have been found in ticks collected from wild boar in the past from countries such as Spain (14,

18) the Czech Republic, Italy, or Germany (61). On the other hand, our negative findings for *C. burnetii* are consistent with earlier research on ticks from wild boars (60). In terms of TBPs identification in wild boar tissues, no reports of *Anaplasma*, *Rickettsia*, or *Babesia* species came from Spanish wild boar tissues, and no reports of *B. burgdorferi* s.l. and *Ehrlichia* species have been made in wild boar tissues to date (35, 50, 61).

Conclusions

Identification of the parasitic agent or infection can be done using direct methods, during the acute phase of the disease, and by molecular and serological methods when in carrier animals the low parasite load makes detection extremely difficult.

Although some diagnostic methods may lack sensitivity and specificity, they are still widely used and useful to support clinical and epidemiological research of all available serological methods, PCR is the technique with the highest sensitivity and specificity, followed by ELISA methods which are suitable for studying the presence in wild boar populations of *Babesia* spp., *Anaplasma* spp., *Ehrlichia* spp., etc.

Molecular diagnostic methods can overcome many of the limitations of other techniques and are essential to identify and distinguish genotypes of pathogens.

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A RARE CASE OF CONJOINED LIZARDS: A CASE STUDY OF TWIN STILLBORN CRESTED GECKOS (*CORRELOPHUS CILIATUS*)

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Summary

Identical twins that maintain their physical connection via head, thorax, abdomen, or limbs are known as conjoined twins. Conjoined human and animal twins have been, for a long time, an interest for the scientific community. These anomalies of congenital duplication can be extremely important in our understanding of embryonic development, mutations and how both of these interact with each other. Literature data is scarce regarding the conjoined reptiles but there is also scarcity in information regarding the mechanism by which these conjoined reptiles are formed. It is well known that, among all clades of vertebrate animals, reptiles are the most prone to such anomalies. Most of the time, oviparous reptile species are designed to produce such eggs, and, implicitly, such abnormal juveniles. Theories that would explain the appearance of this phenomenon accuse aberrant incubation conditions, infections, inbreeding or genetic mutations. Thus, the study of such rare findings in herpetological science can facilitate the understanding of the etiology and pathogenesis of such abnormalities in other fields, like human medicine and animal medicine, as well as providing more information of value to herpetologists and reptile owners or breeders. The pair of conjoined twins described in this case study belongs to the species of Crested Gecko (*Correlophus ciliatus*) and were discovered by a reptile breeder from Cluj-Napoca, Romania. On a visual inspection, the egg had no traces of mold, alteration or any smell. Inside the egg was found a stillborn pair of conjoined twins, which will be further described in this paper. This is the first report of conjoined twins in Crested Gecko. We further present an insight into published study cases of twinning or conjoined reptiles and correlate them with the potential factors that may lead to this malformation.

Keywords: crested gecko, reptile, conjoined, stillborn, twins.

Conjoined twins, characterized by a sustained physical connection through the head, thorax, abdomen, or limbs, represent a unique phenomenon. While identical twins typically arise from the division of an egg into two halves soon after fertilization, the process of separation fails in the case of conjoined twins, the reasons for which this process occurs remain still unknown (8). Reptiles, a diverse and ancient group of vertebrates, exhibit an array of intriguing reproductive strategies that have evolved over millions of years. Among the myriad of reproductive adaptations, one phenomenon that captivates scientific curiosity is the occurrence of conjoined offspring in certain reptilian species. These instances, where two or more individuals share physical connections during development, challenge our understanding of embryonic processes and the genetic factors governing

morphogenesis (2). One study shows that parthenogenetic reptiles have a higher chance of producing neonatal abnormalities (7). Additionally, for oviparous species, their development is notably more influenced by environmental factors (11). The comprehension of congenital malformations and their etiology, particularly within the wildlife domain, constitutes a potent instrument for the comprehensive assessment of the one health paradigm. This knowledge serves as a catalyst, also for discerning substances deleterious to human, non-human, and environmental health, thereby facilitating the implementation of consequential sanitary measures. The class Reptilia manifests extensive species diversity characterized by distinct anatomical features tailored to terrestrial and aquatic environments characterized by hot and humid conditions (6). Consequently, comprehending anomalies in non-avian reptile species can enhance our understanding of climate change effects, gene expression in natural populations, and the impacts of pesticides. Reptiles serve as effective bioindicators of environmental contamination, as highlighted (9).

Conjoined twins, an intriguing anomaly in both human and animal populations, have captivated the scientific community with their potential insights into embryonic development, mutations, and the complex interplay of these factors (8). This case study explores a unique instance of conjoined twins in Crested Gecko (*Correlophus ciliatus*), shedding light on a phenomenon rarely documented in reptiles.

Drawing parallels with documented cases, including conjoined Siamese twins of the Leopard Tortoise (4), reports of dicephalic snakes in India (13), and Siamese twins in the Quince Monitor Lizard (*Varanus melinus) (*14) this study aims to enrich our understanding of reptilian conjoined twins. The diverse nature of these anomalies across reptilian species emphasizes the need for comprehensive studies to unravel the complexities of embryonic development and mutations in reptiles.

The investigation of such anomalies not only enhances our understanding of herpetological science but also holds implications for broader fields, including human and animal medicine (1). By correlating the findings with existing literature, this study serves as a foundation for further research in understanding the etiology and pathogenesis of conjoined twins in reptiles. While knowledge about anomalies is growing for other groups, such as amphibians, it is observed that the same level of attention is not currently directed toward reptiles. However, we predict that this scenario may change in the future, as they, particularly lizards, are becoming models for climate change monitoring (5).

Materials and methods

This case study was made on conjoined twins in Crested Gecko (*Correlophus ciliatus*) (Fig. 1). The conjoined twins, in this unprecedented case, were found to be glued at the cephalic level, presenting a distinctive anatomical connection. Detailed observations and measurements revealed that these conjoined twins were 1 cm smaller than other newborn Crested Geckos, providing crucial

insights into the potential developmental impacts of conjoined twinning in reptiles. This case, the first documented occurrence in Crested Geckos, contributes to the limited literature on conjoined reptiles.

The morphological characteristics of the parents - a dark brown mother and a full pinstripe father - add an intriguing layer to the investigation. This introduces considerations about the compatibility of different morphs in breeding practices and the potential implications for understanding the genetic basis of conjoined twinning in Crested Geckos.

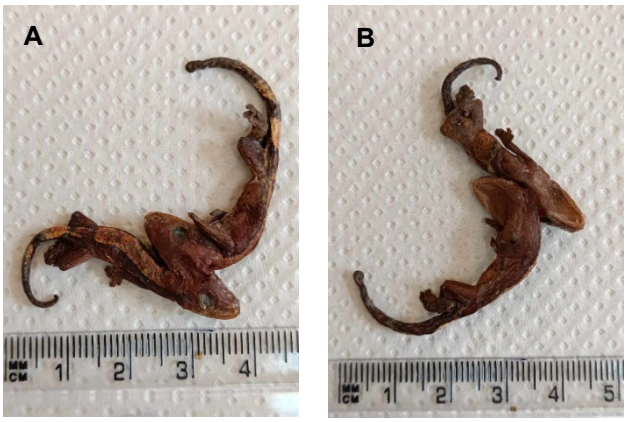

Fig. 1. Conjoined twins of Crested Gecko (*Correlophus ciliatus*): A) ventral view, B) dorsal view

Following the discovery of the conjoined twins, preservation measures were undertaken to ensure their suitability for subsequent examinations. The conjoined geckos were carefully placed in a controlled freezing environment, a common practice in scientific protocols, to halt any potential decomposition processes. This preservation method serves a dual purpose, facilitating both DNA sampling for genetic analyses and the creation of wet specimens. The controlled freezing not only safeguards the structural integrity of the specimens but also allows for a comprehensive investigation into their anatomical features and genetic makeup in subsequent phases of this study.

Speaking of Ethical Considerations, no harm was inflicted on any animals during this study. The conjoined twins were found deceased within their egg, and the egg was carefully opened with scissors for examination. The reptile breeder from Cluj-Napoca, Romania, ensures high standards of animal welfare, maintaining all animals in excellent conditions. The written consent of the owner was obtained.

Results and discussions

This section explores the broader context of conjoined reptiles, drawing on previously published cases and highlighting potential factors contributing to such malformations. Further examination of the anatomical features, including the point of connection and any associated abnormalities, is presented in this section

The conjoined twins identified in this study were Crested Geckos, each measuring between 4 and 5 cm (Fig. 1), falling within the typical size range observed for newborn individuals of this species, which is 5 to 6 cm long, according to our measurements. The conjoined twins under investigation were discovered in a clutch of Crested Gecko eggs produced by a dark brown mother and a full pinstripe father. Over a two-month period, four normally developed eggs were laid in pairs of two, as usual for this species, with three successfully hatchings. Unfortunately, details regarding the initial pair of eggs are unavailable for scrutiny. However, meticulous examination was conducted on a subsequent pair of eggs, from which Siamese twins emerged. Intriguingly, the coexisting egg within this pair yielded a non-anomalous individual, exhibiting the characteristic phenotype of a normal dark brown morph sibling. The remaining larger egg (Fig. 2), upon visual inspection, exhibited no signs of mold, alteration, or odor. Inside, a stillborn pair of conjoined twins was found. Detailed observations and measurements were conducted to characterize the anatomical features and abnormalities of the conjoined twins.

Notably, the conjoined Siamese twins of the Leopard Tortoise emerged in Tanzania and thrived for a noteworthy span of seven weeks. The parental turtles were housed within the confines of a garden with other members of the same species, and interestingly, the identity of the father remains unknown. The circumstances surrounding the incubation of the turtle clutch unfolded discreetly within the garden, with conditions remaining uncontrolled and unobserved. This scenario not only highlights the possibility of incubation in abnormal environmental conditions but also raises the intriguing possibility of consanguinity due to the undisclosed paternity (4). Dicephalic snake case reviews outnumber those of other conjoined reptiles in literature, spanning both wild and captive environments, in comparation with other conjoined families of reptiles which all were found exclusively in captivity. This emphasizes the pressing need for in-depth investigation into the intrinsic factors contributing to the heightened occurrence of these abnormalities in snakes. Also, snakes were the only clade of reptiles that possessed conjoined individuals who were born in the wild.

In our specific case, the parental lineage of the conjoined lizards was identified, with the mother originating from Romania and the father hailing from Hungary. Despite this clarity, the possibility of inbreeding cannot be dismissed outright, given the common practice among Romanian hobbyists of acquiring reptiles from Hungary. However, it's worth noting a contrast with the incubation conditions of Crested Geckos, as they do not demand intricate conditions, unlike turtles, making the reproductive circumstances distinctive. In contrast, the conjoined Quince Monitor

Lizards presented a unique case. The parents were revealed to be siblings, resulting in the production of two clutches. Both clutches exhibited normally developed, viable offspring alongside unfertilized eggs and malformed, deceased individuals (14). Comparatively, both cases involve conjoined individuals, but the circumstances surrounding their development differ. While the conjoined Crested Geckos were born to parents from different locations, possibly related, the conjoined Pacific monitor lizards were a result of sibling parents. Once again, this raises the likelihood that inbreeding plays a significant role in the occurrence of these malformations. What the Siamese conjoined individual of the Quince Monitor and the Crested Geckos under study share is their connection at the cephalic area. The distinction lies in the Quince Monitor twins being additionally conjoined abdominally (referred to as cephalothoracopagus conjoined twins). These monitor twins typically exhibit well-developed entire extremities and tails. Nevertheless, it's noteworthy that the abdominal region of both individuals is open, a feature not observed in our conjoined Crested Gecko. Furthermore, insights into variations among conjoined reptiles are offered by the documentation of dicephalic snakes in India (13). Unlike the case we are examining, the snakes in this study were observed in the wild, alive yet exhibiting limited mobility and locomotion. Additionally, as stated in other article about a dicephalic Western Dusky Rattlesnake (*Crotalus triseriatus*) these dicephalic snakes do not have a prolonged lifespan in their natural habitat but can lead a relatively satisfactory life when kept in captivity (15).

Upon an extensive review of the literature, a noticeable trend emerged, indicating a higher frequency of case reviews documenting dicephalic snakes when compared to another reptilian Order. Notably, this observation extends to instances where wild conjoined snakes have been discovered, distinguishing the snake population from other species previously described solely in captivity. This notable discrepancy accentuates the need for thorough investigation to discern the intrinsic factors contributing to the elevated occurrence of such abnormalities specifically within the snake population. This comprehensive exploration may uncover unique biological, genetic, or environmental elements that render snakes more susceptible

Fig. 2. Conjoined twins revealed after the egg was cut

to the manifestation of dicephalic characteristics than other class of reptiles, even in their natural habitats.

For future research directions, DNA has been successfully collected from the conjoined Crested Gecko twins in this study. Future research will focus on utilizing molecular genetic methods to determine the sex of these specimens, contributing to our understanding of the genetic basis of conjoined twinning in reptiles.

The morphological characteristics of the conjoined twins' parents—a dark brown mother and a full pinstripe father—raise intriguing questions about the compatibility of these morphs in breeding. Further analyses and controlled breeding experiments could shed light on the genetic factors influencing the occurrence of conjoined twins in Crested Geckos. The rarity of conjoined twins in Crested Geckos is underscored by this being the first documented occurrence. Drawing parallels with documented cases in reptilian literature, such as conjoined Siamese twins of the Leopard Tortoise (4), the conjoined Quince Monitor Lizards (14) and records of dicephalic snakes in India (13), aids in a more comprehensive understanding of the morphological variations observed in conjoined reptiles.

While conjoined twins are typically monozygotic, an intriguing instance of heterozygosity non-conjoined twins was documented in the blue tree monitor. After a 211-day incubation period, all four eggs developed a fungus, rendering them non-viable. Upon dissection of the first three eggs, deceased late-term embryos were found, each retaining varying amounts of residual yolk. The fourth egg contained two deceased late-term embryos, notably smaller than their counterparts, yet fully developed. The twins exhibited differences in dorsal patterning, and the presence of two distinct yolk sacs confirmed the unique nature of this twinning event (12).

Various elements contribute to the occurrence of anomalies such as conjoined reptiles. The successful culmination of embryonic development hinges on the intricate interplay between genetic factors and environmental conditions. Any disruptions occurring during this developmental process can potentially impact the genetic and epigenetic regulatory pathways of the embryo, thereby predisposing it to congenital malformations. Notably, many of these anomalies are incompatible with sustaining life (10). Many studies struggle to pinpoint the origins of the observed anomalies. The limited number that has successfully identified potential sources often implicates pesticides and various contaminants. However, temperature has also been linked to anomalies, albeit with an unclear and complex relationship. Despite the ambiguity, there is evidence suggesting a connection between temperature and anomalies in the pholidosis of lizards. It's worth noting that the majority of described anomalies primarily impact the skeletal and nervous systems, underscoring potential limitations in the analysis of the individuals under scrutiny (5).

Significantly, the mutagenic influences exerted on non-avian reptiles were already operative during the ancient period. One study describes a malformed embryonic or neonate choristoderan reptile, identified as *Sinohydrosaurus lingyuanensis*, previously referred to as *Hyphalosaurus lingyuanensis*. Unearthed

from the Lower Cretaceous Yixian Formation in northeastern China, this diminutive skeleton exhibits a rare anomaly - two heads and two necks - with the bifurcation occurring at the level of the pectoral girdle. This fossil marks the inaugural documentation of axial bifurcation in the reptilian fossil record. Given its apparent extreme juvenility, it is surmised that this malformed diapsid from the Yixian Formation likely had a brief, if any, survival duration (3).

Within the realm of conjoined Siamese reptiles, previous studies suggest a higher incidence among parthenogenetic species (7). The intricate interplay of environmental factors, notably temperature and humidity, assumes a crucial role in embryonic development, with temperature possibly standing out as the primary influential element in the manifestation of conjoined reptiles. Authors have further proposed that genetic anomalies, such as inbreeding or gene incompatibility, which might significantly contribute to the observed phenomenon. Beyond this, a spectrum of additional factors, including mold, infections, toxins, and pollution, has been discussed by researchers as potential contributors to the development of conjoined reptiles. This multifaceted understanding underscores the complexity of the mechanisms involved and underscores the need for comprehensive investigations to unravel the intricate web of causative factors in the occurrence of conjoined Siamese reptiles.

Conclusions

In summary, the unprecedented occurrence of conjoined twins in Crested Geckos presented in this study provides valuable insights into the intricacies of reptilian embryonic development. The detailed examination of these conjoined twins, has expanded our understanding of the morphological variations within this species. The rarity of such instances underscores the importance of comprehensive studies to unravel the complexities of embryogenesis and the potential genetic factor.

The investigation into the conjoined twins, carefully preserved for subsequent analyses, offers a foundation for broader implications in the realms of herpetological science, veterinary medicine, and genetic research. The controlled freezing method employed for preservation not only ensures the structural integrity of the specimens but also sets the stage for advanced genetic studies.

Drawing parallels with documented cases of other conjoined twins, enriches our understanding of the diversity of anomalies in reptilian development. These comparative insights contribute to the broader field of comparative embryology and pave the way for further investigations into the etiology and pathogenesis of conjoined twins across different reptilian species.

The fossilized specimen, featuring a two-headed configuration, provides valuable insights into the embryonic development malformations observed in early non-aviary reptiles. The presence of conjoined Siamese twins in this ancient reptilian specimen suggests a distinctive aspect of embryonic development that warrants further investigation. This unique case underscores the complexity and diversity of

developmental processes in non-aviary reptiles, shedding light on the potential factors contributing to the occurrence of conjoined offspring in this taxonomic group.

Furthermore, the morphological characteristics of the conjoined twins' parents - a dark brown mother and a full pinstripe father - introduce intriguing questions about the compatibility of different morphs in breeding practices. The study of these anomalies not only enhances our understanding of herpetological science but also holds relevance for reptile owners, breeders, and enthusiasts who actively engage in the cultivation and conservation of these unique species.

In conclusion, this case study serves as a stepping stone for future research endeavors, promising a deeper exploration of the underlying mechanisms shaping reptilian embryonic development and the factors influencing the occurrence of conjoined twins in Crested Geckos.

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THE MAIN CARDIAC RHYTHM ABNORMALITIES ENCOUNTERED IN DOGS DURING INTERMITTENT HEMODIALYSIS

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Summary

Exploring the cardiac rhythm abnormalities (arrhythmia) that can occur in dogs undergoing hemodialysis, represents an essential aspect in veterinary nephrology and cardiology. Hemodialysis is an extracorporeal renal replacement therapy that can be life-saving for dogs diagnosed with acute kidney injury or chronic kidney disease. Most of the time, hemodialysis can be a challenging therapy in cardiac patients, due to multiple ECG modifications. Identifying, understanding and managing arrhythmias during hemodialysis represents the cornerstone to a successful therapy. A continuous electrocardiogram (c-ECG) is an important monitoring tool and it can be used to detect and manage cardiac rhythm abnormalities, during hemodialysis. The most common arrhythmia observed in patients with acute kidney injury is atrial fibrillation due to hyperkalemia. Severe hyperkalemia, sometimes combined with hypocalcemia, can cause severe brady-arrhythmias in this clinical setting. All chronic patients, submitted to this study, were end stage (stage 4). In this study, the most common finding in patients diagnosed with chronic kidney disease, was ST wave depression due to left ventricular hypertrophy and wide QRS complex, P and T wave modifications due to hyperkalemia in acute or acute on chronic patients.

Keywords: hemodialysis, dogs, nephrology, arrhythmia, hyperkalemia.

Over the years, the scientific view regarding acute kidney injury (AKI), chronic kidney disease (CKD) or acute-on-chronic, has changed to a complex disorder where the kidneys play an active role in the progress of multi-organ dysfunction (13). The expertise of cardiologists and nephrologists, may be the most appropriate approach for patients with concurrent cardiac and kidney diseases or predisposed to cardio-renal syndrome (5). The cardio-renal syndrome (CRS) is defined as a wide spectrum of injuries where both the heart and kidneys are involved (20). Kidney disease is a risk factor for the development of cardiovascular diseases and the links between kidneys and heart from a pathological point of view are better observed in patients with AKI associated with cardiac arrhythmias (12, 21). Chronic mitral valve disease is associated with increased prevalence of CKD (15). Chronic kidney disease is characterized by a progressive and irreversible decline of nephron function and the outcome is mostly dependent on concomitant cardiac pathology (2).

According to the United States Renal Data System (USRDS), the leading cause of death among CKD patients undergoing dialysis is related to cardiac arrhythmias (24).

The pathophysiology of arrhythmias in renal patients it seems to be related to structural cardiac abnormalities caused by CKD, associated with the dialysis procedure itself (6).

Even mild reductions in kidney function can alter the electro-physiological properties of the myocardium and increase the risk of ventricular arrhythmias (17). Electrolyte disturbances, especially hyperkalemia, are defined as a loading factor for arrhythmia generation in such patients (14). Hyperkalaemia is a common complication of end-stage renal disease (ESRD) (9). The electrocardiogram (ECG) abnormality of hyperkalaemia is classically defined by the 'peaked' T-wave (16). It is possible that left ventricular hypertrophy (LVH) may produce tall T-waves (19). Furthermore, hyperkalaemia does not always lead to abnormal T-waves (1). Hyperkalaemic arrhythmic death is thought to be often due to depolarization changes and subsequent bradycardia/asystole. These include widen of the QRS complex and the 'sine' wave ECG which are shown to come before cardiac arrest. The presence or absence of T-wave changes in this setting may provide important prognostic information for patients with ESRD (10, 11). Prolonged QT interval syndrome is one of the known pathophysiological mechanisms of sudden death in renal patients (4).

Hemodialysis is a therapeutic procedure that uses the extracorporeal circulation of a patient's blood and it is used for the management of acute and chronic renal injury that is refractory to conventional medical therapy (23). Is a special procedure that requires an extensive array of sophisticated delivery equipment and specifically trained and dedicated staff to perform, monitor and ensure the integrity and safety of the procedure in critically ill patients (8). Intermittent hemodialysis (IHD) is indicated in cases of acute uremia, electrolyte abnormalities or acidosis, unresponsive to medical management. When patients undergo IHD, their blood is removed from their bodies through an extracorporeal circuit (3). Continuous renal replacement therapy (CRRT) is a continuous process and once it begins, therapy continues until renal function is restored or the patient is transitioned to intermittent dialysis. The continuous operation more closely approximates the functioning of a normal kidney (7).

The electrocardiogram monitoring is mandatory in order to correlate arrhythmias with renal disease associated with electrolyte imbalances that have direct consequences in cardiac rhythm. In patients undergoing hemodialysis, monitoring the electrical changes are mostly associated with the electrolytic disorders (22). Kidney involvement can be progressive to end-stage kidney disease over time and driven by forces associated with the cardiac disease or its management (18).

Materials and methods

The present study was conducted on 30 patients diagnosed with acute kidney injury (Batch 1A, n=15) and acute on chronic kidney disease (Batch 2AC, n=15), both males (n=17) and females (n=13). All patients were mixed breed, aged between 5 and 11 years old.

All patients (n=30), had serum creatinine over 10 mg/dl and blood urea nitrogen (BUN) over 100 mg/dl.

Based on the serum potassium value, patients had the following classification:

- normokalemic: 3.5 - 5.5 mmol/L;
- mild kyperkalemic: 5.6 - 6.0 mmol/L;
- moderate hyperkalemic: 6.1 - 7.0 mmol/L;
- severe hyperkalemic ≥7 mmol/L.

Electrocardiogram (ECG) recording and monitoring was performed using Poly-Spectrum 8 Vet Rhythm, 4 clip electrodes with 6-lead ECG (Fig. 1) and Mindray uMec 12 Vet monitor, 3 clip electrodes with 3/5-lead ECG (Fig. 2) with velocity of 25 and 50 mm/s (1 mm = 0.1 mV, 1 mm = 0.04s). The electrodes were placed on the forelimbs and hind limbs in all patients on lateral recumbency without sedation.

Intermittent hemodialysis was performed with an A/V set and a high flow dialyzer with a surface of 1.5 m^2. The volume of the circuit was 232 ml. Urea reduction ratio (URR) was calculated for 50% and the duration of therapy was 6 hours.

Fig. 1. Dog in lateral recumbency for monitoring and recording electrical changes during intermittent hemodialysis, with Poly-Spectrum 8 Vet Rhythm - 4 clip electrodes with 6-lead ECG (Original)

Fig. 2. Dog in lateral recumbency for monitoring and recording electrical changes during intermittent hemodialysis, with Mindray uMec 12 Vet monitor - 3 clip electrodes with 3/5-lead ECG (Original)

Results and discussions

Hyperkalemia is a prominent factor causing atrial standstill. Atrial standstill is a disturbance of the cardiac rhythm that involves the complete absence of electrical atrial activity, which occurs in severe hyperkalemia, due to nonspecific repolarization abnormalities seen with elevations of serum potassium. Atrial fibrillation is defined as rapid and irregular beating of the atrium, and occurs not as the result of hyperkalemia but rather as the consequence of damage caused by hyperkalemia.

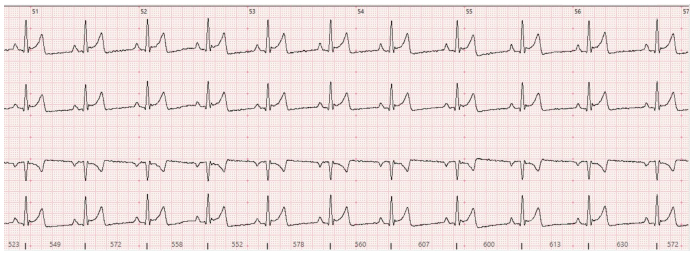

Fig. 3. 'Peaked' T-wave on hyperkalemic patient (Original)

Table 1

Electrolytic abnormalities in patients from Batch 1A (n=15)

Patient no.	Normokalemic (RR: 3.5-5.5)	Mild Hyperkalemic (RR: 5.5-6.0)	Moderate Hyperkalemic (RR: 6.1-7.0)	Severe Hyperkalemic (≥ 7.0)
P1	✓	x	x	x
P2	x	✓	x	x
P3	x	x	✓	x
P4	x	x	✓	x
P5	x	x	x	✓
P6	✓	x	x	x
P7	x	x	✓	x
P8	x	x	x	✓
P9	✓	x	x	x
P10	✓	x	x	x
P11	x	x	✓	x
P12	✓	x	x	x
P13	x	x	x	✓
P14	✓	x	x	x
P15	✓	x	x	x

Table 2

Electrolytic abnormalities in patients from Batch 2AC (n=15)

Patient no.	Normokalemic (RR: 3.5-5.5)	Mild Hyperkalemic (RR: 5.5-6.0)	Moderate Hyperkalemic (RR: 6.1-7.0)	Severe Hyperkalemic (≥ 7.0)
P1	x	x	✓	x
P2	✓	x	x	x
P3	x	x	x	✓
P4	✓	x	x	x
P5	✓	x	x	x
P6	x	✓	x	x
P7	✓	x	x	x
P8	✓	x	x	x
P9	x	x	x	✓
P10	✓	x	x	x
P11	✓	x	x	x
P12	x	x	✓	x
P13	✓	x	x	x
P14	x	✓	x	x
P15	✓	x	x	x

Cardiac arrhythmias were observed by monitoring the electrocardiographic changes during intermittent hemodialysis. Identified arrhythmias were originated as supraventricular or ventricular. Rare ventricular premature complexes and QRS abnormalities such as widening of the complex and ST wave depression (Fig. 5), are classified as ventricular originated.

The most abnormal findings with supraventricular origins were atrial standstill (Fig. 6) and atrial fibrillation.

In both batches (Batch 1A, n=15; Batch 2AC, n=15), patients diagnosed with supraventricular arrythmias had atrial standstill showed on ECG as a missing P wave for every QRS complexes. Also widening of the QRS complexes and depression of ST segment were observed mostly in renal patients diagnosed with hypertrophic cardiomyopathy. We also noticed isolated VCPs (Fig. 4).

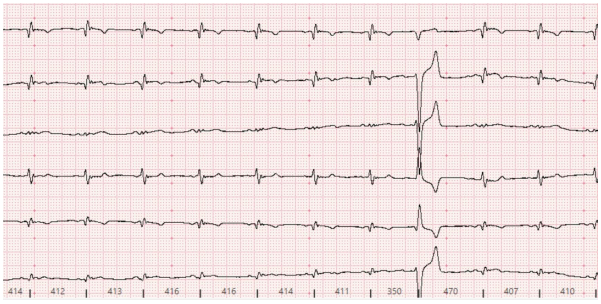

Fig. 4. Isolated VCPs (Original)

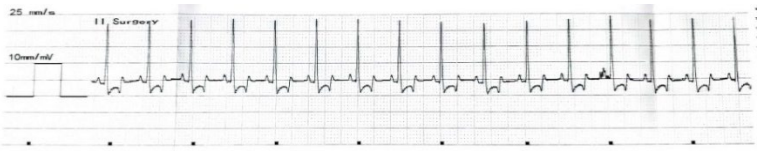

Fig. 5. ST wave depression (Original)

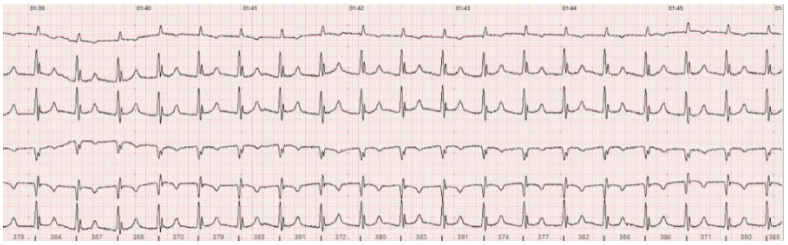

Fig. 6. Atrial standstill (Original)

In Batch 1A (n=15), 53.3% (n=8) patients presented arrhythmias as it follows:

- 1 patient with ventricular arrhythmias and mild hyperkalemia (5.5-6.0 mmol/L);
- 4 patients with ventricular arrhythmias and moderate hyperkalemia (6.1-7.0 mmol/L);
- 3 patients with supraventricular arrythmias and severe hyperkalemia (≥7 mmol/L).

In Batch 2AC (n=15), 40% (n=6) patients presented arrhythmias as it follows:

- 2 patients with ventricular arrhythmias and mild hyperkalemia (5.5-6.0 mmol/L);
- 2 patients with ventricular arrhythmias and moderate hyperkalemia (6.1-7.0 mmol/L);
- 2 patients with supraventricular arrythmias and severe hyperkalemia (≥7 mmol/L).

Table 3

Cardiac rythm abnormalities in patients from Batch 1A (n=15)

Patient no.	Ventricular arrhythmias	Supraventricular arrythmias
P1	x	x
P2	✓	x
P3	✓	x
P4	✓	x
P5	x	✓
P6	x	x
P7	✓	x
P8	x	✓
P9	x	x
P10	x	x
P11	✓	x
P12	x	x
P13	x	✓
P14	x	x
P15	x	x

Table 4

Cardiac rythm abnormalities in patients from Batch 2AC (n=15)

Patient no.	Ventricular arrhythmias	Supraventricular arrythmias
P1	✓	x
P2	x	x
P3	x	✓
P4	x	x
P5	x	x
P6	✓	x
P7	x	x
P8	x	x
P9	x	✓
P10	x	x
P11	x	x
P12	✓	x
P13	x	x
P14	✓	x
P15	x	x

Conclusions

Hemodialysis is a highly effective treatment in patients with acute kidney injury or acute-on-chronic patients. Most of the time, hemodialysis can be a challenging therapy in cardiac patients, due to multiple ECG modifications.

Monitoring and recording ECG abnormalities in renal patients during intermittent hemodialysis is an important tool for identifying, understanding and managing arrhythmias.

Electrolyte imbalance, especially hyperkalemia, is defined as a loading factor for cardiac arrhythmia in end stage renal disease patients.

An accurate ECG diagnosis may be an important monitoring tool and it can be used to detect and manage cardiac rhythm abnormalities, during hemodialysis.

In cardio-renal syndrome, identifying, understanding and managing arrhythmias during hemodialysis represents the cornerstone to a successful therapy with a good outcome for the patient. According to our data, if there will be a ventricular origin arrhythmia, the ventricular tachycardia may lead to cardiac arrest.

In conclusion, the most common finding in patients diagnosed with chronic kidney disease, end stage, was ST wave depression due to left ventricular hypertrophy and wide QRS complex, P wave missing in atrial standstill and T wave modifications due to hyperkalemia in acute or acute on chronic patients.

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ASSESSING PERMEABILITY AND VIABILITY OF MICE JEJUNUM SAMPLES USING THE USSING CHAMBER TECHNIQUE

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Summary

The permeability and the barrier function of the intestinal epithelium can be assessed via the Ussing chamber technique. Considering the electrophysiological parameters, the researcher can gain insight on the processes taking place in the intestine. This study highligts the assessment of the jejunal tissue viability and of jejunal permeability using the Ussing chamber technique, while also underlining some advantages and limitations of working with two types of jejunal specimens harvested from mice (whole-thickness and stripped). Taking into account all of the above, the objective concerns validation of an experimental model for the study of jejunal permeability. The study was conducted on mice jejunum, using the Ussing chamber technique for the assessement of permeability and viability. The electrical parameters (PD, I_{sc}, Rel) recorded for the whole-thickness (J-I) and stripped (J-MS) samples throughout the experiment showed time dependent changes in viability. The mean values for PD were: J-I 5.77 ± 0.87 mV, J-MS 5.23 ± 0.82 mV; for I_{sc}: J-I 199.8 ± 29, J-MS 11 μA/ cm^2; for Rel: J-I 32.2 ± 3.61 Ω/cm^2, J-MS 25.98 ± 2.27 Ω/cm^2. The electrical parameters recorded during the experiment showed time-dependent variations and there are no significant differences of values between J-I and J-MS samples.

Keywords: Ussing chamber, permeability, intestine, mouse, electrical parameters.

The intestinal epithelium has two significant functions: *permeability*, which allows the transport of nutrients and fluids, which can be quantified through the processes of intestinal absorption and secretion; *the barrier function*, which opposes the transfer of some substances through the intestinal epithelium (13, 19).

The two functions are possible due to the epithelial morphology of the intestine. It is made up of many types of epithelial cells tied toghether by junctions, the most important being the tight junctions, which consist of proteic complexes that ensure the sealing of the paracellular space. The intestinal epithelium, like any other epithelia, is characterised by two important properties: polarity and tightness. Polarity is the result of the distribution of structural proteins along the apical and basolateral membranes of the enterocytes, while the tightness is provided by the composition and distribution of proteic units belonging to the tight junctions. Those morphological features make the transepithelial transport possible through two pathways: transcellular and paracellular. It is important to mention the fact that there are morphological dissimilarities between different segments of the intestine which generate variability in permeability (1, 2, 7, 8, 11, 14, 18, 20). Those variations can be evaluated using the Ussing chamber technique (17), by which the potential difference (PD), the short-circuit current (I_{sc}) and the transepithelial resistance (Rel)

can be recorded. PD refers to the electrical potential difference generated by the uneven distribution of ionic species across the plasma membrane of a cell. This parameter points to the health state of the intestinal epithelium. I_{sc} is expressed by the sum of all the currents generated by the movement of ions through the cell membrane. Rel points to the integrity of the intestinal epithelium, providing information about the permeability of the tight junctions, with interest to the paracellular transport. Low values of Rel are a result of high paracellular permeability (3, 16).

This study aims to evaluate the permeability of whole-thickness and stripped mouse jejunum given by electrophysiological measurements using the Ussing chamber technique.

Materials and methods

The study was done on jejunal segments harvested from 10 male adult mice, fed with a standard diet and water provided *ad libitum*. After euthanasia, the small intestine was harvested from each individual. After the harvesting, the jejunum was bathed in cold Krebs Ringer bicarbonate buffer solution (KBR), corrected to a 7.4 pH value, gassed with carbogen (95% oxygen, 5% carbon dioxide) and kept on ice. The carbogen provides the tissue oxygenation, maintaining the partial pressure of oxygen above 400 mmHg. At the same time it ensures a partial pressure of CO_2 similar to that of the venous blood, it maintains the pH value of the solution bathing the tissue constant (pH=7.4), and thus ensuring tissue viability (3, 21).

The jejunum was cut into segments, which were kept in cold KBR buffer solution, at pH=7.4, gassed with carbogen and placed on ice. The prepared jejunal segments were washed with cold KBR buffer and cut longitudinally along the mesenteric insertion (3).

For the measurement of the electrical parameters two types of samples were prepared:

- whole-thickness jejunum (J-I), keeping all 4 layers of the intestinal tissue - mucosal, submucosal, muscular and serosal;
- stripped jejunum (J-MS) - prepared by removing the muscular and serosal layers under a stereomicroscope, while bathing the tissue in carbogen gassed, cold KBR.

The prepared jejunum fragments were cut into rectangular shape (L= 2cm, l= 1cm) and mounted in Physiologic Instruments type 2304 slides, with rectangular aperture and an area of 0.3 cm^2. The slides were kept on ice in Berzelius beakers with carbogen gassed KBR (3).

Using the Ussing chamber technique the electrical parameters – PD, I_{sc} and Rel were measured to evaluate the intestinal permeability, for a period of 90 minutes, using the 2 types of jejunal samples (J-I and J-MS) between recording minutes 10 and 100.

Assessment of tissue viability was made by evaluation of the PD and Rel. The measurements mentioned were recorded for a period of 180 minutes, between the time points: minute 0 to minute 180.

To ensure the same working conditions, the jejunal samples (whole-thickness J-I and stripped J-MS), harvested from the same animal were subjected to simultaneous measurements in 2 separate Ussing chambers, applying the same protocol.

During the whole experiment the animal welfare regulations were met according to law (4).

Results and discussions

Assessment of jejunal tissue viability

The Ussing chamber technique provides a handling of samples that ensures very close to realistic physiological conditions. However, the fact that the specimens suffer an articifial processing must be taken into account, therefore the assessment of tissue viability is crucial. Tissue integrity can be evaluated via molecular markers (mannitol, PEG-4000, inulin, Cr-EDTA), lactate dehydrogenase releasing, D-glucose transport, but also by checking the electrophysiological parameters (12).

Viability of the jejunal segments was assessed based on the potential difference (PD) and transepithelial resistance values over the 180 minutes of the experiment, the time points being minutes: 0, 60, 120 and 180.

Comparing the values recorded for the J-I and J-MS, it seems that the PD values decrease over time. It is noted that at the 120 minute time point the PD drops below 4 mV (Fig. 1), which indicates that the integrity of the tissue is affected. Research conducted by Polentarutti et al. (12) shows that values below 4 mV are correlated with an altered tissue viability. Therefore, it is recommended that after the baseline measurements and the recording of the initial PD (at the begining of the experiment), samples that have a PD value below 4 mV should be excluded from the experiment (15).

PD values recorded at 120-minute time stamp (3.8 mV for J-I and 3.5 mV for J-MS) point out the altering of the intestinal epithelium. Research done by Inagaki et al. (9) on mice shows important morphological changes of the intestinal mucosa at 120 minutes of incubation in the Ussing chamber. The changes described include morphological alterations of the intestinal villi, without any damage inflicted upon the crypts. The same authors report massive destruction of the villi at 240 minutes of incubation in the Ussing chamber, but without morphological changes of the crypts. These tissue alterations are correlated with changes in electrical parameters, including PD which is an indicator of the health state of the epithelium.

After 180 minutes of incubation in the Ussing chamber, the PD values decrease further, respectively 2.7 mV for J-I and 2.3 mV for J-MS.

The analysis of the results shows that the Rel values measured at minutes 0, 60, 120 and 180 of the experiment range within the normal limits of 20 and 50

Ω/cm^2 (Fig. 1) mentioned in the literature (12). Also, the values are relatively constant in the two types of tissue (J-I and J-MS) where the PD falls below the normal values characteristic of the jejunum. The decrease in PD is the result of morphological alterations of the intestinal epithelium, especially the enterocytes. Polentarutti et al. (12) report a 50% reduction in the epithelial surface (edema, shortening of villi, mucosal denudation covering extended surfaces) which would theoretically lead to a doubling of Rel values. The same authors report an increase in permeability to mannitol, indicating structural alterations of tight junctions that generate a decrease in Rel. The overall effect resulted is the maintenance of Rel at constant values.

Recorded values for PD and Rel between minutes 0-180 of the experiment were considered only if there were no morpho-physiological alterations of the samples.

The intestinal integrity changes are in correlation to the timestamp of the experiment. The timestamps marking minutes 120 and 180 point to a decrease in PD value of both J-I and J-MS samples. This translates to the alteration of the intestinal tissues evaluated.

PD, I_{sc} and Rel values recorded between minutes 10 and 100 of the experiment were taken into account for the assessment of intestinal permeability. The PD and Rel values prove that during those 90 minutes of recording, the morphological integrity of the jejunum is preserved, the tissue is viable and has kept its two important functions: permeability and the barrier function.

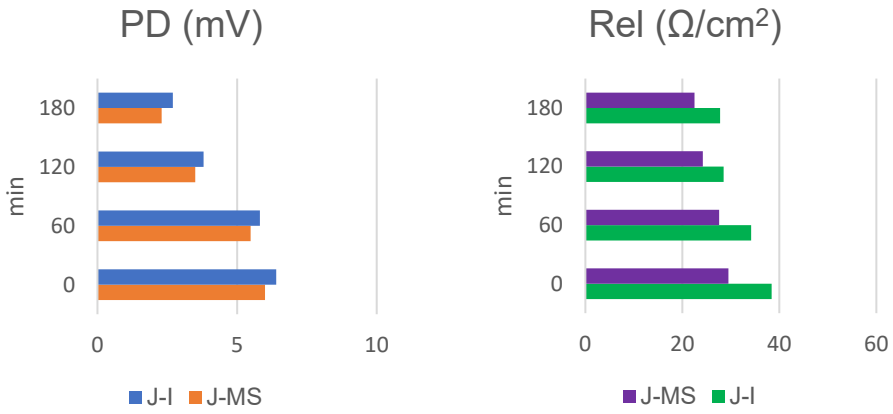

Fig. 1. PD and Rel values recorded at 0, 60, 120 and 180 minute time points

Measuring PD and Rel at the beginning of the experiment (time 0), ensures the experimental use of only viable tissue fragments and the exclusion of those that for various reasons (anoxia, ischemia, improper working conditions) have lost their

viability. Measuring PD throughout the experiment at short time intervals allows permanent monitorization of the tissues' health state.

Assessment of jejunal permeability

Table 1 shows the mean values of PD, I_{sc} and Rel for J-I and J-MS measured during 90 minutes of incubation in the Ussing chamber.

Table 1

Mean values of PD, I_{sc} and Rel for J-I and J-MS measured during 90 minutes of incubation in the Ussing chamber

Electrical parameter	J-I	J-MS
PD (mV)	5.77±0.87	5.23±0.82
I_{sc} (µA/ cm^2)	199.8±29.11	186.1±24.55
Rel (Ω/cm^2)	32.2±3.61	25.98±2.27

Analysis of results showed that the average value of PD for J-I was 5.77±0.87 mV, while for J-MS it was 5.23±0.82 mV. Comparing the two values, it was found that the difference between them is insignificant (p= 0.10) and that the values, for both types of tissues, are greater than 4 mV, which is the minimum value that characterizes the viability of the mouse jejunum (12).

Analyzing the mean values of each jejunum sample (J-I and J-MS) it was found that they range within the characteristic limits for this intestinal segment i.e. between 4 and 8 mV. There were no significant differences between the whole-thickness and stripped jejunum samples. The range between these values, and especially the fact that all PD values were greater than 4 mV, proves the preservation of tissue viability over the 90 minutes work interval.

Regarding the I_{sc} measurements, the average value for J-I was 199.8±29.11 µA/cm^2, and for J-MS was 186.1±24.55 µA/cm^2. Although there are important morphological differences between the tissue fragments (J-I versus J-MS), comparing the results it is observed that within the limit of 90 minutes, the values do not register significant differences (p=0.14). Also, the values are comparable to those determined by Polentarutti et al. (12), who report values between 100 and 250 µA/cm^2 for the small intestine. This demonstrates that within 90 minutes, as long as the integrity and viability of the intestinal epithelium is preserved, processes such as active transmembrane transport take place.

The electrical resistance (Rel) of the intestinal epithelium is the parameter that provides important information about the intestinal integrity, a fact that is proven by Ussing chamber experiments done on freshly harvested tissue samples from animals (after slaughter or biopsies) or cell cultures (3, 6, 10, 16, 19).

The measurement of Rel allows the evaluation of the intestinal epithelium integrity, reflecting the state of the tight junctions (TJ) and basically the paracellular transport of water and micro molecules.

Prior research studies done on tissue harvested from mice small intestine point out Rel values between 20-50 Ω/cm^2 (12). Table 1 shows the average values for each tissue type, over the 90 minutes recordings done during this experiment: 32.2±3.61 Ω/cm^2 for J-I and 25.98±2.27 Ω/cm^2 for J-MS. The comparison of these values reveals significant differences (p=0.0013) between J-I and J-MS. However, noted is the fact that all the values recorded from both tissue samples range within normal limits.

Validation of an experimental model for the study of jejunal permeability

Morphologically, the small intestine consists of the following layers: mucosal, submucosal, muscular, serosal. The meta-sympathetic nervous component is represented by the two nerve plexuses: submucosal and myenteric. The existence of these nerve plexuses, as well as the presence of muscular tissue, can influence the absorption and secretion processes in *ex vivo* conditions. Therefore, it is recommended to remove the muscular and serous layers from the submucosa and mucosa of the intestine by mechanical separation ("seromuscular stripping") (3, 16).

It must be considered that mouse intestinal walls tend to be thinner. This leads to a higher difficulty in removing the seromuscular layers without inflicting damage to the tissue and jeopardizing the transport function of the intestinal surface (the tissue mounted in the Ussing chamber).

Moreover, pathological conditions like denudation, inflammation, hemorrhages, etc. where the intestinal mucosal layer is affected, the separation procedure becomes more difficult, if not impossible sometimes.

The results obtained in this experiment show that under the given conditions, membrane electrical parameters (PD, I_{sc}, Rel) for the two types of tissue J-I and J-MS range within the normal limits mentioned by the literature, provided that viability of the tissue is preserved. Viability can be permanently checked by measurements done throughout the experiment. Shifts regarding the values of these parameters imply important histological changes that lead to tissue death and result in the exclusion of the concerned samples from the experiment.

Therefore, depending on the purpose and objectives of the experiment, both types of tissues (J-I and J-MS) can be used, each of them offering advantages and disadvantages (Table 2).

Taking into account the aspects discussed, the scientific investigator can choose the experimental protocol most fitted for its research purpose.

Table 2

Advantages and disadvantages of whole-thickness vs stripped jejunum samples

	Advantages	Disadvatages
J-I	Short period for sample preparement Preservation of epithelial integrity, especially when working with samples harvested from small animals or when working with pathologically altered samples, where the mucosa might be damaged	The muscular layer toghether with the enteric nervous system generate contractions of the tissue which can influence the intestinal permeability
J-MS	Accuracy in the assessement of intestinal permeability	Long sample preparation time, especially when working simultaneously with several Ussing chambers High risk of mucosal damage while stripping the intesitnal samples High fragility of the tissue samples subjected to the experiment

Conclusions

Assessment of jejunal tissue viability

The viability of the mounted jejunal samples is maintained between the time points 0 to 120 minute of the experiment.

The decrease of PD values below 4 mV between the time points: minute 120 to 180 of the experiment, suggest the loss of jejunal tissue viability of the samples. It is recommended to make the electrophysiological measurements between the minutes: 0 to 120 of the incubation inside the Ussing chambers.

Permeability assessment of the jejunum

PD and I_{sc} values range within normal limits, with no significant differences between whole-thickness and stripped samples.

There are significant differences in Rel values between whole-thickness and stripped samples, but they lie within normal ranges.

Validation of an experimental model for the study of jejunal permeability

There are no significant differences between the two types of samples used in this study, whole-thickness and stripped (J-I and J-MS) jejunum. Thus, depending on the purpose, the objectives and the working conditions of the experiment, the researcher can choose the experimental model that is best fitted.

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MEMBRANES USED IN THE MANUFACTURE OF SAUSAGES – REVIEW

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Summary

In this review we analyze the development of production practices and composition of different types of membranes (whey proteins, collagen, synthetic polymer, cellulose, vegetable), the basic properties of the membranes, the beneficial and toxic effects of the added ingredients used in their manufacture and biological risks in the use of different membranes.
Keywords: sausage, food, membrane, food additives.

In human nutrition, sausages have been an important food due to their price, long shelf life, fast digestion, texture and nutrition.

The diverse assortments of sausages, as well as their processing, have undergone changes due to climate, culture and consumer preferences.

A number of membrane types are used in the current production of sausages: natural collagen, cellulose, synthetic polymers, plastic and the new type of co-extruded coating made of collagen, alginate or alginate-collagen hybrids (20, 21).

Basic properties of membranes

Membranes are soft cylindrical containers used for packing meat or vegetable products. They can be of natural or artificial origin (18).

Sausage fillings are mostly mixtures of minced or shredded meat held together by the casing. Several processing steps may apply, including cooking, smoking, boiling, roasting, roasting and fermentation/drying (24).

The membrane is an integral part of sausages that gives them shape, integrity, that contributes to the volumetric, chemical, structural changes that occur during the production phases. The function of the sausage wrapper starts from the moment of filling to the final consumer (18).

The basic characteristics of the membrane are: mechanical resistance, permeability to water and gases, adhesion and elasticity, chemical inertia, impermeability to grease, uniform diameter, resistance to temperature variations.

Water and gas permeability and mechanical strength are the most important characteristics because they affect the final shape and weight of the sausages (7).

The mechanical properties of the membrane play an important role for consumers as they differentiate between edible and non-edible casing, and for

production, so the elasticity and strength of the casing are important in the filling process (1).

Tensile strength, elasticity, temperature resistance, transparency and gloss are given by the mechanical properties of the shell, namely: structural integrity, shape, size, volumetric changes, texture and appearance of the finished product (18).

The barrier between sausages and the external environment is influenced by the degree of permeability of the shell and depends on the level of exchange of substances from the filling with the environment.

Water loss, compositional changes, fat hydrolysis, pH, fat oxidation and sensory characteristics are affected by the degree of permeability of the carcass to water, gas and light (18).

The role of the membrane in the production of fermented raw sausages is to adapt to changes in the volume of sausages that occur during production, otherwise during ripening it can directly affect the quality of the final product (11).

The final product is the result of the interactions between the membrane and the filler during the manufacturing process. Membrane permeability is reduced by the acidic components of smoke and by lactic acid-producing bacteria. If the membrane cannot adapt to changes in the volume of the sausages, structural defects in the product may result.

Natural or artificial membranes are used for fermented sausages, they must be firm, elastic and retractable, permeable to smoke, water vapor and gases. It is important that during the process they adhere well to the filler during the drying period when the volume of the filler decreases (13).

Natural membranes are strong enough to withstand the pressure during loading and are permeable to water vapor, smoke and gases, adhere firmly to the sausage stuffing and can be tied or cut at the end of the sausages, they are mostly used in the production of traditional sausages (18).

The production processes are completed by measuring, salt water washing, final drying, dry salting and storage. The natural shell does not freeze because it loses its firmness and elasticity (18).

For some technologies, casings are also involved in the flavor and protect the products during storage.

Natural membrane: In the food industry, the first varieties of sausages that appeared on the market had a natural casing obtained from animal intestines, from slaughter. The intestines used are derived from pigs, cattle and sheep (10).

Natural coatings are composed of the submucosa of the small intestine of animals whose content is mainly natural collagen (18).

In Chinese and Western European cuisine most membranes come from pigs, but elsewhere goat, sheep, cattle or horse intestines are also used (10).

Sheep small intestines consist only of the submucosa and are considered edible, while in cattle or horse intestines the muscular layer is not removed and even if they are considered edible they are not eaten because they are hard and difficult

to chew (16).

Intestinal processing should begin immediately after slaughter when the tissue is still warm to avoid bacterial spoilage.

The process of preparing intestines for use as casing includes: washing, scraping and cleaning with water and salt by hand or with special machines. The outer fat and inner mucous membrane are removed during processing, they are salted to reduce water activity, inhibit microbial growth and preserve the coating.

Natural casings have been used in the production of meat specialties for centuries and have remained unchanged in function, appearance and composition (19).

The use of natural casing may pose a risk to human health due to various biological hazards: Salmonella spp., Clostridium spp., Listeria Monocytogenes, prions (5).

Artificial membrane: Fabricated artificial membranes are obtained from cellulose, collagen or synthetic materials and are available in a variety of types and sizes (8).

Depending on the structure and composition, the artificial membranes are divided into two groups: the artificial covering made of natural material and the artificial covering made of synthetic material (8).

In the artificial covering of natural material there are: coverings of cellulosic material of plant origin and collagen coverings (of animal origin). Synthetic coatings are made of polymeric material (plastic) (8).

The first types of artificial membranes were created at the beginning of the 20th century from cellulose, later they were made from collagen, plastic or even vegetarian ones based on 100% plants.

An assortment of artificial membranes is represented by those made from collagen that is derived from bovine skin. Artificial animal collagen coatings are generally edible, they are mainly produced from collagen from beef or pork hides, bones and tendons but can also be derived from birds and fish.

In addition to collagen, the sausage casing was developed using other proteins such as wheat gluten, soy protein, peanut protein, corn zein and feather keratin, pectin and gelatin/sodium alginate and protein isolate from whey (6).

Artificial synthetic polymer membranes are made of: polyamide, polypropylene or polyethylene. Synthetic polymer membranes are not edible and are generally used for non-smoked products. The advantage of using them is that they provide protection against oxidation because they are impermeable to oxygen, significantly helping to increase the shelf life of the products (7).

In recent years, attempts have been made to produce artificial membranes from whey proteins, an edible film that can be used as an alternative casing material for sausages. The production of intramolecular disulfide bonds were responsible for the film structure. A plasticizer was added to give the polymer film flexibility and extensibility. The addition of a cross-linking agent was to form chemical bonds between the molecular chains to form a three-dimensional network of connected

molecules (17, 22).

Cellulose membranes are very durable and can be used on automated systems, due to uniformity and the ability to control the degree of stretch, portion control is easy to achieve. Cellulose membranes are indigestible and must be removed before consumption.

Vegetarian membranes are 100% plant-based and are in development to produce membranes that can be used in Halal or Kosher food manufacturing.

Food additives: In order to minimize the deterioration of the quality of the natural salted coating over time, food additives were used as preservatives. Previous studies on the use of certain additives during the processing of natural sausage casings have identified phosphate as a suitable agent (23).

Some studies observed a marked improvement in the hygienic aspects and mechanical properties of phosphate-treated casing. In particular the effect of trisodium phosphate on the slippage of the carcass during the filling process showed a clear improvement (3, 9, 12).

Nitrites and nitrates are used in meat processing to prevent the growth of harmful bacteria and to develop the color and flavor of the products. The ability of nitrite to limit the growth of bacteria, especially toxin-producing strains of *Clostridium botulinum* has been previously demonstrated (19). Nitrite also promotes the preservation of clean meat due to its antioxidant capacity (2).

Nitrite and nitrate salts are used as food additives in various processed meat products to prevent or reduce the growth of pathogenic bacteria (eg, *Clostridium botulinum*, *Clostridium perfringens, Listeria monocytogene*s (*L. monocytogenes*), *Salmonella spp*.), to extend the of shelf life, to limit oxidation and to contribute to the color and taste of processed products (organoleptic functions) (4, 15).

Compared to nonconsumers, high consumers of nitrate and nitrite food additives had a higher risk of developing several types of cancer: nitrate food additives and nitrite salts were positively associated with the risks of breast cancer and prostate, while no association was observed for nitrites/nitrates from natural sources (9, 14).

In terms of the market, consumers demand the healthiest meat products that are low in fat, salt, cholesterol, nitrates and calories, which additionally contain health-promoting bioactive components such as carotenoids, unsaturated fatty acids, sterols and fiber. At the same time, consumers also accept meat products that contain flavor and taste enhancers, but which are traditionally processed.

Artificial membranes are in high demand in the food industry due to low production costs, reduced manufacturing time and processing of the finished product.

From a hygienic point of view, artificial membranes have an advantage because microbiological contamination is negligible, and during storage and transport the risk of damage is minimal.

Food additives, especially nitrite and nitrate salts are used in the preservation of sausages, reducing the growth of pathogenic bacteria, but frequent

consumption and in large quantities represent a real risk for the development of certain types of cancer.

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PREVALENCE OF BEHAVIOURAL DISORDERS IN DOGS IN TIMISOARA

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Summary

Behavioural disorders are common among dogs. It has been estimated that up to 90% of dogs may exhibit behaviours that their owners find unacceptable. So, for most people, the only solution seems to be to give up the animal to a shelter. The chances of a dog developing a behavioural disorder are dependent on several factors, including breed, age, sex, diet and relationship with the owner. There are numerous reports in the literature on the prevalence of behavioural problems in dogs, based on the caseloads of those who deal with these problems. While this information is useful in itself, it remains largely unknown how it relates to the prevalence of these behaviours in the wider population. This paper aimed to determine the prevalence of behavioural disorders among the clients of several clinics in the Municipality of Timisoara, to determine the percentage of occurrence of behavioural changes, by analysing predetermined variables, to obtain an overview of the predisposition of a breed about the type of behavioural change occurring as well as the percentage of occurrence of a behavioural disorder.

Keywords: behavioural modification, anxiety disorder, clinical ethology.

Dogs have changed, in so many ways, during domestication and now they depend on humans for their well-being. In many ways, modern dogs seem to be better adapted to communicating with humans than other dogs (2, 3). Dog owners are faced with normal but unacceptable behaviours: dogs bark at inappropriate times, urinate on furniture, pull on leashes, walk out the front door and growl at strangers (3). Because of the diversity of phenotypes and functional specializations, dogs represent an extremely diverse biological group that allows only limited and superficial generalizations (16, 19).

Fear is a natural, instinctive feeling of awe resulting from a situation, person or object that presents a perceived external threat. It is the result of the autonomic nervous system preparing the body for the freeze, fight or flight response (10, 18).

It is considered normal behaviour, essential for adaptation and survival. However, the situation surrounding the cause of the fear must also be considered and can be classified as normal/rational or abnormal/irrational and inappropriate.

Anxiety can be defined as the anticipation of future dangers of unknown or imagined origin resulting in reactions associated with fear (4). Separation anxiety is one of the most common forms of anxiety affecting dogs. Other forms of anxiety may be related to noise, travel or unknown scenarios.

A phobia is an excessive or persistent fear of a specific stimulus or situation.

Any animal can develop a phobia of almost any situation or stimulus (12). Events associated with danger can cause fear in pets.

Fear can become a phobia. A phobia is a fear out of proportion to the danger of the real situation. Phobia is defined as a persistent, excessive and irrational fear response to a situation.

Aggression is controlled by the limbic system, which includes the hippocampus, hypothalamus, amygdala, cingulate gyrus, midbrain and thalamus (13). The cerebral cortex interacts with this system. The core of the system's activity is a direct response to inputs from the environment, both external and internal, that may be crucial to the animal's survival, as well as situational analysis of possible or actual threats (12, 13).

The limbic system is involved in triggering positive or negative emotions, sexual drive responses and the need to acquire food (6, 7, 9). It also affects cognitive abilities, selection of situationally appropriate action tactics (learning and recall) and triggers an effective behavioural response (decision-making processes: attack or flight).

The ethology of compulsive behaviour in dogs is not fully understood; however, several prominent risk factors have been identified (10). Compulsive behaviours are most commonly reported in dogs that have been stressed by excessive isolation, exposed to sensory-motor deprivation (e.g., boredom and inadequate exercise), given inadequate attention and social stimulation, or exposed to a high-conflict environment (13, 17).

Stereotypic behavioural patterns may develop as a result of neurobiological stressors; for example, hyperkinetic dogs under the influence of long-term amphetamine treatment may exhibit destructive behaviour or spontaneous barking (8).

Compulsive symptoms also often occur in highly excitable or nervous dogs in which no external causes or stressors can be identified, suggesting that a genetic predisposition may underlie the aetiology of some compulsive behaviour disorders (14, 16, 18).

Once established, the frequency and range of contexts in which compulsive behaviour occurs may increase and expand over time, making early diagnosis and treatment imperative.

Materials and methods

The study was carried out between May 2021 and May 2023 in four clinics in Timisoara. The study was based on the behavioural assessment of all patients who presented to the consultation for behavioural reasons. All dogs whose owners mentioned different behavioural problems during the consultation were taken into the study. From a descriptive point of view, all general characteristics of the patient were noted at an early stage and an observation sheet was drawn up. The observation sheet was then supplemented by a paraclinical analysis of the patient,

where appropriate. A differential diagnosis was made to confirm the suspicion of behavioural change and to rule out any medical pathology, with behavioural change, as a clinical sign. At the end of the study, data were collected from 235 canine patients presented to the practice, out of which only 105 were taken into the study. The 130 canine patients eliminated from the study were confirmed with various medical pathologies and thus, did not present behavioural disorders, and therefore were not included in the final analysis.

For the 105 patients, an Excel table was compiled with individual data, i.e. age, breed, sex, length of ownership by current owner, origin, and number of hours per day. It was also chosen to record the season in which the patient presented to the practice and the patient's history, depending on the suspected problem. Questions specific to the behavioural disorder being analysed were added to allow for the whole analysis. After the first consultation, an ethogram was carried out on each patient over 7 days and the situation was reviewed. At the end of the study, the total number of patients confirmed with behavioural disorders after the ethogram, the most likely reason for the occurrence of the behavioural disorder, the nature of the behavioural disorder and the analysis of a probable race specificity were taken into account.

Once the descriptive phase was completed, with the help of Excel software, the data collected were statistically analysed, analysed by the variables sought, and plotted in graphical and percentage form, to finally represent the final hypotheses on the prevalence of behavioural disorders and possible connections between genetic character, environmental influence and the occurrence of behavioural disorders.

Results and discussions

If we look at the results on the sources of dogs diagnosed with behavioural disorders over twenty years (5), dogs taken from rescues and shelters have become more commonly reported than dogs from breeders.

Unfortunately, provenance information is not known for all dogs presenting to medical facilities, so we do not know whether this reflects a change in the preferences of the general population or whether dogs from shelters or rescue centres are overrepresented in behaviour disorders.

From the 105 dogs, as shown in Table 1 and Fig. 1, 43 (40.95%) dogs were found to exhibit separation anxiety, 22 (20.95%) exhibited phobias, another 22 (20.95%) exhibited excessive licking, redirected aggression was present in 10 dogs (9.52%) and 8 dogs (7.63%) were identified as having destructive behaviour.

Table 1

Behavioural disorders in patients

Nr. crt.	Behavioural disorder	Females	Males	Total
1	Separation anxiety	23	20	43
2	Phobias	10	12	22
3	Obsessive compulsive licking	16	6	22
4	Destructive behaviour	2	6	8
5	Redirected aggressivity	5	5	10

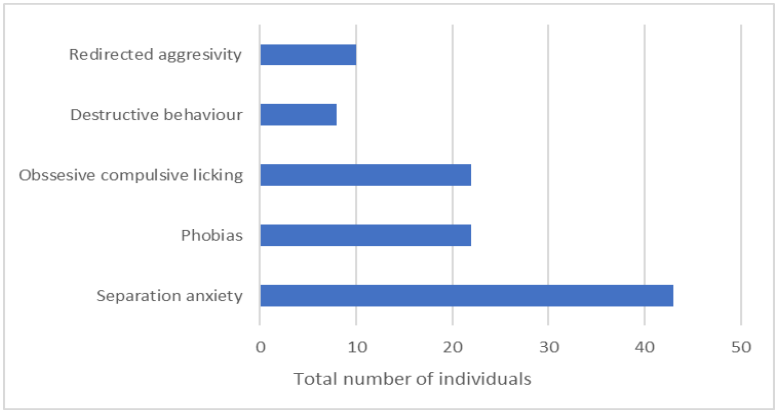

Fig. 1. Distribution of cases based on the behavioural modification

In the case of anxiety, 23 dogs were male (53.48%) and 20 were female (46.52%). Of the purebred category, there were 38 dogs (88.37%) and 5 were mixed breeds (11.63%). In terms of age, the average was 5 years old with a higher percentage of 62.79% (n. 27) being under 5 years old, followed by the 5-8 category (n. 14) with 32.55% and only two dogs (4.66%) being older than 8 years old. Almost half of the dogs (n. 21) showed symptoms of separation anxiety since their owner took them home and the rest developed it later.

In the studies researched, higher rates of separation-related behaviour problems have been reported for dogs living with a single adult, a couple, or multiple adults than for those living with a family with children, but other researchers have found that the presence or absence of children in the home does not affect the prevalence of separation-related problems (2, 12, 19).

Of all dogs diagnosed with this behavioural problem, a higher prevalence was observed in the Bichon and Labrador Retriever breeds, followed by the German Shepherd (Fig. 2, 3).

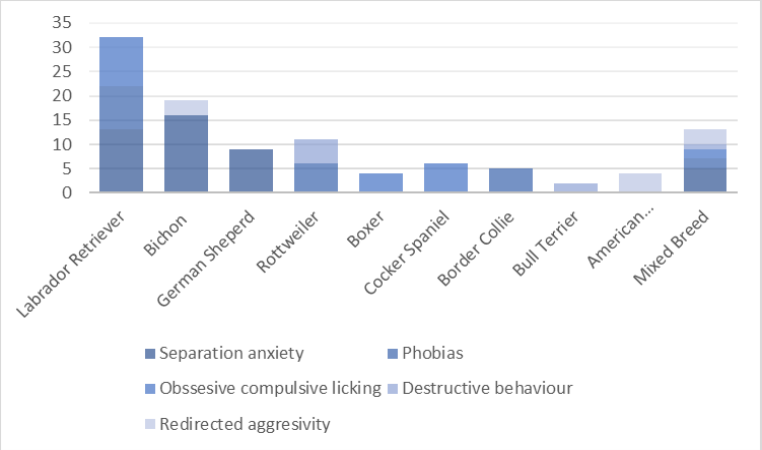

Fig. 2. Breeds distribution

For dogs diagnosed with obsessive-compulsive disorder based on excessive licking, the majority of dogs (n. 16) were female with a percentage of 72.72%, and only 6 males (27.28%) were diagnosed with this behaviour disorder. The breeds of dogs with this problem were diverse, of which a higher prevalence was observed in dogs of the Labrador Retriever, Cocker Spaniel and Boxer breeds.

These dogs ranged in age from 2 to 7 years, with an average age of 4 years. It was also found that 64% of these dogs lived in multi-pet households.

In the case of phobias, the age range of the dogs ranged from 3 to 8 years. Phobias were approximately equally distributed between male (n. 12) and female (n. 10) dogs. The most common breeds associated with phobias were Labrador Retriever, Rottweiler and Border Collie. The most common phobia was storm phobia, which affected 10 dogs (n. 8 females, n. 2 males), meaning 45.45% out of all affected cases. Other phobias included fireworks, vacuum cleaners and strangers. Almost all dogs exhibited phobic behaviour from an early age (n. 18), while 4 developed phobias later in life. Most dogs (n. 20) spend a considerable amount of time with their owners.

In one study (15) it was found that owners reported in 40 dogs that they were afraid of any noise in the past, but not currently (16 fireworks, 17 thunder and 5 gunshots). Of these, 16 indicated that they had not received any advice on how to treat their dog for its behaviour. This suggests that there are a small number of cases where dogs appear to recover spontaneously, although other information recorded in interviews suggests that about half of these animals' 'recovery' was associated with the onset of hearing loss.

At present, there is little evidence on the prevalence of phobic behaviour in dogs following exposure to loud noises. Unpublished data reported by the ASPCA (17) suggest that up to 74% of respondents to an online survey had a dog with

some type of aversive reaction to noise, although owners of affected dogs may be more likely to participate in such studies. Some indication of the extent of the problem results from case reports from specialist behavioural clinics, where between 10 and 20% of dogs present due to noise-related fear-phobic reactions.

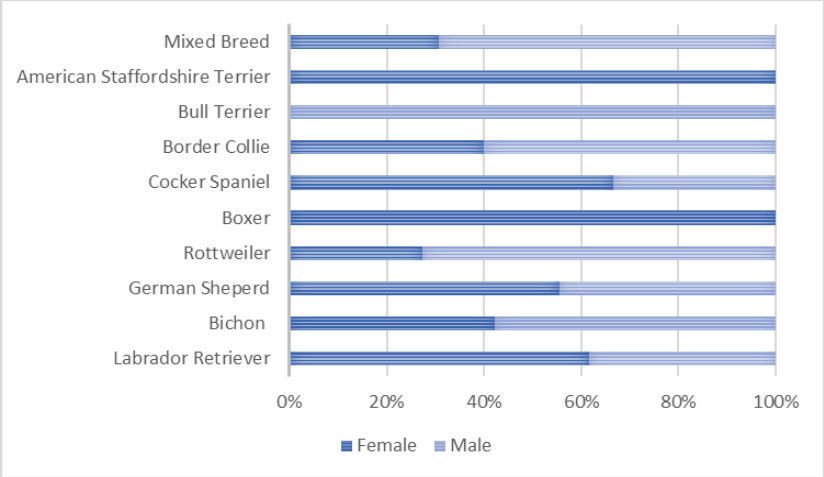

Fig. 3. Percentage of male and female patients

A total of 8 dogs were identified as having destructive behaviour. The ages of these dogs ranged from 6 months to 4 years. Male dogs were more prone to destructive behaviour. The affected breeds were Rottweilers and Bull Terriers, but the low number cannot be conclusive statistically for further interpretation. A study of 100 dogs who visited a veterinary clinic for reasons other than behavioural problems found that 82% of dogs exhibited behavioural problems, most often destructive behaviour, barking and aggression towards humans (7).

As for redirected aggression,10 dogs were diagnosed with this behaviour disorder. In a study of aggression (7), the authors reported that, for all types of aggression, measured variables explained a relatively small amount of variation between aggressive and non-aggressive animals, suggesting a much greater importance of factors specific to the individual dog's experience in the development of aggression. The age range of these dogs ranged from 8 months to 5 years. No significant difference was found in the gender distribution of this disorder.

The 10 patients diagnosed were dogs of the breeds Doberman Pinscher, Staffordshire Terrier and Bichon. The study found that 70% of these dogs were kept in strict isolation for long periods, and 50% of the cases started after painful medical procedures.

The distribution of cases (Fig. 2) in this dataset is consistent with several previous studies (5, 8, 9, 15) that have examined referrals to behavioural

veterinarians, with separation anxiety being the most common problem.

Regarding breed-related findings, it has been assumed in the literature (1, 11) that mixed breeds and German Shepherds present more often for behavioural problems.

Differences between our results and owner-reported data may be, in part, related to correlations between dog size and potential risk posed. For example, an owner might be more motivated to seek treatment for a larger dog (German Shepherd) than for a smaller dog (Chihuahua).

Conclusions

The study showed that the prevalence of behavioural problems in dogs varies. The total prevalence of behavioural disorders was 44.68%. For separation anxiety, the prevalence was 40.95%, in phobias 9.52% the same percentage as licking disorder, the percentage of destructive behaviour was 9.52% and for redirected aggression, it was 7.63%.

The study also highlighted the importance of understanding the specific time when behavioural problems began, as this information can provide invaluable insight into potential triggers or causes of such behaviour.

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CONGENITAL TORTICOLLIS IN CHINESE GOSLINGS – CASE REPORT

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Summary

Torticollis is a contracture of the neck muscles, mainly involving the sternocleidomastoid muscle, with multiple causes including inbreeding. We present the case of two Chinese goslings, originating from geese raised in an extensive system, that presented torticollis in the first week after hatching. We tried to treat them with massage, vitamin therapy and physical therapy, but without result.

Keywords: torticollis, congenital, inbreeding, Chinese goose, treatment.

Torticollis is a degenerative myopathy, represented mainly by the contracture of the neck muscles, mainly of the sternocleidomastoid muscle, being present degenerative lesions at their level (4, 5, 9, 12, 13, 14). As a result of the muscle contracture, the head deviates to the side and balance difficulties occur (4). The causes of torticollis are multiple and it can appear at any age depending on the etiology, congenital torticollis usually occurs in the first weeks in humans and in the first week in birds (1, 4, 5, 6, 8, 9). We present the case of two goslings that presented torticollis in the first days after hatching, their treatment was attempted but without result.

Materials and methods

We present the case of two Chinese goslings, hatched at the end of June, 2023, from eggs from a breeder in Dâmbovița County. From 15 purchased eggs, 11 goslings hatched, after hatching none of them showed any clinical signs. From the 3rd day the first gosling started showing torticollis, and the next day the second one started too. The owner contacted the breeder from whom the eggs came and found out that he practices inbreeding to select certain characters and has had such cases in the past.

Goslings with torticollis presented with head deviation to the side, one of them to the right side and the other to the left side, they started to have loss of balance, difficulty in maintaining a straight line while walking and difficulty in returning to a normal position when standing on the back (Fig.1). After observing the clinical signs, the treatment was tried with massage, physiotherapy and oral administration of vitamins B1, B3, B6 and E.

Results and discussions

The treatment did not lead to any results, although in the first days there seemed to be an improvement, with time the clinical signs worsened.

Fig. 1. The two goslings at the age of 10 days

Over time, the two affected goslings began to develop more and more difficult and the condition to degenerate. The two remained much smaller compared to the other goslings, this being also due to the difficulties encountered at the time of feeding, they presented difficulties in prehension, thus affecting their development. Also the degree of deviation of the head and neck began to increase, and difficulties in walking and balance to worsen (Fig. 2).

Fig. 2. One of the goslings 2 months after the appearance of clinical signs

Torticollis has multiple causes and is a muscular contracture of the neck muscles followed by a sideways head turn and various mobility difficulties (4, 5, 9, 12, 13, 14). Among the multiple causes of torticollis in both humans and birds are the congenital factor and inbreeding (1, 4, 9). It has been observed that in inbred bird populations, torticollis also occurs in newly hatched chicks, usually in the first week, in a small percentage (4, 5).

Degenerative lesions occurring in the muscles are similar for both humans and animals, thus studying this condition in animals makes possible a better knowledge of this condition in humans, its congenital causes being still little known (4, 5).

The treatment used in this case, the massage, physiotherapy and oral administration of vitamins B1, B3, B6 and E, was similar to the treatment used in other cases of congenital torticollis, but the goslings shown no improvment after it (2, 3, 7, 10, 11, 15).

Conclusions

Among the multiple causes of torticollis in humans and animals, there is also the genetic factor. Congenital torticollis occurs, most of the time, in the first weeks after birth in humans and in the first week after hatching in birds. Degenerative lesions in the neck muscles are similar in both humans and birds, the causes of their onset are still little known. The goslings studied come from inbred birds with a history of torticollis. They showed clinical signs on the 3rd and 4th day after hatching.

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MICROBIOLOGICAL CONTAMINANTS OF HONEY BEE

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Summary

Due to its particular composition, honey has the ability to inhibit or even destroy microorganisms. However, certain types of microorganisms persist in honey, usually in a dormant state and some yeast species, due to their pronounced osmophilicity, can multiply and cause fermentation of the product, making it unfit for human consumption. The research was performed between 2022 and 2023 and a total of 552 samples from 5 sorts (acacia honey, lime honey, sunflower honey, rapeseed honey and polyfloral honey) were collected and examined. The laboratory analyses aimed to determine the total number of yeasts and moulds using the colony counting technique (cfu/g). The honey samples examined were found to have minimal contamination, with some samples showing the presence of conditionally pathogenic or pathogenic bacteria, and some samples had yeast and mould levels above 100 cfu/g, with sunflower and rapeseed honey having the highest numbers of fungus.

Keywords: honey, bacteria, yeast, fungus.

Honey, unlike other foods is not a favourable substrate for the survival and multiplication of microorganisms; due to its particular composition, honey has the ability to inhibit or even destroy them. For this reason, microbiological examination of honey has not been a concern for honey specialists (2, 8, 9).

The ability of honey to inhibit or even destroy microorganisms is due to a complex of factors, the most important of which are: high sugar content and very low free water, which causes dehydration, shrivelling and death of microbial cells, acidity (pH ≤ 4.5), the glucose oxidation system in honey, which causes the formation of hydrogen peroxide, a toxic product to microorganisms, known long before identification as "inhibin", very low protein content and high C/N ratio of honey, the lack of oxygen in honey, as atmospheric oxygen cannot penetrate the honey because of its viscosity (most contaminating microorganisms need oxygen in order to grow), the presence in honey of chemicals and enzymes that are unfavourable to the growth of microorganisms (pinocembrin, lysozyme, phenolic acids, terpenes, benzyl alcohols, various volatile substances), the unfavourable electrical charge created by the reducing sugars in honey, which inhibit the multiplication of moulds and aerobic bacteria (1, 11, 15, 17).

However, rare types of microorganisms persist in honey, usually in a dormant state, and some yeast species can multiply and alter the product, making it unfit for human consumption. This has recently led producers and specialists to pay increasing attention to the microbiological quality of honey and to require its

microbiological examination before marketing (1, 4, 14, 16).

Contamination of honey with microorganisms occurs at the hive level, by bees, during harvesting and nectar deposition, as well as after harvesting and primary processing of honey. Contamination during the harvesting and primary processing of honey is of the most important hygienic and sanitary significance, as it often includes pathogenic or potentially pathogenic microorganisms from the persons carrying out these operations. It is caused by unhygienic working conditions and inadequate microbiological quality of the water used. Moreover, the poor microbiological quality of the water used by bees can also contribute to contamination during harvesting and nectar deposition in honeycombs (5, 10, 15).

According to current data, primary and industrial processing can increase or decrease the total number of microorganisms in honey (2, 6).

Materials and methods

Samples of honey were taken from different marketing outlets located in the Bucharest area (city and suburbs), the research work aimed to include different assortment types, produced by different economic agents and marketed in different establishments. For this study, five honey varieties were chosen: acacia honey, lime honey, sunflower honey, rapeseed honey and polyfloral honey.

The samples were placed in sterile containers and transported under optimal temperature conditions. Sample processing was carried out in the food microbiology laboratory according to STAS microbiological rules and ISO standardized methods (10, 11, 13, 20).

The study was conducted over a period of 1 year (from 2022 to 2023), analyzing a total of 552 samples (125 samples of lime honey, 114 samples of acacia honey, 96 samples of sunflower honey, 89 samples of rapeseed honey and 128 samples of polyfloral honey).

To determine the total number of mesophilic and aerobic germs, decimal dilutions were made in peptone water, from each dilution, 1 cm^3 of each was distributed with sterile pipettes in 2 Petri dishes. Melted agar cooled to 40 - 45°C was poured into each plate, homogenized and incubated for 24 h at 37°C. The average number of colonies/g produced was determined (1, 3, 11, 19).

Decimal dilutions were made to determine the probable number of coliform bacteria, with 1 ml of each dilution being placed in 3 tubes of BBLV medium (lactic broth with bile salts and brilliant green) and Durham tube. Incubation for 24 to 48 hours at 37 °C. Interpretation was done by gas production and calculation of the 3-digit mean (based on the score received by each of the 3 tubes of BBLV medium), the mean obtained was interpreted using the Mac Grady table. In parallel, the method of determining the number of coliform bacteria by colony counting (ISO 4832) was also used (1, 4, 10, 12).

From the decimal dilutions, 1 ml was distributed with sterile pipettes in 2 Petri dishes. Pour VRBL medium (agar with lactose, bile salts, crystal violet and

indicator), melted and cooled to 45°C, into each plate. The prepared plates are incubated at 35°C for 24 hours. After incubation count the red-violet colonies and calculate the mean with the formula:

$$\sum \frac{c}{n1 + 0{,}1\ n2} \times d$$

where Σ c is the sum of colonies counted, n1 is the number of boxes of the first dilution retained for counting, n2 is the number of boxes of the second dilution retained for counting; d is the dilution rate corresponding to the first dilution used (1, 3, 5, 11, 16, 17).

Determination of the number of pathogenic staphylococcus was done by a technique similar to that used for NTGMA determination, using Chapman's agar or Baird Parker agar as solid medium (3, 4, 8, 9).

The determination of the number of sulfite-reducing bacteria was carried out on sodium sulfite and iron citrate medium, melted and cooled to 45°C in which 1 ml of decimal dilutions were seeded. It was incubated anaerobically at 37°C for 24 to 48 h, after which black colonies were counted (1, 2, 3, 11).

For the determination of pathogen species and pathogen conditioners, standardized ISO methods were used: for the identification of *Salmonella* bacteria - SR ISO 6597, for the identification of plasmo - coagulazo - positive staphylococcus - STAS ISO 6888, for the identification of Proteus bacteria - SR 2356/1, for the identification of *Escherichia coli* bacteria - STAS ISO 4832, for the determination of the probable number of E. coli - SR ISO 7251, for the identification of *Bacillus cereus* species - SR ISO 7932, for the determination of the number of moulds - STAS 12965 - 91, for the determination of the number of yeasts - STAS 12964 - 91, etc (1, 11, 12, 18).

All isolated bacterial strains that were presumptively identified as belonging to pathogens or conditionally pathogenic were biochemically tested for confirmatory diagnosis, thus rigorously demonstrating the scientific data obtained (6, 7).

Results and discussions

The results obtained from the complete bacteriological analysis of each sample collected and processed were statistically processed by calculating averages for each product type.

Microbiological analysis to assess the presence and number of coliform bacteria, *Escherichia coli* and coagulase-positive staphylococci showed that a number of samples had such microorganisms. Microbiological analysis to assess the number of sulphite-reducing bacteria and *Bacillus cereus* showed the presence of these bacterial species in the samples studied; although they do not represent an absolute hazard to consumer safety, biochemical processes may be generated leading to fermentation and denaturation of the products concerned. The number of samples analysed in which the different species or genera of microorganisms were

recorded are shown in (Table 1), with statistical analysis of the variations for the number of samples analysed from each assortment of honey.

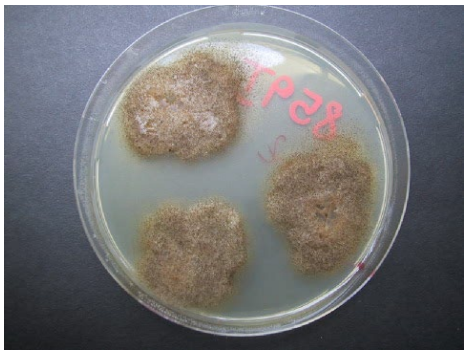

Fig. 1. Positive sample for *Mucor sp.* in polyfloral honey

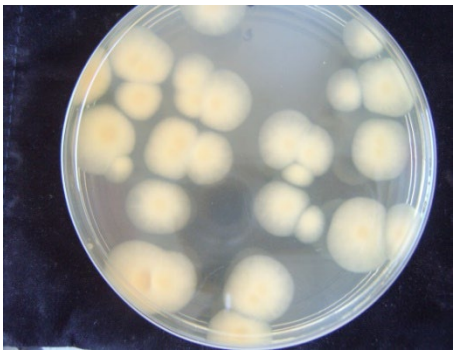

Fig. 2. Positive sample for *Aspergillus sp.* in polyfloral honey

Table 1

The microbiological analysis results for honey bee product samples

Type	Bacteriological parameter investigated									
	Coliform bacteria		*E.coli*		Coagulase-positive staphylococcus		*B.cereus*		Sulphite-reducing bacteria	
	No. of samples	%	No. of samples	%	No. of samples	%	No. of samples	%	No. of samples	%
Lime honey	12	9.68	4	3.22	15	12.09	4	3.23	1	0.81
Acacia honey	17	14.78	7	6.09	21	18.26	2	1.74	5	4.35
Sunflower honey	5	5.10	1	1.02	5	5.10	2	2.04	-	-
Rapeseed honey	4	4.59	-	-	2	2.29	1	1.15	2	2.29
Polyfloral honey	28	17.83	11	7.01	22	14.01	4	2.55	10	6.37

Statistical analysis of the results showed that all honey varieties had an inadequate number of samples.

No *Salmonella* bacteria were isolated from the total samples analysed. Instead, bacteria belonging to the genus *Proteus, Clostridium*, or samples with a yeast and mould load (NTDM) greater than 100 were isolated. Some cheeses were also found to contain mould species that either produce toxic compounds (*Aspergillus*) (Fig. 2) or degrade the biochemical structure of proteins and lipids and alter organoleptic indicators (*Mucor* sp.) (Fig. 1). The number of positive samples and the percentage determined of the total samples analysed for each

assortment is shown in (Table 2).

Table 2

The microbiological analysis results for honey bee product samples

Type	Bacteriological parameter investigated									
	Proteus		*Clostridium perfringens*		NTDM		*Aspergillus sp.*		*Mucor sp.*	
	No. of samples	%	No. of samples	%	No. of samples	%	No. of samples	%	No. of samples	%
Lime honey	-	-	2	1,61	2	1,61	-	-	-	-
Acacia honey	1	0.64	2	1.27	4	2.55	1	0.64	-	-
Sunflower honey	2	2.56	4	5.13	-	-	4	5.13	7	8.97
Rapeseed honey	1	1.79	1	1.79	4	7.15	2	3.57	-	-
Polyfloral honey	4	3.57	2	1.79	2	1.79	2	1.79	1	0.89

NTDM – număr total de drojdii şi mucegaiuri

Based on the results of bacteriological analyses performed it is shown that a number of products are contaminated either during transport or directly at the point of sale, due to storage in inappropriate conditions or due to storage at temperatures inappropriate to the type of product.

The presence of *Aspergillus* and *Mucor* species has been observed in sunflower honey and polyfloral honey, which practically leads to the frequent development of fermentative biochemical phenomena (4, 5, 6, 7).

Conclusions

All the assortments tested showed microbiologically unsatisfactory samples. Three assortments in particular stand out: linden honey, polyfloral honey and rapeseed honey, which recorded the highest number of inadequate samples for most of the microbiological parameters analysed.

Microbiological analysis showed that none of the products had samples contaminated with *Salmonella* germs.

The microbiological examination for the identification of bacteria belonging to the genus *Proteus* revealed the presence of these bacteria in 8 samples, of which 4 samples belonging to a single type of product (polyfloral honey).

Clostridium perfringens species was identified in 16 samples (2.88% of the total samples examined), the highest number of positive samples being recorded in sunflower honey samples (4.16%).

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PREVALENCE AND RISK FACTORS OF SUBCLINICAL MASTITIS IN DAIRY COWS FARMS IN THE PORO REGION (IVORY COAST)

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Summary

Establishing an early diagnosis of subclinical mastitis is particularly important in order to reduce the economic losses caused by the evolution of this pathology in dairy herds. The present study aimed to determine prevalence of subclinical mastitis in traditional cattle farms in the Poro region (northern Ivory Coast). A total of 360 lactating cows in 45 traditional farms in the four departments of this region (Dikodougou, Korhogo, M'bengue and Sinematiali) were taken into study. Somatic Cell Count (SCC) with Delaval Cell Count (DCC) and California Mastitis Test (CMT) were used to analyze the collected milk samples. These tests revealed cases of mastitis in the four departments of Poro region at frequencies varying from 54.17% to 97.92%. The prevalence was 75.5% and 80% (positive at score ≥ 2) using DCC and CMT respectively. There was no significant difference between prevalence obtained with these two tests (p = 0.312). The prevalence of mastitis in crossbred and zebu cows (100%) was significantly higher (p = 0.003) than that of local cow breeds with 90%, 78.88% and 75% respectively for N'dama, Méré and Baoulé. In addition, month of lactation and number of calving had no effect on occurrence of mastitis (Odds ratio = 0.279; 95% CI: [0.474-0.083]); (Odds ratio = 0.164; 95% CI: [2.341-2.683]). However, the risk of onset of subclinical mastitis is higher in older cows (Odds ratio = 10.09; 95% CI: [0.283-0.148]).

Keywords: subclinical mastitis, prevalence, CMT, DCC, Ivory Coast.

Livestock in Ivory Coast still remains a secondary economic activity with a contribution of around 4.5% to agricultural gross domestic product (GDP) and 2 % to total GDP (10). Dairy farming is a sector with a strong socio-economic impact. Indeed, it occupies a non-negligible dimension in human food by production of milk and red meat in particular; it is also a source of income for producers and farmers (14). The deficit in animal protein, particularly in milk and dairy products, is a permanent problem in Ivory Coast. Production of this country covers 51% of national meat consumption, and only 17% of that of milk and dairy products (14, 20). Thus,

in order to resolve this ever-increasing need for milk and derived products, the Ivorian State committed to developing local dairy production (13). This solution also aims to reduce this country's dependence on milk and bovine protein on Sahelian and European countries, which gives rise to products (7). However, despite all efforts, the results of improved milk production are mixed. If the quantity of local milk is insufficient, the hygienic and sanitary quality of milk produced could be improved in order to make the local sector competitive. Local dairy production faces a number of constraints that prevent its competitiveness, such as mastitis, which has an effect on the quantity and quality of milk, particularly the chemical composition (11). Indeed, diagnosis of clinical mastitis will be relatively easy thanks to visible clinical signs that of subclinical mastitis will be less so. However, with the Somatic Cell Count and California Mastitis Test, screening for subclinical mastitis becomes possible and useful (15). Due to its impact on milk production and quality, as well as on public health, subclinical mastitis control remains a common concern for dairy producers. Also, the identification of the bacterial strains responsible for mastitis is essential to the therapeutic choice (1).

By definition, mastitis is characterized by the presence of pathogenic germs and the presence of abnormally high numbers of somatic cells in milk (11, 19, 21). Due to affecting the quality and quantity of milk production, the profitability of dairy farms, therefore, depends on the control of subclinical mastitis, and this through a better knowledge of risk factors and screening for these mammary infections.

All these being exposed, the objective of this study was to determine the prevalence and risk factors of subclinical mastitis in traditional dairy cow farms in Poro region (northern Ivory Coast) by California Mastitis Test (CMT) and DeLaval Cell Counter (DCC).

Materials and methods

The studied area

The Poro region is a territorial community in northern Côte d'Ivoire whose name comes from the cultural practice of the peoples (Sénoufo) of this locality. It is located between 8°30 and 11° North latitude, 5° and 6° West longitude and covers a total area of 13400 km² (Fig. 1). The population, mostly rural, practices agriculture and livestock; animal husbandry is the main activity that characterizes this region (18). The cattle breeds observed in the Poro region are Baoulés, Méré, N'dama, Zébu and Métis; three of which have different phenotypes of cattle which constitute the herd and are made up of bullfighting (Bos taurus) N'Dama and Baoulé, the Méré breed (a cross between male Zebus and female Baoulé), zebus (Bos indicus) which is the cross between the Zebu (Gobra x Goudali) and crossbreeds from crosses between zebus and taurines or crosses between (local breeds x exotic breeds). Breeding is practised in a traditional way in the dairy farms visited at the level of each locality.

Fig. 1. Map of the Poro region showing the different localities taken into study

Samples examination

Three hundred and sixty (360) dairy cows were taken into study from 45 traditional farms in the Poro region (northern Côte d'Ivoire).

A questionnaire was used to collect information on the typology of farms (age, breed, month of lactation, number of calvings). Within the 360 cows studied, five types of breeds were distinguished: 322 cows of the Méré breed, 20 cows of the N'dama breed, 8 cows of the Baoulé breed, 6 cows of the Zebu breed and 4 cross-bred cows (crossing between Gobra and exotic European breeds). Regarding the lactation period, 300 of the dairy cows were between 1 and 3 months of lactation, 52 were between 4 and 8 months of lactation, and 2 were 12 months of lactation. Then, 86 were in the first calving (primiparous), 130 were in the second calving, 84 cows in the third calving and 60 cows had calved more than three times. Finally, 144 were between 3 and 5 years old, 182 were between 6 and 9 years old, 24 were between 10 and 12 years old, and 10 were between 13 and 15 years old.

Individual and mixing milk were collected in sterile 30 ml bottles and kept cool in a cooler.

The California Mastitis Test (CMT) was used as the field method to quantify results at each farm visited; it is inexpensive, reliable and easy to use in breeding. The test was applied according to the protocol provided by the manufacturer (24). The presence and appearance of the flocculation are noted by transparency on a scale of 0 (healthy cow = normal consistency) to 4 (sick cow = thick gel, consistency of egg white). The result is considered positive at a score ≥ 2, apparently healthy (negative ≤ 1) and doubtful at CMT=1, then negative if the score is equal to 0 (3).

Somatic cell counting was performed by the fluorescence method (DeLaval

Cell Counter (DCC), Tumba, Sweden). the stages of the work technique were carried out according to the protocol developed by the manufacturer. The measurements were carried out using cassettes containing a reagent, which makes milk cells fluorescent. The interpretation of the results is presented in Table 1.

Table 1

Interpretation of the results of the DeLaval DCC® device (8)

Cell counts (cells/mL)	Interpretation
< 300.000	Healthy
Between 300.000 and 800.000	Doubtful
> 800.000	Contaminated (subclinical mastitis)

Statistical analyzes

The Excel spreadsheet was used to record and collect the data. The comparison of infection according to breed, age, month of lactation and number of calvings was made using the Khi2 test of independence with the XLSTAT software version 2019. The difference between these variables was considered statistically significant with $p < 0.05$.

Results and discussions

The percentage of positive cows by CMT, respectively with subclinical mastitis (positive ≥ 2) was 80 % and the one of apparently healthy (negative ≤ 1) was 20 %.

The prevalence of positive cows in the departments visited is presented as follows: M'Bengué 97.92%, Korhogo 89.58%, Dikodougou 77.78% and 54.17% in Sinématiali (Fig. 2).

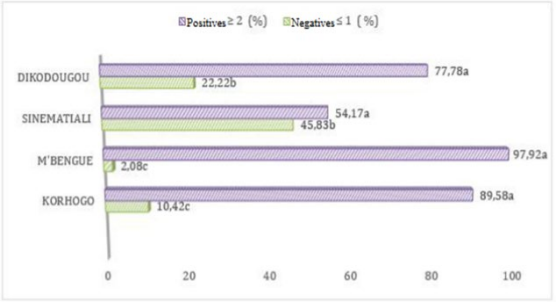

Fig. 2. The prevalence of subclinical mastitis in studied localities

NB: a, b and c of the proportions of the same line affected by different letters are significantly different at the 5% level

Prevalence of mastitis by CMT

The results of the CMT (California Mastitis Test), depending on the cattle breeds, are presented in Table 2. The results demonstrated that mixed breeds were more commonly affected by subclinical mastitis than local breeds ($p < 0.05$).

Table 2

Prevalence of mastitis depending on breed

Prevalence %	Meres	N'Dama	Baoules	Zebus	Metis	P-value	Total
	N= 322	N= 20	N= 8	N= 6	N= 4		N= 360
Positive %	78.88%	90%	75%	100%	100%	0.03	80%
Doubtful %	9.32%	10%	0	0	0		8.88

The prevalence of subclinical mastitis during the lactation period is presented in Fig. 3. The Odds ratio test showed that there was a lower risk for cows in the advanced month of lactation being infected (Odds ratio = 0.279; 95% CI: [0.337-1.880]).

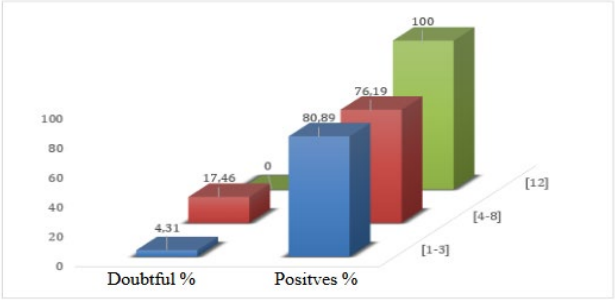

Fig. 3. Prevalence of subclinical mastitis by CMT, during the month of lactation

The two-by-two comparison of the variables between them interval of (1-3) months of lactation and (4-8) months of lactation ($\chi 2= 7.70$; $p=0.005$), interval of (1-3) months of lactation and (12) months of lactation ($\chi 2= 5.17$; $p= 0.022$), (4-8) months of lactation and (12) months of lactation ($\chi 2= 20.49$; $p< 0.0001$).

Although the prevalence of cows with calving greater than 3 is 86.66%, no significant difference was observed (Table 3). Cows with high calving had a higher risk of subclinical mastitis at the same time than cows with low calving ($p > 0.05$).

Table 3

Prevalence of the CMT test according to calving

Prevalence %	Primiparous	2nd calving	3rd calving	> 3 calving	Total
	N= 86	N= 130	N= 84	N= 60	N= 360
Positive %	74.42%	76.92%	85.71%	86.66%	80%
Doubtful %	8.14%	13.08%	3.33%	6.66%	8.88%

The two-by-two comparison of the variables between them primiparous and 2nd calving (χ2= 0.87; p=0.352), primiparous and 3rd calving (χ2=2.57; p=0.109), primiparous and more than 3 calving (χ2= 0.42; p=0.516), 2nd calving and 3rd calving (χ2= 6.2; p=0.0012); 2nd calving and more than 3 calvings (χ2= 2.60; p=0.106), 3rd calving and more than 3 calvings (χ2= 1.01; p=0.032).

Regarding age, the results demonstrated that older cows had a higher risk of disease than younger cows (p > 0.05) (Figure 4). The Odds ratio test also showed that there was no high risk for older cows of being infected (Odds ratio = 10.09; 95% CI: [0.28-0.148]).

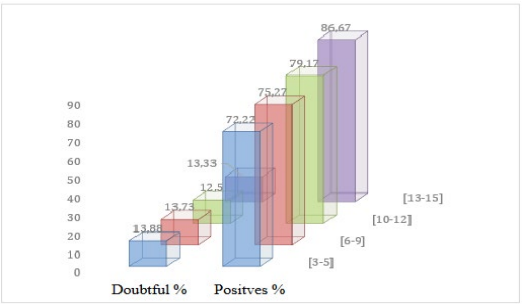

Fig. 4. Prevalence of mastitis in the CMT test according to age

The two-by-two comparison of the variables with each other, interval of (3-5) years and (6-9) years (χ2= 0.08; p=0.766), interval of (3-5) years and (10-12) years (χ2= 0.22; p=0.631), (3-5) years and (13-15) (χ2= 11.87; p=0.0006); interval of (6-9) years and (10-12) years (χ2= 0.03; p=0.856), interval of (6-9) years and (13-15) years (χ2=10.351; p=0.001), (10-12) years and (13-15) (χ2=9.50; p=0.002).

Prevalence of subclinical mastitis by DeLavale Cell Counter (DCC)

The prevalence of subclinical mastitis diagnosed by the DCC DeLaval or CSS method did not vary significantly according to each breed (p >0.05), as indicated in Table 4. For the CSS test, the Odds ratio test showed that there was a high risk for cows with a higher farrowing rank (Odds ratio = 0.092; 95% CI: [0.892-

1.749]).

Table 4

Prevalence of mastitis in the DCC test according to breed

Prevalence %	Meres	N'Dama	Baoules	Zebus	Metis	P-value	Total
	N= 322	N= 20	N= 8	N= 6	N= 4		N= 360
Positive %	74.53%	90%	50%	100%	100%		75.55%
Doubtful %	13.66%	10%	25%	0%	0%	0.0709	13.33%

Cows in advanced lactation months did not have a higher prevalence of disease than cows with lower lactation months (Fig. 5) ($p > 0.05$). The Odds ratio test showed that there was a lower risk for cows in advanced lactation months to be infected (Odds ratio = 0.279; 95% CI: [0.275-0.017]).

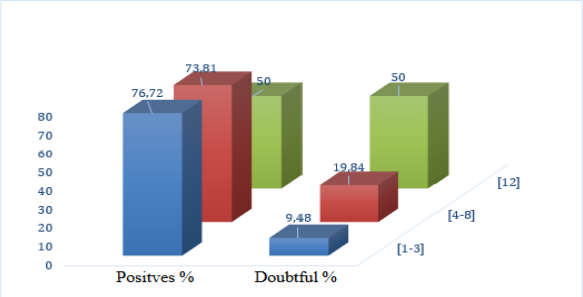

Fig. 5. Prevalence of mastitis in the DCC test according to the month of lactation

The two-by-two comparison of the variables between the interval of (1-3) months of lactation and (4-8) months of lactation ($\chi2=3.41$; $p=0.064$), the interval of (1-3) months of lactation and (12) months of lactation ($\chi2= 32.39$; $p< 0.0001$), (4-8) months of lactation and (12) months of lactation ($\chi2= 17.413$; $p< 0.0001$).

The prevalence of subclinical mamitis in cows with greater than 3 calving is 86.67%, no significant difference was observed (Table 5). Cows with high calving had a higher risk of disease at the same time than cows with low calving (Table 5) ($p > 0.05$).

Table 5

Prevalence of the DCC test according to the number of calvings

Prevalence %	Primiparous	2nd calving	3rd calving	> 3 calving	Total
	N= 86	N= 130	N= 84	N= 60	N= 360
Positive %	68.60%	73.07%	82.14%	81.66%	76.36%
Doubtful %	13.95%	16.92%	8.33%	11.66%	12.86%

The two-by-two comparison of the variables between the primiparous and 2nd calving (χ2= 0.106; p=0.742), primiparous and 3rd calving (χ2=0.70; p=0.401), primiparous and more than 3 calving (χ2= 0, 37; p=0.542), 2nd calving and 3rd calving (χ2= 1.41; p=0.273); 2nd calving and more than 3 calvings (χ2= 0.91; p=0.337), 3rd calving and more than 3 calvings (χ2= 0.05; p=0.812).

Older cows had a higher prevalence of disease than younger cows (p > 0.05) (Fig. 6). The Odds ratio test also showed that there was no great risk for older cows of being sick (Odds ratio = 5.54; 95% CI: [0.638-0.228]).

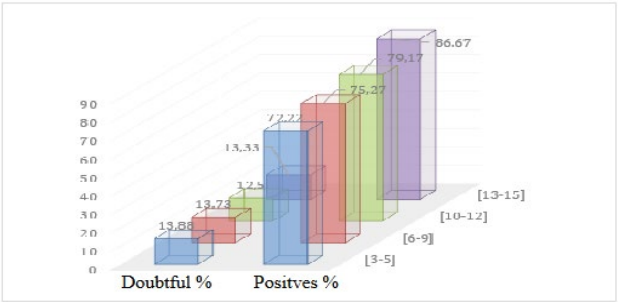

Fig. 6. Prevalence of mastitis in the DCC test according to age

The two-by-two comparison of the variables with each other, interval of (3-5) years and (6-9) years (χ2= 0.01; p=0.899), interval of (3-5) years and (10-12) years (χ2= 0.21; p=0.641), (3-5) years and (13-15) (χ2= 0.é_; p=0.591); interval of (6-9) years and (10-12) years (χ2= 0.11; p=0.732), interval of (6-9) years and (13-15) years (χ2= 0.16; p=0.681), (10-12) years and (13-15) (χ2=0.003; p=0.950).

Comparison of the prevalence of subclinical mastitis according to tests used

Table 6 shows that the DCC test is capable of detecting more cases than CMT (p<0.05). On the other hand, after the comparison of the two tests used, both tests showed the same sensitivity in revealing positive cases.

Table 6

The prevalence of subclinical mastitis by DCC and CMT tests

Test	Positive %	Doubtful %	x2 -	P-value
CMT	80	8.88	1.02	0.31
DCC	75.55	13.33		

The prevalence of mastitis of 80% in traditional farms in Poro region according to our study remains high and close to that carried out in Maghreb and East Africa where the prevalence can reach up to 89% (4, 5). Similarly, Shyaka et al. (23) obtained a lower prevalence of 68.75% in peri-urban area of Dakar. Our results are superior to those obtained in local breeds of Niger with a prevalence of 44.2% (2), as well as the prevalence of 44.71% found in Benin in local breeds (16). The high prevalence obtained in our study could be due to sampling mainly increased by local Méré breed. With DeLaval DCC direct method, which facilitates somatic cell counting (SCC) of quarter milk, the prevalence of (75.5%) was almost the same with the CMT test (80%), therefore did not vary significantly ($p>0.05$). Similarly, Shyaka et al. (23) and Kalandi et al. (17) obtained about the same concordance of results with the two tests carried out in dairy cows in regions in Senegal with 63.28% and 68.75% then the prevalence of 10.9% and 11.9% respectively by CCS and CMT tests. In European countries, where the milk quota is imposed on producers, the milk cell count is an important element for payment or sale of milk. It is an excellent indicator of health status of herd. Of 360 dairy cows, 288 (80%) were positive with a score greater than or equal to 2 and 72 were negative with a score greater than or equal to 1 in CMT test. The percentage of cows with a reported critical case (score equal to 4) was 31.25% (90/288). The overall prevalence of subclinical mastitis obtained in another study, in two different farms was 58.53% (15). This percentage remains high compared to those obtained in Senegal for half-breeds and for local breeds in Niger, which are respectively 46.2% and 44% (6). This high rate could be justified by the fact that breeds selected by humans for their milk production are predisposed to mastitis (9). This is in accordance with our study; the positive cows belonged to several breeds, including half-breeds (100% affected) with subclinical mastitis, there is a significant difference between breeds (Baoulé, Zebus, Méré, N'dama) and half-breeds, concerning the CMT test. The analysis after mapping, concerning the mastitis detection zone, revealed a large number of positive cases in CMT test in departments of Korhogo, Dikodougou and M'bengué compared to the department of Sinématiali in the Poro region (northern Ivory Coast). The cartographic analysis, concerning the Poro region, revealed a large number of cases in the different departments. According to Gambo et al. (12), mastitis can occur in cows of different breeds, depending on age or month of lactation, but more in the mixed breed have been reported and are

largely affected by subclinical mastitis. Because for these authors, milk from first week of lactation is characterized by a high concentration of somatic cells. Also, some studies show that mastitis infection is mostly symptomatic and asymptomatic and that the treatment proposed by the veterinary practitioner is based on the epidemiological model of herd established from documents of breeding and a bacteriological survey (22). The treatment of subclinical mastitis is done at the drying off with rare exceptions, during lactation. The cure rate for subclinical mastitis during lactation is 50% on average compared to 70 to 80% at drying off (6).

Regarding the month of lactation; our study showed that there is an influence on appearance of disease after CMT test in young cows of 76.39% of positive cases and fluctuates up to 100% for older cows. These results agree with Gambo et al. (12) work, which stipulates that the increase in cell rate at the end of lactation could be explained by an increase in the cell concentration physiological drop in a low volume of milk and contamination of animals during or between milking. The increase in the cell count with lactation number may be linked to the drop in natural defenses in mammary gland of older cows (12). Thus, the susceptibility of the udder to infection increases with the number of lactations (9). Highly positive data can therefore be obtained with older dairy cows or cows at the end of lactation.

Conclusions

Mastitis screening in traditional dairy farms in the Poro region shows a correlation between the results of CMT and DCC tests. The prevalence of positive cows is different from one department to another: M'Bengué 97.92%, Korhogo 89.58%, Dikodougou 77.78% and 54.17% in Sinématiali.

By using the CMT test, 80% of the studied dairy cows were positive for subclinical mastitis, while DCC test revealed 75.55% of cases.

The breed, age, and calving are factors that influence the prevalence of the pathology. Mixed breeds were more commonly affected by subclinical mastitis than local breeds. Older cows had a higher prevalence of disease than younger cows. Cows with high calving had a higher risk of disease than cows with low calving.

The prevalence of mastitis observed in traditional farms of each department of the Poro region makes it possible to say that it is necessary to consider effective management of cows with subclinical mastitis and, finally to increase milk production.

Acknowledgements

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RETROSPECTIVE CLINICAL STUDY ON CHRONIC COUGH IN DOGS

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Summary

The study aimed to analyse the underlying causes of chronic cough in dogs, aiming to refine and prioritise diagnostic strategies and methods for determining the aetiology of chronic cough. This study was conducted on 92 dogs with chronic cough, the aetiology of which was established by corroborating data obtained at clinical examination (inspection, palpation, auscultation) with those provided by paraclinical examinations (imaging examinations and laboratory tests). Chronic bronchitis and heart failure were the main pathologies associated with chronic cough in dogs, especially those aged 8-14. The haematological profile did not show significant changes from the reference values nor among the conditions causing chronic cough in the dogs in this study. Chest radiography should be the first step in the clinical approach to identifying the cause of chronic cough.

Keywords: chronic cough, dogs, clinical examination, paraclinical examination.

Coughing is a vital physiological reflex (initiated by cough receptors in the larynx, trachea and bronchi) that serves as a defense mechanism by evacuating foreign or own material from the airways, improving mucociliary cell motility and protecting the airways against accidental aspiration of material from the oral cavity (6, 10).

Despite its apparent protective function, chronic unproductive cough can damage the airway mucosa, as well as patient discomfort and reduced quality of life. In general, chronic cough in dogs may be a secondary respiratory and/or cardiac disease symptom (17). Treating these underlying problems takes priority over simply suppressing cough, although determining these underlying causes can be challenging (4, 21).

The European Respiratory Society (ERS) defines *cough* as "a forced expelling action" or "action against the closed glottis that is associated with a characteristic sound or sounds". In veterinary practice, cough is a meaningful clinical sign that may indicate an underlying disease process (8, 13). Despite its apparent protective function, chronic unproductive cough can damage the airway mucosa, as well as patient discomfort and reduced quality of life. In general, chronic cough in dogs may be a secondary symptom of respiratory and/or cardiac disease (3, 9).

Localization of lung disease requires corroboration of the results of physical examinations and imaging, visualization of the large airways and evaluation of specific airway samples or parenchymal tissue (1, 2). Chronic bronchitis usually

affects middle-aged to elderly dogs and is defined as a daily cough lasting over two months (5, 19). In some patients, respiratory sounds and abnormal breath sounds such as wheezing and lung crackles can be heard, but lung auscultation reveals no pathological changes (12, 15).

Materials and methods

The study was conducted from January 2019 to May 2022 at the Internal Medicine Clinic of the Faculty of Veterinary Medicine Timisoara. A total of 1196 records were evaluated for the research, from which 92 cases presenting with chronic cough were selected. The inclusion criteria for this study were as follows: cough older than two months; a chest X-ray or cardiological examination; a diagnosis justifying the onset of cough. Clinical records for this study were completed during consultation and included information on history, clinical signs, imaging and laboratory findings, diagnosis and treatment. The clinical examination consisted of inspection, palpation and auscultation, and the paraclinical examination consisted of imaging examinations (radiological, ultrasound) and laboratory tests.

Radiological imaging investigations were carried out in Timisoara's Diagnostic Imaging Service of the Faculty of Veterinary Medicine, using the Siemens MULTIX Swing digital radiology machine. The complete blood counts were performed using the ProCyte DX™ (IDEXX, USA) automated haematology machine. The collected data were statistically analysed using the computer program SPSS (Statistical Products of Services Solution), version 20 and are presented in the paper as mean ± standard deviation.

Results and discussions

To carry out this study, 1196 records were evaluated, from which 92 cases that presented symptoms of chronic cough were selected, representing 7.69% of the total cases evaluated.

In this study, one of the criteria considered was the age of the patients, which ranged from less than one year to more than 14 years. For this reason, the dogs taken in the study were divided into six intervals: <1 year, 1-3 years, 4-7 years, 8-10 years, 11-14 years, and>14 years. The prevalence of chronic cough according to age was observed to be highest in dogs aged 11-14 years (42%), followed by those aged 4-7 years (21%) and very close to that in those aged 8-10 years (18%), while the lowest prevalence was found in dogs under one year (2%) (Fig. 1).

Out of the total of 92 patients, 52% of the patients seen were male, and 48% were female. The very close percentage values resulting from the data analysis revealed an approximately equal distribution of the occurrence of this symptom according to gender.

These results are close to those of Hawkins et al. (7) obtained in 115 dogs with chronic cough study. Thus, the mean age of the dogs was 9.4+/- 3.6 years, with no gender-related predisposition identified.

The breed is an essential clinical feature in cardio-respiratory diseases evolving with chronic cough, some breeds being predisposed to lung diseases that may trigger the occurrence of this symptom in breed-specific pathologies. Figure 2 shows that out of the total of 92 patients, the dogs with the highest percentage of cough were the mixed breed (44%), followed by the Bichon (13%), Poodle (6%), Shih-Tzu (5%), Brac (5%) and other breeds with meagre percentage (Fig. 2).

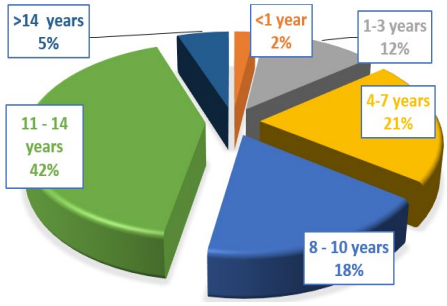

Fig. 1. Prevalence of chronic cough in dogs according to certain age ranges

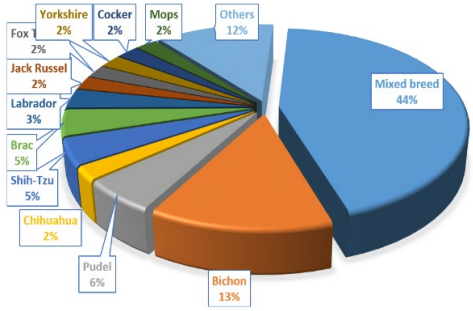

Fig. 2. Prevalence of chronic cough in dogs according to breed

In a study of 115 dogs with cough, Hawkins et al. (7) observed that the highest incidence was in small breed, toy breed and Cocker spaniel patients. An essential aspect in assessing breed predisposition would be the general distribution of breeds in the study area. As a syndrome, chronic cough in dogs is a relatively unaddressed topic in the veterinary medical literature. However, it involves a complex approach in terms of diagnostic conduct and therapeutic approach.

The etiological diagnosis is critical in treating chronic cough because only after identifying the causative factor can a therapeutic protocol be established to eliminate or control the cause.

Following the evaluation of the dogs included in this study, the most common diagnosis identified was chronic bronchitis in a percentage of 47%, followed by heart failure in different stages (45%). Almost the same results were obtained in the 2008 study conducted by Varsheney, J.P. (20) in which the most common diagnosis was heart failure, in 5 out of 10 dogs taken in the study.

The mean values of complete blood count parameters did not show changes outside the reference values but showed more or less apparent fluctuations between the cardiopulmonary conditions diagnosed (Table 1).

Table 1

Average values of blood parameters in the most common pathologies associated with chronic cough

Pathology	RBC M/ µL	Hematocrit %	WBC K/ µL	Neutrophil K/ µL	Monocyte K/ µL	Eosinophil K/ µL	Platelet K/ µL
Bronchial asthma	6.57	42.20	10.03	6.78	0.72	0.57	216
Bronchopneumonia	7.47	51	10.70	6.30	0.20	1.30	247
Chronic bronchitis	6.76	44.10	8.94	5.27	1.18	0.66	330.75
Heart failure	6.39	39.66	13.96	10.56	0.83	0.43	273.43

The mean erythrocyte values were within normal limits for each condition presented, with the highest erythrocyte value in bronchopneumonia (7.5 mil/ µL) and the lowest mean value in heart failure (6.39 mil/ µL). Mean hematocrit values were within the reference range (35-57%). However, they had the same erythrocyte pattern, with the highest value seen in bronchopneumonia (51%) and the lowest mean value in heart failure dogs (40%), this being consistent with the fact that in heart failure a degree of anaemia occurs (16).

In this study, mean values of leukocyte counts did not vary outside the reference range (5.0-14.1 thousand/µL), with the highest mean value being identified in dogs with heart failure (13.96 thousand/µL). Similar results were also obtained by Hamilton-Elliott (6), who found a significantly increased mean leukocyte value (12 thousand/µL) in dogs with congestive heart failure (CHF) compared to the control group in which the mean leukocyte value was 9000/µL (6). In another study, leukocytosis was found in the acute and chronic bronchopneumonia (14).

The mean neutrophil count was within the reference values (5.0-14.1 thousand/µL), with the highest mean value in dogs with heart failure (10.56 thousand/µL) and the lowest in dogs with chronic bronchitis (5.27 thousand/µL). Regarding the percentage value of monocytes, it can be seen that the highest average value (1.18 thousand/µL) was recorded in dogs with chronic bronchitis. Monocytosis is a haematological change suggestive of the course of chronic inflammatory conditions, representing an essential indicator in the diagnosis and prognosis of these conditions (18).

In this study, dogs diagnosed with heart failure, chronic bronchitis and bronchial asthma showed mean eosinophil values within the reference range (0.06

- 1.2 thousand/µL). In contrast, in those diagnosed with chronic bronchopneumonia, the mean eosinophil value was at the upper limit of physiological values.

Increased eosinophil counts in lung pathology can frequently be associated with allergic, parasitic, eosinophilic bronchopneumonia, etc., requiring additional investigation methods such as bronchoalveolar lavage and paraclinical examination of lavage fluid. Varshney's study observed a significant increase in eosinophils in cases diagnosed with dirofilariosis (20).

The mean platelet count is within the reference range (211-621 thousand/µL) for all diagnosed conditions. It should be noted that the highest mean value was recorded in dogs with chronic bronchitis (330.75 thousand/µL), followed by dogs with heart failure (273.43 thousand/µL). In comparison, the lowest value was recorded in dogs with bronchial asthma (216 thousand/µL).

Radiographic evaluation of the chest, including the lung parenchyma, tracheobronchial tree, pulmonary vessels and heart, provided an essential database for determining the causes of chronic cough. Thus, in dogs with chronic cough of cardiogenic cause, chest radiographs revealed the presence of cardiomegaly associated with enlargement of the left atrium, the presence of variable amounts of oedema fluid in the bronchoalveolar space and pulmonary venous distention. In the study carried out by Varsheney (20) on ten dogs, changes in lung parenchyma and arterial pathology were also found in terms of chest radiographs. Likewise, in another study by Jessica L. Ward (11) in 2019 involving 100 dogs with chronic cough, chest radiographs revealed airway collapse, cardiogenic pulmonary oedema and bronchitis as common causes, as with our results.

In dogs with myxomatous mitral valve disease, the cough is caused by compression of the left atrium on the main bronchi. In dogs with congestive heart failure, the cough is triggered by the entry of oedema fluid into the airways and pulmonary venous distention stimulating the juxta pulmonary receptors. When the receptors are stimulated, reflex bronchoconstriction occurs, and increased mucus secretion occurs, ultimately resulting in coughing (1).

Conclusions

Chronic bronchitis and heart failure were the main pathologies associated with chronic cough in dogs, especially those aged 8-14 years.

The haematological profile did not show significant changes from baseline nor among the conditions causing chronic cough in the dogs in this study.

Chest radiography should be the first step in the clinical approach to identify the cause of chronic cough.

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INTEGUMENTARY TUMORS IN PET HAMSTERS

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Summary

Pet hamsters are very popular pets worldwide, but their short lifespan can interfere with general knowledge regarding frequent pathologies. While scientific reports on tumors in laboratory hamsters are common, reports on tumors of pet hamsters are uncommon, as they are mostly case reports that include a high incidence of integumental neoplasms such as mammary tumors, atypical fibroma and papilloma, round cell tumors such as lymphoma, mastocytoma and mesenchymal tumors, mostly cutaneous hemangiosarcoma and other soft tissue sarcomas. In this study, conducted in the department of Pathological Anatomy at the Faculty of Veterinary Medicine from Bucharest, we examined five cases of adult pet hamsters (aged 1,5-2,5) submitted for investigations on suspected external tumors by gross examination (5 cases), cytopathological (M.G.G. stain) (4 cases), histopathological (H.E. and H.E.A. stains) (3 cases) and necropsy examinations (2 cases). The results included cutaneous and subcutaneous nodular lesions, frequently ulcerated with a diameter between 0.5-3 cm. The affected anatomic regions were the ventral thoraco-abdominal region in three cases, one case with dorsal cervical lesion and one in the external ear. Histopathological examination confirmed two cases of mammary carcinomas with multiple metastasis in the lung for one case submitted to necropsy. One hamster with a cervical subcutaneous nodule was diagnosed with a sarcoma tumor on cytopathological examination. The tumoral cells in the smears presented a high grade of cellular pleomorphism, frequently multinucleated (up to 12 nuclei) and a round to oval shape of nuclei with 1-2 nucleoli. Two cases of integumentary lesions included epithelial tumors located in the auricular pavilion and another in the ventral abdominal region with nodular, whitish, irregular surface diagnosed as trichoepitheliomas. Markedly expanding the dermis are multifocal, well demarcated, neoplastic masses with a wall of stratified squamous cells and central accumulation of concentric stratum corneum. No viral inclusions were visible in light microscopy in correlation with hamster polyomavirus, which is considered the main cause for such lesions in hamsters. In conclusion, integumentary nodular lesions of pet hamsters included mostly epithelial tumors with mammary epithelium origin or the superficial layers of the epidermis.

Keywords: pet hamsters, neoplasia, mammary tumors, epithelial tumors.

Hamsters, members of the Rodentia Order, are commonly embraced as pets. Among them, the Syrian or golden hamster (*Mesocricetus auratus*) stands as the most prevalent species. Dwarf hamsters, such as the Djungarian hamster (*Phodopus sungorus*), Campbell's dwarf (*Phodopus campbelli*), Roborovskii hamster (*Phodopus roborovskii*), and Chinese hamster (*Cricetulus griseus*), are also noteworthy. The average lifespan varies from 24 to 36 months depending upon species and individual traits (14, 16).

While there is vast scientific literature concerning tumors in laboratory

hamsters, documentation on spontaneous tumors in pet hamsters remains scarce. The literature comprises mainly case reports on this pet animal category. Most common tumors in pet hamsters include lymphoma, mastocytoma, and mesenchymal tumors (primarily cutaneous hemangiosarcoma), and epithelial tumors- papilloma and different types of carcinomas (10, 15). A singular study from Japan offers a more extensive examination, documenting spontaneously developing tumors in 85 pet hamsters. The authors note that integumental neoplasms presented different percentages between hamster species, represented by mammary gland tumors, atypical fibroma, and papilloma were almost exclusively found in Djungarian hamsters, but hematopoietic tumors, such as plasmacytoma and lymphoma were widespread in Syrian hamsters (10, 13, 16).

This study aims to evaluate spontaneous integumentary neoplasms in pet hamsters, involving data collection, macroscopic and microscopic evaluation in order to provide additional knowledge for both veterinary clinicians and pathologists regarding these increasingly popular animals.

Materials and methods

In this study, conducted in the department of Pathological Anatomy at the Faculty of Veterinary Medicine, University of Agronomic Sciences and Veterinary Medicine of Bucharest, we examined five cases of adult pet hamsters (aged 1,5-2,5) that belonged to private owners. Animals and samples (cytopathological samples obtained by fine-needle aspiration or imprints and excisional biopsies) were submitted for investigations for suspected external tumors and were represented macroscopic and microscopic evaluation and postmortem examinations that included lesional evaluation and complete necropsy. The study included gross examination (5 cases), cytopathological evaluation (4 cases) using May-Grunwald-Giemsa stain (M.G.G.), necropsy examinations (2 cases) and histopathological evaluation after standard processing of samples (3 cases) of biopsies and after postmortem sample collection, represented by Hematoxylin-Eosin stain (H.E.) and Hematoxylin Eosin Methylene Blue stain (H.E.A.).

Results and discussions

The data regarding anamnesis included one Syrian hamster, 3 Dwarf hamsters and one case of unknown exact species for a surgical excised nodule submitted to diagnosis. The animals were all adult hamsters with a medium age of 18 months.

The time between observation and investigations was between 2-3 months, and most owners describe a double in size and ulceration after 2-4 weeks of evolution, except one ear lesion that remained similar in size during the period between observation and surgery. Notably, there is a delay in diagnosis due to a lack of frequent manipulation for some pet owners or associated with a relatively low

number of veterinarians specialized in exotic animals in order to decide what is the best approach to integumentary lesion that is suspected to be of neoplastic origin in these exotic pet animals (1, 7).

The most commonly affected anatomical regions were the ventral thoraco-abdominal region (three cases), the dorsal cervical region (one case), and the external ear (one case). The results included cutaneous and subcutaneous nodular lesions, frequently ulcerated with a diameter between 0.5-3 cm.

Fine-needle aspiration is commonly used as initial diagnosis and cytopathology plays an important role in determining whether the lesion belongs or not to an epithelial origin and for some extent to characterize the presence of malignancy cellular features (12, 13).

Cytopathology was performed in two female hamsters cases that presented nodular subcutaneous lesions on the ventral abdominal region and on lateral thoracic integument. Cytopathologic examination revealed clumps of round-oval cells of epithelial cell origin, moderate anisocytosis, and anisokaryosis, a large nucleus with 1-2 nucleoli and mitotic figures. Some cells presented basophilic, vacuolar cytoplasm associated with active secretory function (Fig. 1). Based on the localization and cytopathologic aspects a mammary carcinoma diagnosis was formulated, followed by histopathologic examination of surgical excision in one case and a necropsy examination for the other.

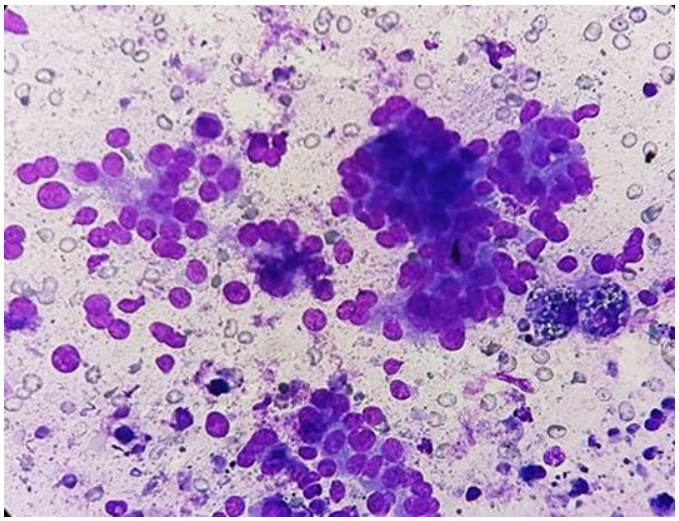

Fig. 1. Epithelial cell aggregates, round and oval shape, with moderate anisocytosis and anisokaryosis, some exhibiting granule-vacuolar basophilic, secretory material (M.G.G., ob. 40)

Histopathological examination confirmed two cases of mammary

carcinomas. One case submitted to necropsy presented multiple metastasis in the lung. The neoplasms were well differentiated, with a main tubulopapillary morphologic pattern and focally, adenocarcinomas exhibited increased mitotic activity with nuclear atypia, multilayered epithelium and infiltrative growth into the surrounding adipose tissue (Fig. 2).

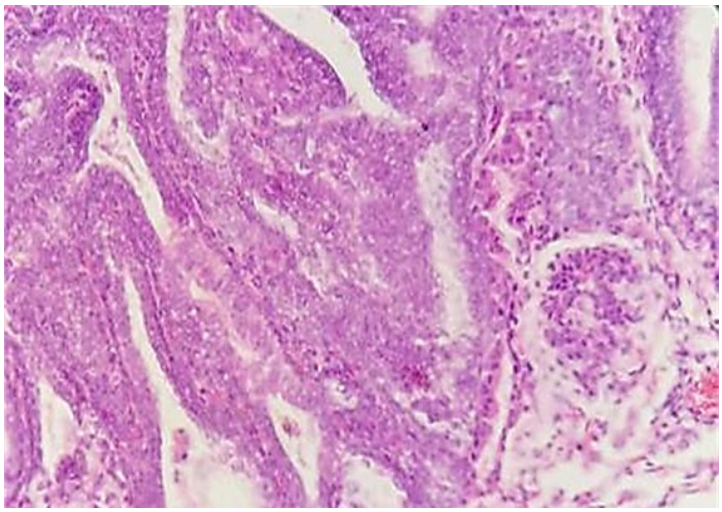

Fig. 2. Mammary carcinoma with tubulopapillary pattern and tumor embolus in a vessel (H.E., ob.20)

The literature data mention mammary tumors as common in domestic and laboratory Dwarf or Djungarian hamsters (9, 10, 19, 20). Mammary tumors are one of the most important diseases among females of all species, including humans. The morphology and biological behavior of mammary tumors vary between each species, and interspecies comparative studies may contribute significantly to the understanding of human mammary gland cancer (11, 13, 20).

Additional observations in the histopathologic examination included hepatic congestion, renal congestion and enlarged filtration space and pulmonary edema and congestion. The case submitted to postmortem investigations revealed at a histopathologic examination the presence of metastatic disease in the form of multiple metastasis of carcinoma in the lung, previously inapparent at a gross examination. Proliferation with tubulopapillary morphologic pattern of epithelial neoplastic cells was observed in both mammary nodules and the lung (Fig. 3).

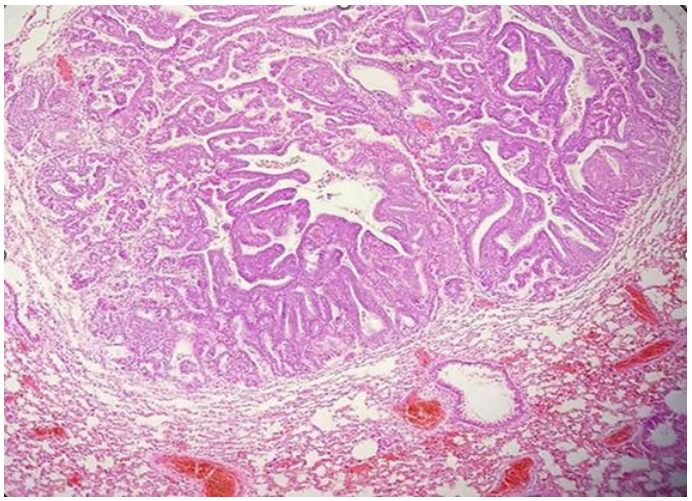

Fig. 3. Carcinoma metastasis in the lung with atelectasis and congestion on the surrounding pulmonary tissue (H.E., ob. 40)

In one clinical case, a cervical subcutaneous nodule with a diameter of 1 cm and elastic consistency was diagnosed by cytopathologic investigation as a malignant mesenchymal tumor. The tumoral cells in the smears presented a high grade of cellular pleomorphism, frequently multinucleated (up to 12 nuclei) and a round to oval shape of nuclei, finely stippled chromatin and 1-2 nucleoli (Fig. 4). Blood pollution of cytopathology smears was evident and it was the result of both aspiration difficulties due to the small size of the animal and to the tumor vascularisation itself.

Two cases of integumentary lesions included epithelial tumors located in the auricular pavilion and another in the ventral abdominal region with nodular, whitish, irregular surface diagnosed as trichoepitheliomas.

Postmortem investigations in one Campbell's dwarf male hamster (*Phodopus campbelli*) revealed a large exophytic, irregular mass located on the ventral abdominal region. The mass presented multiple layers of crusts and multiple foci of necrosis. The consistency of the mass was friable and on the cut surface multiple colors were observed, from yellow to pink and dark red, associated with keratin formation, hemorrhage and necrosis of the tissue (Fig. 5). The lesional tissue was 15.62% of the total body mass of the pet hamster (10 g of tumor and 64 g of body mass). Regional subcutaneous edema, ulceration and necrosis were the consequence of the large size of the mass and less likely due to aggressive tumoral behavior, which was confirmed by histopathologic examination (Fig. 5). Additional findings in the necropsy were a good body condition, with full contents in the digestive tract and single, intraparenchymal hepatic cyst and pulmonary edema (Fig. 6).

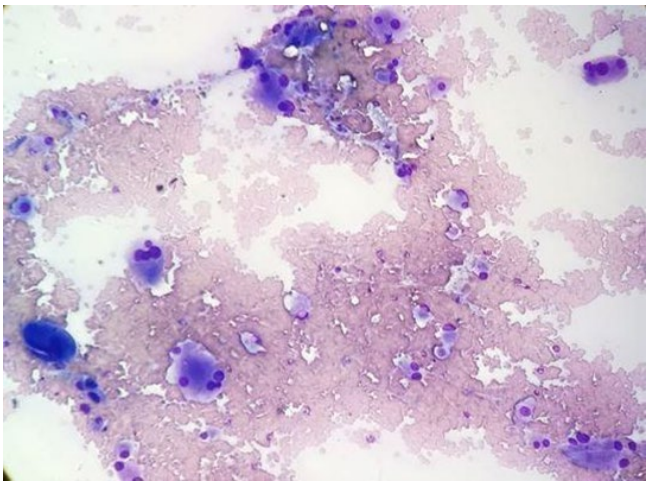

Fig. 4. Mesenchymal malignant cells exhibiting cellular pleomorphism, basophilic cytoplasm and frequent multinucleation among numerous erythrocytes (M.G.G., ob. 10)

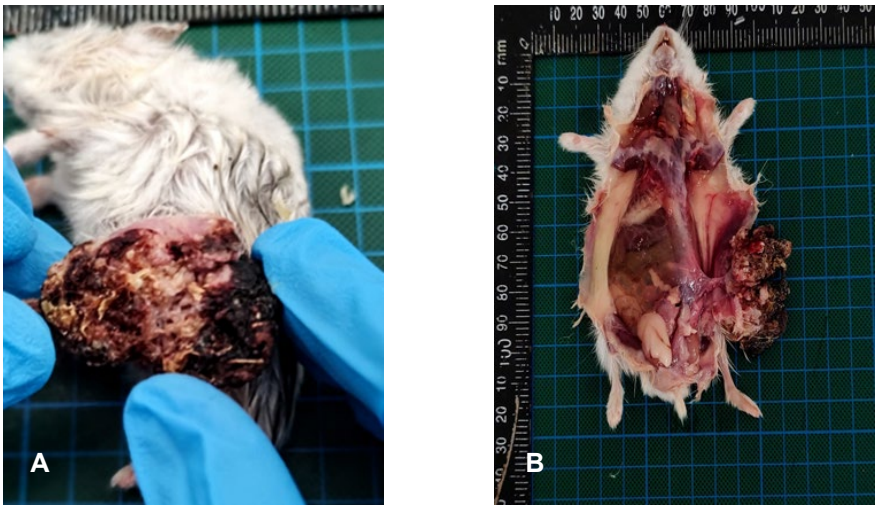

Fig. 5. A 4 cm diameter irregular, ulcerated, exophytic neoplastic mass with central necrosis, located on ventral abdominal region without peritoneal wall invasion (A and B)

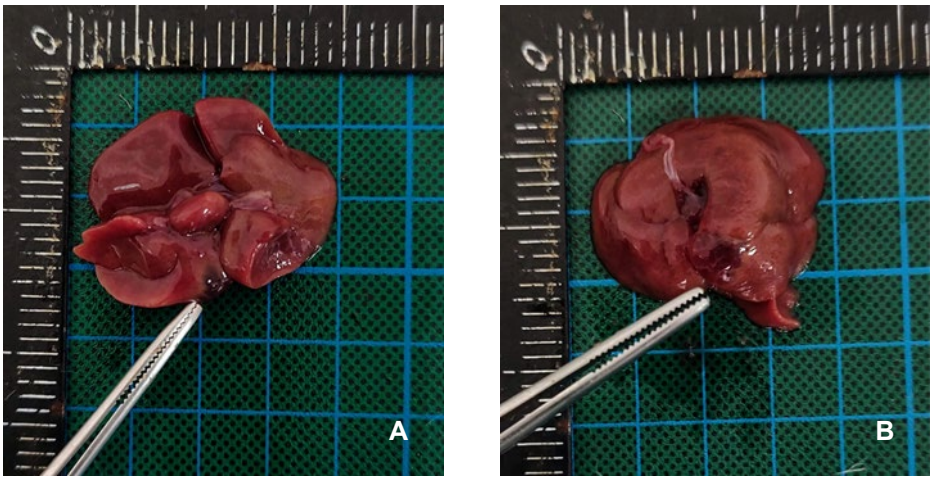

Fig. 6. Additional finding in necropsy- liver cyst – in the case with integumentary abdominal tumor (A and B)

Epithelial tumors in the auricular pavilion and another in the ventral abdominal region were classified as trichoepithelioma in two cases of integumentary lesions. Histopathologically, the epithelium is multifocally, markedly expanded by unencapsulated, well-demarcated, exophytic papillary masses composed of well-differentiated squamous epithelium that is supported by a thin fibrovascular stalk. The neoplastic epithelial cells are polygonal and have distinct cell borders, a moderate amount of cytoplasm, a central round or oval nucleus with 0-1 prominent nucleoli, and finely stippled chromatin. There is discrete anisocytosis and anisokaryosis. Cystic centers resemble dilated infundibulum and have a wall of stratified squamous epithelium with keratohyalin granules and orthokeratotic stratum corneum which accumulates centrally (Fig. 7). In this case, no viral inclusions were apparent in light microscopy in association with hamster polyomavirus, which is thought to be the primary cause of such lesions in hamsters (2, 5, 7, 8, 21).

Hamster polyomavirus (*Mesocricetus auratus polyomavirus* 1, HaPyV) was discovered as one of the first rodent polyomaviruses at the end of the 1960s in a colony of Syrian hamsters (*Mesocricetus auratus*) affected by skin tumors. Natural HaPyV infections have been recorded in Syrian hamster colonies due to the occurrence of skin tumors and lymphomas. HaPyV induces hair follicle keratinocyte proliferation, especially of the hair root epithelium (17, 18). Tumors develop on the head and neck, back, belly, and feet and sometimes, multiple nodules converge to massive confluent layers. Virus particles are present especially in the cornified layer but are absent in the proliferating cells of the basal layer and the spinous layer. A hallmark of HaPyV is the capacity to infect both undifferentiated keratinocytes as well as lymphocytes and thereby generate hair follicle and lymphoid tumors (3, 4, 6, 8).

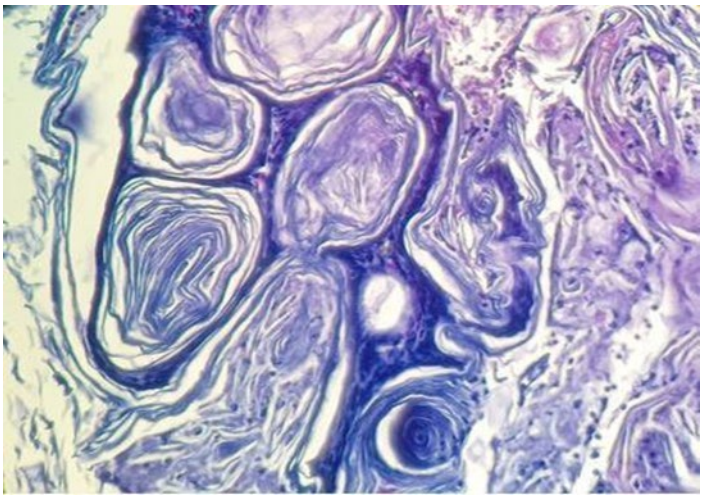

Fig. 7. Multilobular intradermal masses with a central accumulation of concentric keratin (H.E.A., ob. 20)

Conclusions

Integumentary nodular lesions of pet hamsters included mostly epithelial tumors represented by mammary carcinomas and trichoepitheliomas, and only one case of mesenchymal origin.

Microscopic examination of integumentary lesions provides insights into cellular and tissue features and distinguishes between benign and malignant neoplasms in order to provide information on the outcome for pet hamsters.

Dimension of the integumentary tumor, ulceration and necrosis are not indicative factors of the aggressiveness of the tumor itself. Tumoral lesions with dimensions over 2 cm are prone to self-mutilation, ulceration and subsequent necrosis and represent complications for the general condition of the animal and lead to a poor prognostic.

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THE SPOILAGE RATE OF FRESH POULTRY MEAT FROM CONVENIENCE STORE VERSUS SLAUGHTERHOUSE

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Sumarry

Poultry meat is considered a basic food, thanks to its affordable price, high nutritional value and cultural acceptance in some countries, being a highly perishable product, with a limited shelf life period, regardless of storage time. It is well known that poultry meat has a higher initial contamination rate, which can occur at any level, as each stage of the poultry production and processing systems. This has its own challenges, during the entire sequence of food handling from the producer to the final consumer and microorganisms can affect food quality and human health. As such, this article aimed to identify, through monitoring, how the freshness parameters of poultry meat, purchased directly from the slaughterhouse and from the convenience store, are affected. For the two batches, several freshness and quality indicators were used to perceive changes in freshness quality while storing chicken breasts. Changes in organoleptic characteristics, pH, water activity, total volatile basic nitrogen (TVBN) were monitored for six weeks.

Keywords. poultry meat, intensive system, freshness, quality, safety.

The term poultry refers collectively to domestic birds, especially those prized for their meat and eggs, such as chickens, turkeys, ducks, and geese. Only a few studies have considered the health impact of meat in relation to the animal species of origin. Nutritionally, regular consumption of poultry meat has been associated with many positive aspects. Poultry meat, mainly chicken and turkey, is considered more nutritionally valuable than other types of meat. Poultry is available fresh or frozen, as whole birds or as joints (pieces).

The term "poultry meat quality" is not standardized and there are many definitions in the interpretation of poultry meat quality. To produce a good quality carcass, the carcass must have a maximum yield of meat with a low fat content. Postmortem pH changes have been postulated to be responsible for variations in meat quality. Regarding poultry meat quality, it has been suggested that it consists of safety, nutritional value and sensory characteristics.

It has also been defined that the quality of poultry meat must meet consumer demand and this can be achieved by controlling the production chain from the farm to the processing facility and using technologies to reduce risk factors along the chain. production to enable better quality poultry meat production and consequently reduce losses.

Histological and biochemical changes due to genetic progress have placed more stress on the growing bird and affected some meat quality traits. Defects

usually occur in fast-growing birds rather than slow-growing ones. Several studies have reported that fast-growing hybrids have a high incidence of deep pectoral myopathy or green muscle disease. Although this condition was first recognized in the broiler turkey and in the muscles of adult chickens, it has become more common in the broiler chicken. These myopathies can have profound implications for poultry meat quality and specific disease incidence (11).

The appearance, texture, juiciness, wateriness, firmness, tenderness, odor and flavor are the most important and perceptible meat features that influence the initial and final quality judgment by consumers before and after purchasing a meat product.

Furthermore, for processors, manufacturing value added meat products, quantifiable properties of meat such as water holding capacity, shear force, drip loss, cook loss, pH, shelf life, collagen content, protein solubility, cohesiveness, and fat binding capacity are indispensable to acquire excellent functional properties that will ensure a final product of exceptional quality and profitability.

Materials and methods

Two batches were used, each batch having eight chicken carcasses. The first lot was made up of carcasses provided by a private slaughterhouse operator in Vrancea County, and the second lot was made up of two convenience store. The samples were transported in cooling bags with no refrigeration (approximately 30 minutes from the store to the laboratory, for each transport, and approximately 3 hours from the slaughterhouse to the laboratory)

The determinations followed the fluctuation of the organoleptic, physical and chemical characteristics, in a known and established time interval, performing the determinations in the following days:

- 24 hours;
- 48 hours;
- 72 hours;
- One week;
- two weeks;
- three weeks;
- four weeks;
- six weeks;

The determinations were made in the laboratory of the discipline of "Inspection and control of products and foods of animal origin" of the Faculty of Veterinary Medicine Bucharest.

The broiler carcasses weighed between 1.5 and 2 kg and to prepare the samples, meat was taken from the breast and thighs.

Regarding the organoleptic examination, the external appearance of the meat, the appearance on the section, the color, the texture, the consistency and the smell were observed.

The appearance and color are appreciated in daylight. On the surface, the appearance and color of meat and fat, tendons, connective tissue, articular cartilages, synovial fluid, periosteum can be observed. To assess the deep layers, deep cuts are made that include all the muscle layers.

The color of the meat may vary, depending on the type of muscles. The intensity and shade of the color is given by the content of myoglobin, hemoglobin and the chemical state of the muscle pigment. Within the same species, the color is influenced by the age, the state of health, the physiological state before the slaughter of the animal, but also by the conditions for keeping the meat.

The smell and taste are determined by the particularities of the feed, sex and species, the characteristics depending on the ammonia and sulfur content of the meat. The smell is appreciated at room temperature, both on the surface and in the deep layers.

The tenderness is determined by the content of the meat in connective tissue, as well as by the quantity and quality of the adipose tissue that gives the meat a certain degree of marbling and marbling, but also by the quality of the muscle fiber. In young animals, the connective tissue being less developed and the sarcolemma of the muscle fiber thinner, the tenderness of the meat is more pronounced compared to adult animals. There is a positive correlation between meat tenderness and water retention capacity.

The juiciness or flavor of the meat results from the ability of the boiled or roasted meat to retain a quantity of the intrafibrillar, interfibrillar and interfascicular liquid. The juiciness of the meat and its tenderness are two important components of the organoleptic examination of the meat. Juiciness can be appreciated by palpation and with the help of filter paper.

The consistency of the meat differs depending on the state of fattening, age and sex, as well as the storage conditions of the meat. The chilled meat has a firm consistency, slightly elastic when pressed, the refrigerated one in the first days is pronounced elastic when pressed with the finger, the resistance is significant, the depressions are difficult to form, they are superficial, and after the pressure ceases, they quickly and completely return to their original shape. For frozen meat, the trace left by the pulp of the finger held for a few seconds on the surface of the meat, must be bright red, proof of freshness, the brown shade indicates that it has been kept for a long time, and the grayish-yellowish shade indicates the presence of a meat very old.

The TVB-N and pH measurements were carried out according to SR ISO 2917:2007, SR 9065-7:2007, SR 9065- 7:2007/C91:2009 and an AOAC method.

pH

The pH is defined as the negative logarithm of hydrogen ion concentration in a subs& solution and it denotes the alkalinity or acidity of the solution/substrate. You have already studied that after death, muscle pH comes down from normal physiological pH of around 7.2 to 5.5 - 5.6 and this is mainly due to the conversion of muscle glycogen to lactic acid and its accumulation in the muscle. This happens

due to lack of oxygen in the muscle tissues. This anaerobic condition prevails in animal body after exsanguinations. As the accumulated lactic acid is not removed by the circulatory system which is no longer in operation, the muscle gradually acidifies. The final pH reached (5.5-5.6.) is known as ultimate pH. This pH fall is achieved in the muscles of well-fed and unstressed animal. The ultimate pH varies between muscles within the sarne carcass depending upon the glycogen reserve, temperature, glycol ytic enzyme activity etc. in the muscle. Depending upon the pre-slaughter conditions of the animals and stresses during slaughter, the fall of pH can follow any of the three patterns as described below. It should be noted that the rate and extent of fall of muscle pH in the first two hours have considerable influence on the color, texture, water holding capacity and tenderness of meat.

A normal pH decline pattern is characterized by a gradual decrease from approximately 7.2 in living muscles to a pH of about 5.6 to 5.7 within 6 to 8 hours after slaughter and then to an ultimate pH of around 5.4 to 5.6 within 24 hours after slaughter. This generally occurs in healthy, well-fed, rested animals.

Determination of total volatile nitrogen

The method is valid only for fresh or frozen meats and not for bacon and other cured meats. Meat should give a value of less than 20 mg percent calculated on a fat-free basis, values over 30 mg percent are considered to correspond to staleness. Total volatile bases comprise the amino groups resulting from decomposed proteins, as well as free ammonia. Free ammonia should not be confused with total volatile bases, as it only represents a part of it (7).

Since water activity is an important condition for the development and growth of microorganisms in meat, drying is a classic method of food preservation. If the product is dry enough (aw <0.900) and stored in a low relative humidity (RH) environment so that it remains dry, bacteria will not grow. Drying can be done in four main ways (3):

a) Air drying. Dry products often have added solutes to increase the antimicrobial effect of physical water removal. Shells hung in chillers are air-dried because the cooled air circulating around the shell effectively reduces the axis of the shell surfaces. Dry aging of beef at low temperatures and low relative humidity in a ventilated atmosphere is an example of drying that leads to a luxurious final product with a distinct flavor and aroma, which is considered a delicacy intended mainly for the gourmet market.
b) The addition of dissolved substances (NaCl, sugars) to food (meat products, but also jams, jellies, vegetables, pickles) decreases aw.
c) Freezing is also a method of reducing aw because it turns free water into ice, making it unavailable for microbial growth.

MERV - meat extract release volume

The technique was first described in 1964, has been shown to be a value in determining incipient spoilage in meat as well as in predicting refrigerator shelf life. The technique is based on the volume of aqueous extract released by homogenate of meat when allow to pass through the filter paper for a given period of time, by this

meat of good organoleptic and microbial quality release large volume of extract, whereas meat of poor quality releases smaller volume or none (8).

Results and discussions

Organoleptic examination refers to the assessment of the quality of a food product through the human senses, such as taste, smell, visual appearance, texture and general appearance.

Color is an important characteristic of meat that influences consumer purchasing decisions because consumers use meat color as an indication of freshness and quality. Meat color is related to the concentration of pigments (mainly myoglobin and its chemical state), the antioxidant potential of meat (mainly vitamins and carotenoids), the fiber structure and physical state of muscle proteins, and the type and level of intramuscular fat (13).

Visual defects are factors that can significantly impact the appearance of the carcass or meat, but they may not be directly related to the pigments, physical properties, or chemical properties of the skin or meat. The most significant visual defects are associated with bruising and bleeding, and for each defect there is a specific judgment (Table 1).

Discoloration of muscle tissue caused by bruising or the accumulation of blood due to hemorrhages has a negative effect on the appearance of the product. In severe cases, bruises and hemorrhages can lead to the condemnation or rejection of the product by consumers. Bruising occurs as a result of physical trauma without laceration, causing the rupture of capillaries and the escape of blood into the surrounding tissue. Initially, a bruise will cause a red discoloration in the damaged tissues, which will then darken to a blue-black color, and eventually turn green and possibly yellow as the hemoglobin compounds degrade. Hemorrhaging refers to the rupture of any capillary or blood vessel, resulting in blood pooling in the meat or beneath the skin. Therefore, bruises are caused by the aging of capillary hemorrhaging in the tissue due to physical trauma, while hemorrhages simply refer to any accumulation of blood.

Table 1

Some examples of most frequent abnormalities and judgment at post-mortem inspection (6)

Disease/condition	**Etiology**	**Lesions at post-mortem inspection**	**Judgement**
Bruises	Trauma	Bruises in poultry can be localized or generalized and are frequently associated with bone fractures or ruptured ligament tendons. On postmortem examination of bird carcasses affected with bruises and fractures, the following judgement should be observed: (a) the fractures associated with bruises are *removed* and affected tissue is	

		condemned, (b) in compound fractures with damaged skin, the fractured site and surrounding tissue are condemned; (c) in simple fractured without bruises and damaged skin, the affected portion *may be approved* for mechanical and manual boning operations. If the lower part of bone is fractured, the bone may be *removed* by cutting above the fracture. A carcass affected with extensive bruises is *condemned* on postmortem examination. A slightly or moderately bruised carcass is *approved* if no systemic changes are present. Affected tissues are *condemned*	
Various traumas	Bruising, fractures, joint dislocations, occurred antemortem (in the farm or during catching and transport) or post-mortem (electrical stunning, plucking).	*Ante-mortem* traumas are associated with red/blue haemorrhage. In older traumas the color is greenish. *Post-mortem* traumas are not associated with bleeding	
Abnormal color	Many different etiologies can cause abnormal coloration of the carcass. In addition to many pathological conditions, abnormal coloration can be associated with the slaughter process.	Coloration of the carcass can vary greatly depending on the species, breed, age, sex and nutrition of the bird. Dark/bluish: sepsis, cachexia, ascites. Yellow: hepatitis, contamination by faeces or bile. Red: acute sepsis, uncut birds.	Total condemnation in case of associated bacterial contamination evidence, systemic infection, acute condition, generalized condition.
Overscald	Decreased line speed in slaughterhouse, or exceeded scalding temperature	Carcass shows cooked appearance of flesh, due to excessive high temperature scalding, or exceeded exposure time	Total condemnation of the carcass and organs
Deep pectoral myopathy (Oregon disease, green muscle disease)	An ischemic necrosis due to inadequate blood supply of the deep pectoral muscle. Associated with exercise such as wing flapping.	In early cases swollen, pale and oedematous deep pectoral muscle. In older lesions green paleness, necrosis and atrophy of the muscle.	The lesion can be unilateral or bilateral. Condemn affected parts.
Emaciation/cahexia	In emaciation the wasting is due to malnutrition. In cachexia the wasting is	It is usually impossible to separate the conditions from each other in meat inspection.	Total condemnation of the carcass and organs

	due to a pathological condition but specific pathogens are normally not detected in these birds	Regression of body tissues. Prominent sternum, as the pectoral muscles are wasted. The coronary and body fat deposits are missing/gelatinous. The carcass is dark and dehydrated.	
Septicaemia/ toxaemia	The presence of the pathogens and/or their toxins in the blood system. - *Escherichia coli* - *Pasteurella multocida* - *Staphylococcus aureus* - *Streptococcus spp.* - *Erysipelothrix rhusiopathiae (turkeys)* - *Riemerella anatipestifer (ducks)* - *Listeria monocytogenes*	The carcass is usually dark and dehydrated. Petechial haemorrhages in the muscles and serous membranes of the liver, heart, lungs and spleen. Airsacculitis, perihepatitis, pericarditis, arthritis, splenomegaly, pale kidneys, focal necrosis of liver.	Total condemnation of the carcass and organs
Cellulitis	Associated with scratches caused by other birds during the grow-out period. Secondary bacterial infection enters through the damaged skin and causes infection. *E. coli* is the most commonly detected pathogen associated with this condition.	Two forms: wet and dry. Locally restricted inflammation of the subcutaneous tissues in the abdominal and inguinal area. The skin is swollen at the site of an inflammation and sometimes scratches and/ or scabs can be detected. The wet form of cellulitis consists of subcutaneous deposits of jellylike caseous exudate. The dry form of cellulitis consists of a subcutaneous sheet	Total condemnation of the carcass and organs

		of yellow, fibrino-caseous plaques.	

Post-mortem TVN levels are dependent on the level of spoilage microbial and enzymatic activities; therefore, they are used as indices of meat freshness and food safety (15).

In living animals, gut microbiota and endogenous enzymes continually produce a wide range of volatile and non-volatile compounds through amination and transamination of aldehydes and ketones or decarboxylation of amino acids (arginine, lysine, and arginine or ornithine). The compounds have aromatic and heterocyclic structures and include biogenic amines such as methylamines (MA), dimethyl-amine, N-nitroso dimethylamine, putrescine, and cadaverine (15). Although all naturally produced biogenic amines are assimilated for storage in the animal and the human body (3), a well-known group of biogenic amines including dopamine, norepinephrine, epinephrine, histamine and serotonin play a vital role as neurotransmitters. Others have an important role as precursors for the synthesis of alkaloids, nucleic acids, hormones and proteins (19). Some of the metabolites, such as TMA, TMAO and other naturally occurring muscle compounds including choline, betaine and carnitine, contribute to TVB-N generation during post-mortem storage. Post-mortem biochemical and chemical activities due to exogenous and microbial enzymes can also lead to the generation of ammonia, organic acids, and sulphur compounds from amino acids, hypoxanthine from ATP degradation, and acetate from lactate (5, 21).

Moderate levels of biogenic amines are essential and beneficial to regulate physiological activities in the human body. For example, biogenic amines have crucial roles in nucleic acid and protein synthesis, regulation of certain immune functions, stabilization of cell membranes and inhibition of some chronic diseases (6). For example, spermine, spermidine, and cadaverine play important roles in growth regulation, and serotonin is crucial for neural transmission. Histamine and tyramine are inflammation mediators and are essential components in neurotransmission and vascular permeability functions. However, there are toxic thresholds for biogenic amines (12) that could lead to a wide range of illnesses including severe headaches, hypertension, abdominal pain, tachycardia, allergenic reactions and death in some severe cases (6).

Pre-slaughter formation and accumulation of volatile and nonvolatile nitrogenous compounds cannot be ignored in the discourse of factors determining the quality of muscle foods. This fact has been extensively documented in fish and shellfish (9;10) and to a lesser extent in meat, where freshness, sensory properties and acceptability were shown to be correlated with these metabolites. TVB-N compounds also accumulate following microbial and enzymatic degradation of non-protein-nitrogen compounds (21). Although the majority of ‘gut produced TVB-N compounds’ can be excreted (e.g., aliphatic amine emissions), significant amounts were reported to be assimilated in the body or stored in the hepatic system and other tissues (18).

For instance, assimilated TMAO was shown to be further converted by TMA oxide aldolase to formaldehyde (assimilated as carbon source) and ammonium (assimilated as nitrogen source) via intermediate monomethylamine (4). Similarly, TMA is oxidised to TMAO by hepatic trimethylamine monooxygenase (flavin-containing monooxygenase 3 [FMO3]) (3, 17). The major dietary precursors of TMA and its products (DMA, formaldehyde and TMAO) in ruminants include L-Carnitine, choline (phosphatidylcholine and associated phospholipids) and betaine (2).

Therefore, the dietary intake of these compounds will influence the generated amounts of methylated amines in the animal tissues. Pre-slaughter practices that influence meat pH (12) will play an important role in determining the final pH (this will directly affect the activities of microbial populations and enzymes in the meat) and the amount of carbohydrates available for bacteria before being forced to metabolize protein compounds for energy (14). The rapid increase was attributed to microbial degradation related deamination of adenine nucleotide and conversion of TMAO in the meat to TMA, DMA, and formaldehyde.

The spoilage microflora is initially dominated by aerobic bacteria, which are gradually replaced by anaerobic microbes that can breakdown the proteins and amino acids to generate amine compounds and release CO_2.

The TVB-N content of chicken meat is shown to increase with storage time, temperatures and muscle type, with the finding that the rate of TVB-N accumulation across 15 days storage at 4°C was greater for thigh meat when compared to the breast – although this observation may be confounded by the different microbial loads for each cut. Further, this study observed that TVB-N values were significantly different only after the expiration dates for the chicken samples and were therefore useful only to detect the later stages of deterioration (17). It was interesting to note that similar, but not identical trends to TVB-N, observed for other bioactive amine quality indices originally proposed for tuna were calculated for the same chicken samples. When we transfer the acceptable limits for these indices (bioactive amines < 50 mg/kg, we can observe that breast meat was unacceptable after 15 days (when TVB-N was equal to 34.4 mg/100 g) whereas thigh meat remained acceptable, although its TVB-N level was 46.5 mg/100 g and there was substantial variance around these mean values. (2)

Water activity has become one of the most important intrinsic properties in predicting the survival of microorganisms in food due to its direct influence on product quality and stability. (20)

In both batches it was found that a small part of the carcasses presented bruising and hemorrhages.

For the batch from the slaughterhouse, it was found that increasing the storage time of the carcasses influences the values of pH, total volatile basic nitrogen, water activity and the meat extract relase volume changed as follows:

a) The pH increased gradually, from the values within normal parameters (5.8 – 6.2) obtained in the first three days, respectively the first week post-slaughter, up to values between 6.8 – 7.9, obtained between two weeks and

six weeks; for the first two weeks the meat was fresh, in the third week the meat was relatively fresh and in the sixth week the meat was spoiled (Table 4)

b) TVN – poultry meat is considered fresh when it contains up to 25 mg% ammonia, relatively fresh with values between 25-35 mg %, and spoiled with values above 35 mg% ammonia; it was found that three weeks post-slaughter, the amount of ammonia increased, showing the incipient spoilage of the meat; (Table 5)
c) Water activity - for the extract from fresh meat, the filtration was carried out in a continuous stream, light pink in color, clear, with a pleasant specific smell; for the extract from relatively fresh meat, the filtration was in discontinuous jet, with opalescent filtrate; for the extract from the spoiled meat, the filtration was drop by drop, the filtrate being cloudy, brick-pink in color (Table 2).
d) The Meat extract release volume - one month after the slaughter, the incipient spoilage was observed - intermediate decomposition, then at six weeks the spoilage of the meat was noted. (Table 2)

For the carcasses from convenience store s-a constat the freshness parameters changed as follows:

a) The pH increased gradually, from the values within normal parameters (5.8 – 6.2) obtained in the first three days, respectively the first week post-slaughter, up to values between 6.8 – 7.9, obtained between two weeks and six weeks; for the first two days the meat was fresh, in the third day the meat was relatively fresh and from the third week the meat was spoiled (Table 3)
b) TVN – poultry meat is considered fresh when it contains up to 25 mg% ammonia, relatively fresh with values between 25-35 mg %, and adulterated with values above 35 mg% ammonia; it was found that three weeks post-slaughter, the amount of ammonia increased, showing the spoilage of the meat; (Table 6)
c) Water activity - for the extract from fresh meat, the filtration was carried out in a continuous stream, light pink in color, clear, with a pleasant specific smell; for the extract from relatively fresh meat, the filtration was in discontinuous jet, with opalescent filtrate; for the extract from the spoiled meat, the filtration was drop by drop, the filtrate being cloudy, brick-pink in color (Table 3).
d) The Meat extract release volume – one week after the slaughter, the incipient spoilage was observed - intermediate decomposition, then at three weeks the spoilage of the meat was noted (Table 3).

Table 2

Values obtained water activity and meat extract release volume from slaughterhouse carcass

Sample/determination	Water activity		Meat extract release volume	
24 hours post slaughter	0.998	0.998	71 ml	72 ml
48 hours post slaughter	0.997	0.998	70 ml	70 ml
72 hours post slaughter	0.997	0.997	70 ml	70 ml
One week post slaughter	0.989	0.990	65 ml	66 ml
Two weeks post slaughter	0.985	0.983	60 ml	60 ml
Three weeks post slaughter	0.974	0.972	58 ml	40 ml
Four weeks post slaughter	0.968	0.970	23 ml	25 ml
Six weeks post slaughter	0.958	0.962	17 ml	20 ml

Table 3

Values obtained water activity and meat extract release volume convenience store carcass

Sample/determination	Water activity		Meat extract release volume	
24 hours	0.997	0.997	70 ml	72 ml
48 hours	0.994	0.995	69 ml	70 ml
72 hours	0.989	0.990	67 ml	69 ml
One week	0.984	0.986	60 ml	66 ml
Two weeks	0.980	0.980	50 ml	48 ml
Three weeks	0.970	0.970	27 ml	25 ml
Four weeks	0.967	0.967	21 ml	23 ml
Six weeks	0.949	0.951	15 ml	18 ml

Table 4

Evaluation of freshness with digital pH-meter Slaughterhouse carcass vs convenience store carcass

		Storage time							
	Sample	24 hours	48 hours	72 hours	One week	Two weeks	Three weeks	Four weeks	Six weeks
Slaughterhouse carcass	Sample 1	fresh	fresh	fresh	Fresh	fresh	Relatively fresh	Relatively fresh	Spoiled
	Sample 2	fresh	fresh	fresh	Fresh	Relatively fresh	Relatively fresh	Relatively fresh	Spoiled
	Sample 3	fresh	fresh	fresh	Fresh	fresh	Relatively fresh	Spoiled	Spoiled
	Sample 4	fresh	fresh	fresh	Fresh	fresh	Relatively fresh	Spoiled	Spoiled
Convenience store carcass	Sample 1	Fresh	Fresh	Relatively Fresh	Relatively Fresh	Relatively fresh	Spoiled	Spoiled	Spoiled
	Sample 2	Fresh	Fresh	Relatively fresh	Relatively fresh	Relatively fresh	Spoiled	Spoiled	Spoiled
	Sample 3	Fresh	Fresh	Relatively fresh	Relatively fresh	Relatively fresh	Spoiled	Spoiled	Spoiled
	Sample 4	Fresh	Fresh	Relatively fresh	Relatively fresh	Relatively fresh	Spoiled	Spoiled	Spoiled

Table 5

Values obtained from the determination of TVBN slaughterhouse carcass

Storage time	Values obtained (mg/100g sample)	
24 hours post slaughter	6.9	6.8
48 hours post slaughter	6.8	6.8
72 hours post slaughter	11.9	10.2
One week post slaughter	17	15.3
Two weeks post slaughter	20.4	18.7
Three weeks post slaughter	25.5	23.8
Four weeks post slaughter	32.3	34
Six weeks post slaughter	40.8	42.5

Table 6

Values obtained from the determination of TVBN convenience store carcass

Storage time	Values obtained (mg/100g sample)	
24 hours	17.7	18.1
48 hours	25.5	24.8
72 hours	28.9	28.2
One week	30.6	29.9
Two weeks	32.3	32.7
Three weeks	35.7	35.7
Four weeks	38.4	37.8
Six weeks	43.5	44.3

Conclusions

Aspects such as animal welfare, genetic improvement and proper industrialization have a strong positive impact on the quality of the product to be offered to the final consumer. It is understood that each market has its own cultural specificities, and each study should be based on these characteristics. However, what we cannot ignore is the nutritional quality of the products and the constant search for new alternatives and studies to understand how parameters such as freshness and temperature fluctuations along the food chain influence the quality of poultry meat. The results of this study showed a significant difference between the batch from the slaughterhouse and the batch from the convenience store, although the organoleptic examination between the two batches revealed no notable differences.

In conclusion, more studies should be carried out related to the rate of deterioration of fresh poultry meat in stores, as well as consumer awareness regarding the storage and inadequate handling of meat by him can negatively influence the quality of the food.

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PRELIMINARY RESEARCH REGARDING ANTHELMINTIC RESISTANCE DETECTION BY PCR IN ALBA AND HUNEDOARA COUNTIES

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Summary

Resistance to anthelmintics is widespread in the world, especially in the digestive strongyles of ruminants and horses. As a result of this risk, the parasitological control programs must be adapted in order to prevent this phenomenon. Even if FECRT remains the gold standard for resistance detection, molecular biology methods, in the case of PCR, provide much more pertinent information on the phenomenon. This work aimed to identify resistance to benzimidazoles, a phenomenon frequently encountered in Europe, but less described in Romania. Four samples of adult nematodes were used, identified on the base of morphological characteristics and sequencing as three *Haemochus contortus* (two from Alba County and one from Hunedoara County) and one as *Chabertia ovina* (from Hunedoara County). The following pairs of primers were used: CN30/CN24 and CN30/CN25, respectively. One *H. contortus* sample (Alba County) proved to be resistant and *Ch. ovina* proved to be susceptible.

Keywords: anthelmintic resistance, PCR, digestive strongyles.

Considering the important economic losses caused by parasitism with gastro-intestinal nematodes, anthelmintic treatments are still the most used to control these infestations (5, 6, 7, 14, 17, 19, 21).

Benzimidazole (BZs) anthelmintics are still widely used, especially in less developed countries, but are still also available in Europe, despite the significant spread of the resistance phenomenon (1, 9, 26). In the UK the prevalence of resistance was 64-77%, affecting most species of gastrointestinal nematodes (22).

It has been known for more than two decades that resistance to BZs is correlated with the appearance of a mutation in the isotype 1 β-tubulin genes. This mutation was responsible for obstructing the binding of the anthelmintic, being initially noticed in *Heamonchus contortus* and *Trichostrongylus colubriformis* (11, 15).

The objective of this study was to identify the presence of resistance to BZs in two flocks of sheep from Alba and Hunedoara Counties by the PCR method.

Materials and methods

The polymerase chain reaction (PCR) is an in vitro enzymatic reaction of specific primer-mediated amplification of short regions of the genome.

From sheep slaughtered in the two counties, samples were collected consisting of adult nematodes which were examined with a stereomicroscope in order to highlight the specific morphological characters of each species. They were identified as *Haemonchus contortus* and *Chabertia ovina*, respectively. Three *H. contortus* (two from Alba County and one from Hunedoara County) and one *Ch. ovina* (from Hunedoara County) were selected for PCR.

Afterwards, the samples were subjected to molecular analyses in order to identify the parasitic DNA. Extraction was performed using the Bioline tissue protocol kit (BIOLINE®). During successive steps, other cellular components were removed, so that at the end of the process the DNA was precipitated, thus obtaining a purified DNA without RNA contamination. The obtained DNA was stored in a freezer at –20°C until processing by PCR.

The PCR reaction was performed according to the technique described by Gasser et al. in 1993, with some minor changes (10). The amplification itself was performed by classical PCR and was based on the creation of several copies of a ~300 bp ITS-2 gene sequence. The primers used were: forward NC1: 5'-ACGTCTGGTTCAGGGTTGTT-3' and reverse NC2: 5'-TTAGTTTCTTTTCCTCCGCT-3' (10). Amplification was carried out according to the protocol described in the article, modified according to the requirements of the mixture. A Master Mix MyTaqTM Red Mix (BIOLINE®) was used to perform the reaction. The final volume of the PCR reaction was 25 µl, of which 12.5 µl MyTaqTM Red Mix (BIOLINE®), 1 µl primer NC1, 1 µl primer NC2 (diluted to a concentration of 10 pmol/µl, according to the protocol described by manufacturer), DNA extracted from the sample to be analysed and ultrapure water. The amplification program was carried out with the thermocycler My Cycler (BioRad®).

This program included DNA denaturation steps at 95°C for 1 minute in 35 cycles of: denaturation at 95°C for 30 seconds, hybridization at 55°C for 30 seconds and extension at 72°C for 30 seconds, followed by incubation at 4°C.

In cyathostomins, the resistance mechanism to benzimidazoles involves more than one mutation (TTC / TAC), so beta-tubulin isotype 1 codons 167 and 200 are considered to be important for resistance (13, 16, 31). The primers used to identify resistance to anthelmintics were: Reverse - CN30R (nonspecific) with the sequence 5' AGC AGA GAG GGG AGC AAA GCC AGG 3' and Forward - CN24FS (specific alleles susceptible) with the sequence 5' GGT TGA AAA TAC AGA CGA GAC TTT 3' or - CN25FR (specific resistant alleles) with the sequence 5' GGT TGA AAA TAC AGA CGA GAC TTA 3'.

Identification of benzimidazole-resistant strongyles was accomplished using the primer set CN25FR/CN30R, while the primer set CN24FS/CN30R was used for benzimidazole-sensitive strongyles. Analysis and control of amplicons was

performed by horizontal electrophoresis in a submerged electrophoresis system in 1.5% agarose gel, with the addition of the fluorescent dye RedSafe (iNtRON Biotechnology, Korea). 100 bp DNA Ladder marker was used in the first well of the gel, in an amount of 5 µl.

After migrating the samples in the agarose gel, the image of the gel with the migrated DNA fragments was captured using a UV photodocumentation (UVP®) system. For species confirmation, PCR products were sequenced and compared to those available in the GenBank database using BLAST alignment.

Results and discussions

Fig. 1 shows the image of the gel with the migrated DNA fragments, confirming the susceptibility of the existence of the phenomenon of resistance to albendazole.

Wells 1, 6, 11, 16 represent the 100 bp molecular marker. Wells 2, 3, 4, 5 show positive samples (samples 1, 2, 4 are from *Haemonchus* spp., and sample 3 is from *Chabertia* spp.).

Fig. 1. 1.5% agarose gel electrophoresis image of amplicons resulting from amplification

In wells 7-10, there are samples examined for benzimidazole-susceptible alleles with primers CN30/CN24, sample 3 (*Ch. ovina* from Hunedoara County) being identified as positive. Wells 12-15 reproduce the samples examined for benzimidazole-resistant alleles with primers CN30/CN25, sample 2 (*H. contortus*

from Alba County) being positive. This is the first evidence for the resistance susceptibility in *Ch. ovina* in western Romania.

Nowadays, it is accepted by the majority of the researchers that the major molecular target site for the BZs within nematodes is β-tubulin (2, 23, 28). For all trichostrongyles, the major genetic determinant of BZ resistance is considered to be a single nucleotide polymorphism (SNP) in the parasitic isotype 1 β –tubulin. The spindle is represented by the replacement of phenylalanine with tyrosine at codon 200 (F200Y), encoded by a change from TTC to TAC (15). Even if other single nucleotide polymorphisms were discovered at codon 167 and 198 respectively, F220Y seems to remain the most important in relation to the resistance phenomenon (12, 29).

Resistance to BZs is widespread in Western European countries such as France, Spain, Norway, but the most severely affected are the countries that make up United Kingdom (4, 8, 18, 20, 30). Moreover, multiple resistance (to BZs, levamisole, ivermectin) was also described to which moxidectin is added, which was used without success to control these resistant strains (3, 27). For the UK, the most frequently reported resistant species was *Teladorsagia circumcincta* to all mentioned anthelmintics, followed by *Trichostrongylus* sp and *Nematodirus* sp. while in the other European countries *T. circumcincta*, *Haemonchus* sp. and *Trichostrongylus* sp. were found to be frequently resistant, and *Cooperia* sp. and *Nematodirus* sp. less resistant (3, 4, 24, 25, 27).

Conclusions

Two samples, one from Alba County (*H. contortus*) and one from Hunedoara County (*Ch. ovina*) proved positive following the PCR test. This is the first indication of resistance susceptibility for *Ch. ovina* in western Romania.

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QUESTIONNAIRE ON THE WELFARE CONDITIONS AND LEVEL OF VETERINARY MEDICAL CARE RECEIVED BY RURAL DOGS AND CATS

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Summary

In most Romanian households from rural areas, there are domesticated dogs and cats. In general, these animals are not perceived as pets, and the level of care from the medical point of view is very different compared to the one pets receive in urban areas. This survey was taken in order to understand better the resources that the owners from rural areas are able or willing to invest in order to take care of their domesticated carnivores and also the level of animal welfare. The questionnaire used is original, consisting of 16 questions. The answers were anonymously recorded. One hundred fifty-nine persons from two villages from Teleorman County answered. The responses were recorded by the interviewer on paper and subsequently transferred to Google Forms. Of the respondents, 53.2% (83/159) are men, 26.1% (41/159) are between 36 and 45 years old, and 23.6% (37/159) are between 46 and 55 years old, 38.8% (59/169) went to high school and 27% (41/159) only went to elementary school. More than half have another animal besides a dog or a cat. For a cat's treatment, the most common sum the owners are willing to pay is between 10 and 50 RON, the equivalent of 2 to 10 euros, and for a dog's treatment, between 50 and 100 RON, the equivalent of 10 to 20 euros. Most dogs and cats, 81% (128/159), are not sterilized. Moreover, of the dogs, 46.5% (73/159) are chained, and of those, 11.5 % are never unchained to move free in their yard.

Keywords: welfare, rural area, dogs, cats.

The owner-pet bond is an essential factor in seeking veterinary care. Studies suggest that the stronger the bond, the higher the level of veterinary care the pet will receive and the more compliant the owner will be to the vet's instructions (11). Dog owners are more attached to their dogs than cat owners (8,9). This relationship impacts the level of care or the willingness to pay for that care. Dogs visit the vet more often, and the owners are willing to spend more money on their dogs, even if both species are in the same household (11). Dog owners are more likely to bring their pets in for vaccination and physical exams than cat owners (7).

Regarding parasite control, ESCCAP (European Scientific Counsel Companion Animal Parasite) has guidelines for both ectoparasites and endoparasites. Owners might only be aware of pets' flea infestations once they see clear signs or are directly affected. Also, they do not know that fleas and other ectoparasites can cause health and welfare problems, given that most owners from rural areas do not treat their dogs or cats with anti-flea products at least once a

year (6). In areas with high, continual re-infestation risk, as in the mixed-pet households with unlimited outdoor access, it is recommended to have sustained flea control, year-round and environmental treatment for immature stages (6). For tick infestations, treatment is recommended for constant protection, at least during "tick season" or all year round if the winter is not clear cold (5). ESCCAP has guidelines based on risk factors for endoparasite infection in dogs and cats (4,19,20). Studies indicate that most pets are not being dewormed four times per year or more, indicating a significant concern (12,13). Also, animal owners are poorly informed about appropriate parasite control and human and animal health risks associated with endoparasites (14,10).

Regarding sterilization and vaccination, dog owners or owners of both species are more likely to pursue those treatments for their pets than only cat owners (2). The highest proportion of roaming animals, dogs and cats, have owners, and prevention of roaming might reduce the number of animals, given that sterilization rates are low (1,17,18). In Romania, sterilization of mixed breed dogs is mandatory by law, and owners should take care of this aspect when puppies are between 4-6 months of age for females and 6-8 months for males (16). Tethering, the practice of chaining or other restraining forms, imposes risks. Dogs tethered for long periods tend to be highly aggressive (3). Also, tethering dogs for long periods can change their behavior and cause them to fail to meet their basic needs (15).

Materials and methods

The present study compares the level of care that dogs receive vs. cats and farm animals in rural areas.

a. Study population: The 159 participants, all living in rural areas in two villages from Teleorman County, participated voluntarily.

b. The questionnaire was initially designed specifically for this study. It is composed of 16 questions. The answers were recorded on paper and transferred to Google Forms for a more straightforward interpretation.

Results and discussions

a. Demographic results

Of the respondents, 53.2% are men, 26.1% are between 36 and 45 years old, 23.6% are between 46 and 55, and 19.1% are between 21 and 35 (Fig. 1). Most of them, 38.8%, went to high school, 27% attended elementary school, and 19.7% have post-secondary education (Fig. 1).

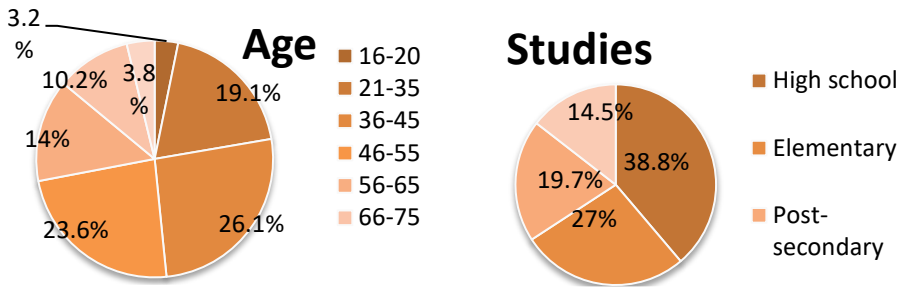

Fig. 1. Age distribution among respondents and educational level

b. Distribution of animals in households

Most respondents own at least one dog, 93.7%, and at least one cat, 75.5%. Most of them own at least one other animal species: 59.1% pigs, 11.3% sheep, 8.8% goats, 10.7% horses, and 14.5% poultry (Fig. 2).

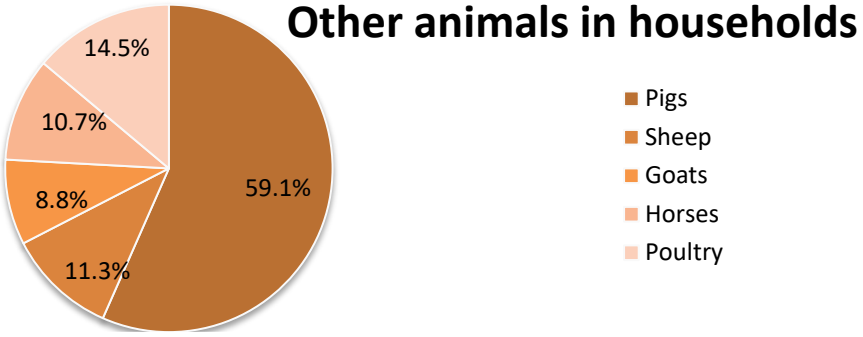

Fig. 2. Other animal species distribution in households

c. Attitude toward seeking medical care for the animals

Asked if they call for the veterinarian if one of the animals is unwell; 88.7% of the respondents answered "yes," and only 11.3% answered "no". Most respondents, 78.8%, said they call for the veterinarian for their dog or cat as fast as they do for other animals, and 21.2% answered "no". We can clearly distinguish between the availability of care for farm animals and that of domesticated carnivores (Fig. 3).

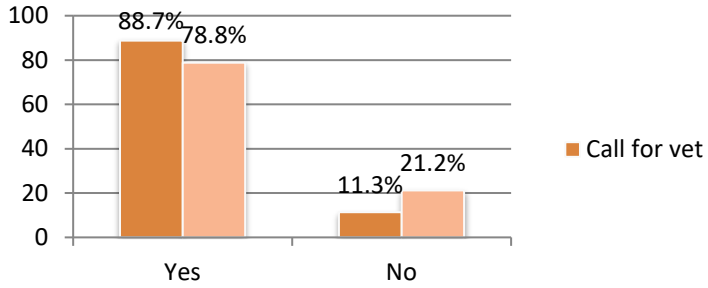

Fig. 3. Difference in seeking medical care for carnivores and other animal species

Moreover, 31.4% of cat owners and 15.7% of dog owners are willing to pay between 10 and 50 RON for their cat treatment, equivalent to 2 and 10 euros. Of the dog owners, 38.9% are willing to pay between 50 and 100 RON, equivalent to 10 and 20 euros, and only 25.7% of the cat owners are willing to pay the same amount for their cat's treatment. Only 20.1% of dog owners and 10% of cat owners are willing to pay more than 200 RON (40 euros) for the treatment. We want to distinguish that in the rural area, the final price includes treatment, travel, and labor costs. So, the amount they are willing to pay covers everything, including consultation, prescription drugs, and labor costs (Fig. 4).

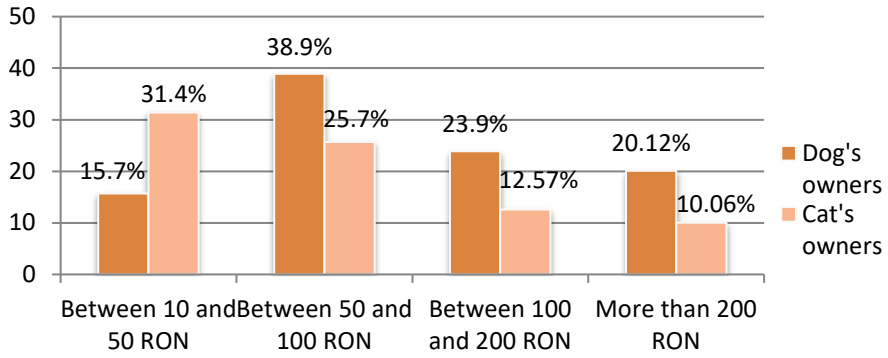

Fig. 4. Amount of money for dogs and cats' medical care

Regarding other farm animals, respondents are willing to pay much more for medical care. Of them, 46.4% are willing to spend between 100 and 200 RON (20 to 40 euros), 25.8% more than 200 RON (40 euros), 7.9% less than 400 RON (80 euros), and 6.6% less than 500 RON (100 euros) (Fig. 5).

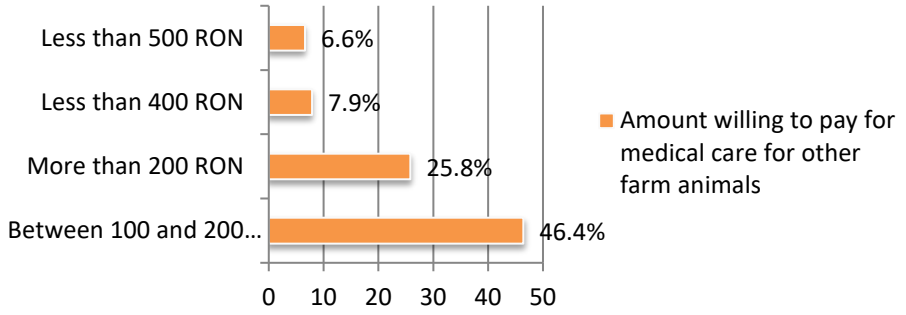

Fig. 5. Amount willing to pay for other farm animals' medical care

d. Medical care for dogs and cats

All dogs above three months old should be vaccinated against rabies in Romania. In rural areas, this vaccine is free of cost for the owners, so most dogs are vaccinated annually. Most dog owners vaccinate their puppies with the first vaccine from the vaccination schedule against distemper and parvovirus. Moreover, some follow with the second dose of DHPP (distemper, adenovirus, parainfluenza, and parvovirus). Asked if their dogs are vaccinated with other vaccines than the free-of-charge annual anti-rabies vaccine, 71.1% answered "yes," and 28.9% answered "no, " meaning that the dogs living in those households never received a vaccine from the vaccination schedule.

Regarding parasite control, 28.9% of the owners say they are deworming (pills with internal action) their dogs annually, 25.8% every six months. Of the respondents, 15.1% do not know if they deworm their dogs, and 5% never deworm them. We keep in mind that most dogs and cats are outdoor animals, and the recommendation is to be dewormed every three months, and only 25.2% of the respondents respect that schedule (Fig. 6).

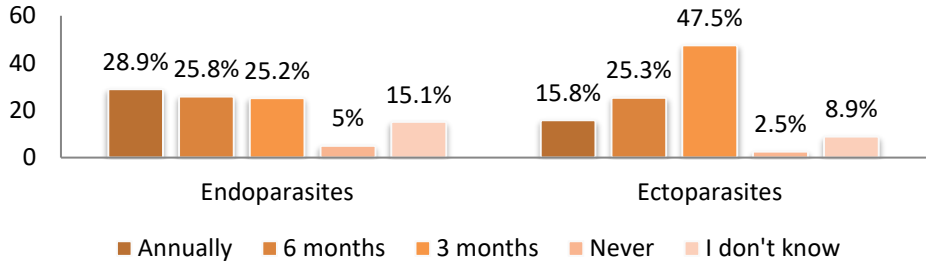

Fig. 6. Frequency of parasite control

Regarding ectoparasites control, 47.5% of the owners say they give treatment every three months, 25.3% every six months, and 15.8% annually. It is a concern that 8.9% of owners do not know how often they treat ectoparasites, and 2.5% never do that (Fig. 6). Flea infestations are more prevalent in rural areas than urban areas; therefore, prevention should be done correctly combined with environmental control. Also, ticks are endemic throughout almost all of Europe, and with climate changes, their seasonal activity has changed, and they can be found almost all year. Those two types of ectoparasites are more prone to be treated because they can affect humans. Usually, they seek treatment after the family members are affected.

Another concerning fact is the lack of sterilization among the dog and cat population. Of the owners, 81% declared that their dog/cat is not spayed/neutered, even though in Romania, the owners must take care of that aspect regarding mixed-breed mongrels. It seems that 56.6% do not know that sterilization for those breeds is mandatory, and even though 43.4% know about the regulations, they did not take the animal to be spayed/neutered. The lack of sterilization impacts the population of free-roaming dogs and cats in rural areas.

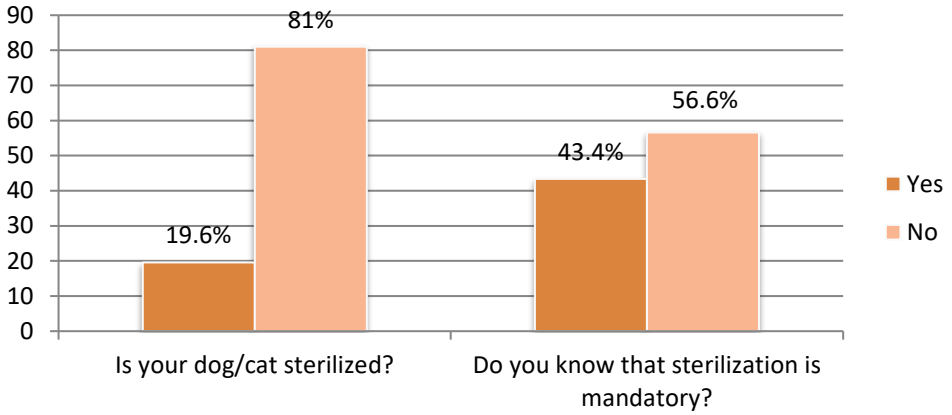

Fig. 7. Percentage of sterilized animals and the level of information among owners

e. Welfare conditions

Most participants admit that they feed their dog/cat with leftovers 54.4% and with bread 36.7%. Although 58.9% report that they buy commercially packed food for their animals. We can only assume that the quality of the store-bought food is deficient if we compare the amount of money they are willing to pay for medical care, which is not a constant payment with the food prices because the average price per kilo is less than five RON (1 euro).

Regarding the living conditions, 46.5% of owners responded that their dogs are chained, 44.6% said the dogs are free in their yard, and 24.2% have special

enclosures. Of the chained dogs, 22.1% are unchained daily, 18.3% every other day, 20.2% weekly, 12.5% monthly, 17.3% rare, and 11.5% never unchained.

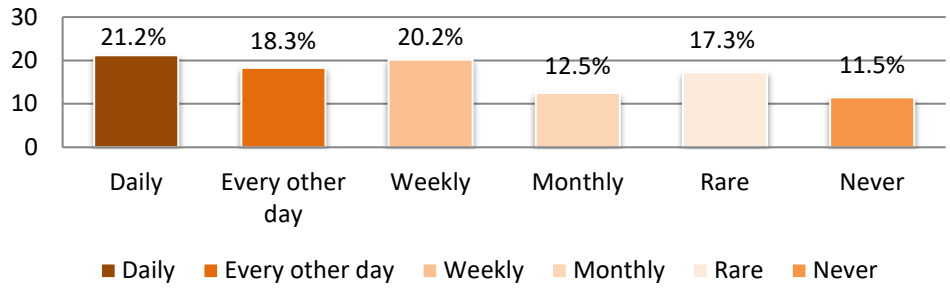

Fig. 8. Frecquency of unchaing dogs

Conclusions

Most respondents care for at least one species of animal and, therefore, are familiar with different healthcare problems that can occur. The majority, 88.7%, call for the vet when one of the animals is unwell. However, 21.2% do not call with the same rapidity regarding dogs or cats. The financial aspect is alarming; half of the respondents would spend a maximum of 100 RON for a dog or cat treatment. For other species, almost half of the respondents, 46.4%, would spend between 100 and 200 RON and 25.8% more than 400 RON. Comparing the amount of money available for different species, we conclude that domesticated carnivores will receive less treatment.

The frequency of parasite control needs to be improved according to the standards of care for outdoor carnivores. Most households have unsterilized dogs/cats, increasing the stray carnivore population as the cats and dogs roam around the village. Data shows that 44.6% of the owners keep the dogs chained. It is concerning that 11.5% declare that they never unchain the dogs, 17.3% do it rarely, and 12.5% do it monthly. There is a need for veterinary care for domesticated carnivores in rural areas, but the financial aspect has a substantial negative impact. Most owners spend money on medical care for other domesticated species, such as swine, bovines, equines, and even poultry, rather than on dogs or cats.

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EMPYEMA OF THE GUTTURAL POUCH IN A NONIUS FILLY – A CASE REPORT

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Summary

Empyema in the guttural pouch typically arises as a consequence of upper respiratory tract infections, often attributed to Streptococcus equi. This study aims to present a case of guttural pouch empyema, with a specific focus on the diagnostic and treatment approaches. A 2-month-old Nonius filly, was admitted to the clinic due to breathing difficulties and noticeable swelling in the retropharyngeal area. Upon clinical examination, the following symptoms were observed: labored breathing, difficulty swallowing, lymph node enlargement, unilateral swelling in the retropharynx, and the presence of mucopurulent nasal discharge. Furthermore, endoscopic assessment confirmed the existence of purulent material in the left guttural pouch, ultimately leading to a diagnosis of empyema. To facilitate bacterial culture and antibiogram assessment, samples were obtained through a transcutaneous puncture procedure. Subsequently, lavage was performed using an endoscope. The treatment plan involved administering procainbenzylpenicillin, along with flunixin meglumine, probiotics, gastric protectants, vitamin supplements, and lactated Ringer's solution, while awaiting the results of the bacterial culture and antibiogram. The outcome of the bacterial culture ultimately confirmed the presence of Streptococcus, which exhibited sensitivity to penicillin, amoxicillin, and marbofloxacin. Twelve days after the lavage, with the procedure itself performed ten day, the filly was discharged. A thorough evaluation of the case's resolution was conducted during a follow-up assessment several days after discharge.

Keywords: guttural pouch empyema, equine, endoscopic lavage.

Guttural pouch empyema is characterized by the presence of mucopurulent exudate, with or without chondroids (3, 5, 8, 9, 10). This condition arises due to an infection of the upper respiratory system, with *Streptococcus equi* being the primary etiologic agent responsible for this disorder (7, 13, 16). However, guttural pouch contamination may also occur due to other bacteria or through the drainage of abscesses at the retropharyngeal lymph node into the guttural pouches (3, 5, 13, 17).

This paper aims to report the non-surgical treatment of a guttural pouch empyema, by flushing the guttural pouches with saline solution, mucolytics and removal of the purulent material using a basket forceps through the endoscopic channel.

Materials and methods

A two-month-old Nonius filly was brought to the Emergency Department at USL in Timisoara, Romania, experiencing respiratory distress and evident swelling in the retropharyngeal region. The physical examination unveiled several symptoms, including dyspnea, dysphagia, lymphadenopathy, distension, and discomfort in the left parotid region. Additionally, stertorous breathing, unilateral mucopurulent nasal discharge, a rectal temperature of 38.9 °C, a heart rate of 80 bpm, and a respiratory rate of 50 breaths per minute were observed.

The endoscopic evaluation was conducted while the filly was subjected to standing sedation, facilitated by the administration of Domosedan® (detomidine 10.0 mg) and Butomidor® (butorphanol 10.0 mg) (4, 6). The KARL STORZ endoscope was utilized for the endoscopic procedure, being introduced through the left nostril. Utilizing biopsy forceps, access to the guttural pouch was successfully attained through its ostium.

In preparation for the transcutaneous puncture, the clipping and aseptic measures involving the application of betadine, chlorhexidine, and alcohol were conducted within the confines of the Viborg's triangle. Subsequently, a needle of dimensions G18 (1.30 X 88 mm) was employed for the purpose of collecting pathological specimens.

The specimen retrieved from the cavity was introduced into an Amies, Deltalab® transport Swab with medium. In the laboratory setting, the material underwent inoculation onto a blood agar culture medium.

The systemic therapeutic approach encompassed the administration of procainbenzylpenicillin, concomitant with flunixin meglumine, probiotics, gastric protectants, vitamin supplements, and lactated Ringer's solution (2, 4, 12). This regimen was implemented while awaiting the results of bacterial culture and antibiogram analyses (15).

Subsequently, the purulent material was removed using a basket forceps through the endoscopic channel, followed by irrigation with a warm saline solution (0.9%) and 30 mL of acetyl cysteine once per day during 10 days (15) (Fig. 1).

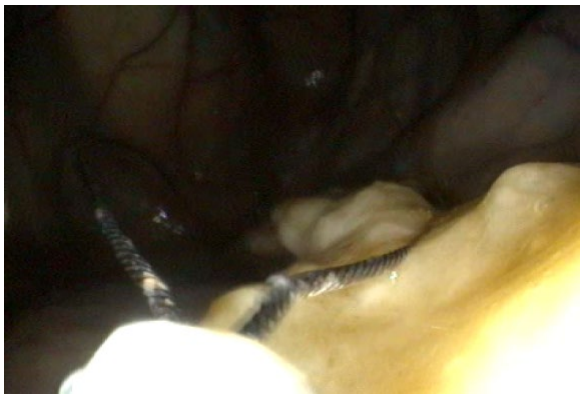

Fig.1. Endoscopic images demonstrate the removal of purulent material using transendoscopic basket forceps

Results and discussions

In the course of the endoscopic examination, the presence of purulent secretion emanating from the left pharyngeal orifice was observed, accompanied by the identification of a purulent collection within the left guttural pouch (Fig. 2). Subsequently, the clinical diagnosis established at this juncture was indicative of left guttural pouch empyema.

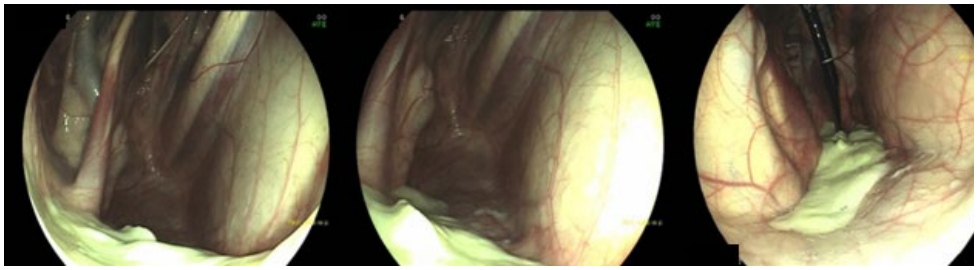

Fig. 2. Left-sided guttural pouch endoscopic imaging, revealing the presence of a purulent collection

The bacterial culture identified a strain of *Streptococcus* sensitive to penicillin, amoxicillin, and marbofloxacin. Eight different antimicrobial agents were tested, including rifampicin, florfenicol, erythromycin, enrofloxacin, and azithromycin.

Lavage was performed for a duration of 10 days, and the filly was discharged from the hospital after 12 days (1). A comprehensive assessment of the case's resolution was established through a follow-up evaluation several days after discharge.

Differing viewpoints exist regarding the non-surgical management of guttural pouch empyema (11). Certain studies challenge the approach outlined in the current case, citing the limited efficacy of non-surgical treatments for guttural pouch empyema. Furthermore, some authors highlight complications associated with inflammation and rupture of the guttural pouch, stemming from the buildup of content in the pouches or excessive distension during lavage procedures (5, 16).

Regarding inflammation, there is evidence suggesting that the use of antimicrobials and mucolytics may contribute to an elevation in mucosal inflammation within the guttural pouches. Additionally, there is a potential risk of causing harm to the nerves situated inside the pouches, leading to complications such as dysphagia (7, 8, 10).

The non-surgical treatment involves flushing the guttural pouches with a 0.9% saline solution, antimicrobials, and mucolytic agents (11, 13).

Using antimicrobials and mucolytics during lavage procedures offers several advantages, including the suppression of pathogens and enhancement of the drainage of both exudate and chondroids, respectively (3).

The implementation of non-surgical treatment implies the avoidance of post-operative complications, including hemorrhages, cellulitis, and dysphagia originating from iatrogenic injuries to the vagus, hypoglossal, and glossopharyngeal nerves (7, 9). Additionally, it results in lower costs for the owner and significantly minimizes interference with the horse's return to athletic activity (13, 14).

Conclusions

In summary, while the non-surgical treatment of guttural pouch empyema, is not yet a firmly established protocol, it has shown effectiveness and potential applicability in this case. Our treatment ensured healing, so at the end of the treatment, the animal was healthy.

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AN EPIDEMIOLOGICAL STUDY OF COLIBACILLOSIS IN A TURKEY BROILER FLOCK

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Summary

Pathogenic strains of *Escherichia coli* are recognized as the causative agents of avian colibacillosis, associated with acute deaths, high mortality, and important economic losses in the affected flocks. Interventions in disease treatment are made difficult by the emergence of antibiotic resistant strains. Taking these considerations, the study aimed to investigate the occurrence of bacterial infections, with special emphasis on avian colibacillosis, in a group of 113 turkey broilers in the first three weeks of life. The turkey broilers were reared under household conditions in Alba County, between March-April of 2021. During this period, from the total of 24 dead broilers, 8 were randomly selected and examined for the presence of avian colibacillosis using classical bacteriological techniques combined with necropsies in order to monitor the presence of specific anatomo-pathological lesions. In addition, the testing of the antimicrobial susceptibility profile of the isolated *E. coli* strains were performed using the Kirby-Bauer disk diffusion technique. Post-mortem examinations revealed characteristic lesions associated with avian colibacillosis. Thus, the most frequently encountered were: fibrinous pericarditis (n=7), perihepatitis (n=5), and aerosaculitis (n=4), followed by uric acid deposits on the ureters (n=2), omphalitis (n=1), and pulmonary congestion (n=1), respectively. All strains (n=8) expressed multiple antimicrobial resistance phenotypes (resistance from 3 to 10 antimicrobials). The highest resistance was observed towards tetracycline and doxycycline (87.5%), followed in descending order by erythromycin (75%), spectinomycin (62,5%) amoxiclav (37,5%), and novobiocin (25%). Total susceptibility was noticed against: florfenicol, gentamicin, lincomycin, enrofloxacin, and ciprofloxacin. The study demonstrated that avian colibacillosis, produced by *E. coli* with multiple antimicrobial resistance, is an important disease implicated in the mortality of turkey poults. Further studies, focusing on the zoonotic potential of the *E. coli* strains are still necessary, in order to evaluate the public health importance of the isolates.

Keywords: colibacillosis, antimicrobial resistance, turkey broilers.

Poultry farming is a vital and rapidly growing industry, with significant importance in satisfying the nutritional needs of humanity and contributing to economic development. This branch of animal husbandry focuses on the breeding and management of domesticated birds, such as broilers, layers, and turkeys, to produce large quantities of poultry meat and eggs (14). The rearing of turkeys constitutes a pivotal component within the avian sector, yielding manifold advantages. It serves as a valuable reservoir of sustenance, wherein turkey meat emerges as a significant protein source characterized by a low-fat content (27). These birds exhibit a favorable growth-to-feed ratio, rendering them more efficient in resource utilization compared to other avian species (17, 18, 25, 31).

However, poultry farming is constantly threatened by numerous hazards, including bacterial infections, among which infections with *E. coli* are included. Bacterial infections pose a significant challenge in poultry rearing, particularly in the growth of turkey broilers, often resulting in severe consequences for their performance and health, leading to substantial economic implications. The importance of bacterioses in turkey chick rearing stems from morbidity and mortality, the negative impact on growth and development, compromising the immune system of turkey broilers, the risk of secondary infections, and the associated costs of treatment and prevention (7, 12, 23).

Over time, it has been demonstrated that birds and poultry products constitute an immense reservoir of bacteria, such as *Campylobacter* spp., *Salmonella* spp. or *E. coli*, which, due to its pronounced zoonotic risk, can cause severe episodes of intoxication in humans. The issue of *E. coli* infections in birds is of both economic importance, due to the damages incurred, and sanitary importance, related to public health. It is under the scrutiny of national, European, and international specialized forums. The antibiotic resistance of *E. coli* strains holds particular significance, as colibacillary infections in both animals and humans represent infectious entities where antimicrobial therapy plays a crucial role (21, 22, 28).

Poultry colibacillosis is a systemic poultry disease caused by Avian Pathogenic *Escherichia coli* (APEC), which causes economic losses in the poultry industry. Due to the fact that the management and vaccination procedures are not always effective, antimicrobial therapy is considered the most common intervention method to reduce poultry colibacillosis. However, *E. coli* strains are becoming increasingly resistant to a wide range of antimicrobial drugs, indicating that the treatment of colibacillosis in poultry may become challenging in the near future (8, 10, 32).

Considering all these aspects, this study aims to investigate the progression of bacterial infections and their impact by monitoring an episode of avian colibacillosis in the early weeks of life of a batch of turkey broilers raised in a micro-breeding facility in Alba County. The study further aims to microbiologically analyze the collected samples, highlight the main anatomopathological lesions that occurred, and assess the strains' sensitivity to specific antimicrobials.

Materials and methods

The research conducted in the period of March-April 2021 took place on a farm located in the Aiud locality, Alba County, where a total of 113 turkey broilers, specifically the BIG 6 hybrid, originating from Hungary, were raised. These birds were acquired at one day old from specialized suppliers in poultry trading.

Following the acquisition, the turkey broilers were reared on the ground in an enclosed, improvised shelter specifically set up for their growth. This facility was isolated from the area where other birds were raised. Throughout the monitoring

period, clinical and anatomopathological examinations, laboratory tests, and daily mortality records were conducted. The birds were weighed on a weekly basis as part of the study. The epidemiological investigation focused on identifying factors that could contribute to the occurrence of health or management issues in the flock of BIG 6 turkey broilers under study.

Daily clinical examinations were conducted on the entire batch of subjects to detect symptoms indicating changes in the general condition, as well as the presence of digestive, respiratory, or nervous symptoms.

Anatomopathological examinations were performed at least once every three days, with eight carcasses being necropsied. During the necropsy, the corpses that were altered, crushed, or showed other mechanical changes (12.5% of the corpses) were excluded, as these modifications can impact the results and the accurate interpretation of the causes of death. By excluding these corpses, it ensures that the anatomopathological examinations focus on cases that exhibit natural and relevant pathological changes for the investigation (26).

For the bacteriological examination, samples were taken from the long bones of the remains that exhibited specific lesions associated with infectious diseases of bacterial nature. The isolation and characterization of *E. coli* isolates were conducted in accordance with standard methodology. Upon sample collection, each sample was cultured on Columbia agar with 5% sheep blood (MLT, Romania), incubated at 37°C for 24 hours under aerobic conditions. In total, 15 bacteriological cultures were performed. The characterized strains were purified and placed on nutrient agar (MLT, Romania) to obtain fresh cultures required for performing biochemical tests using the API 20E system (12). The API 20E system is a widely used biochemical test kit designed for the identification of *Enterobacteriaceae* and other related Gram-negative bacteria. This system consists of a series of miniaturized biochemical tests, each targeting different metabolic pathways of bacteria. Bacterial isolates are inoculated into the API 20E strip, and the reactions with various substrates are observed. The results are then interpreted using an API 20E profile index, which provides a numerical code that corresponds to a particular bacterial species. This method is valuable in clinical microbiology for the rapid and accurate identification of *Enterobacteriaceae* strains, aiding in the diagnosis and treatment of bacterial infections (3, 6, 29).

An antibiogram was performed to assess the resistance profiles of isolated bacterial strains against a spectrum of antimicrobial agents. This comprehensive analysis provides valuable insights into the susceptibility patterns of the isolated strains, aiding in the monitoring of microbial resistance (30). By exposing the bacterial isolates to various antimicrobials and observing their responses, the antibiogram serves as a crucial tool to make informed decisions regarding the selection of effective antibiotics for treatment (9). The antibiotics chosen for antimicrobial resistance testing were selected based on their frequency of use, cost-effectiveness, and solubility characteristics. The disk diffusion method is a laboratory technique commonly employed to assess the efficacy of antimicrobial

substances, such as antibiotics or antiseptics, against bacteria. In this procedure, a culture of bacteria is evenly spread on a solid agar medium within a petri dish. Small paper discs, each impregnated with a known quantity of an antimicrobial agent, are then strategically placed on the agar surface (16). Following an incubation period, during which the bacteria proliferate, the zone of inhibition— the region surrounding the discs where bacterial growth is restrained—is observed and measured. A larger zone of inhibition typically signifies greater effectiveness of the tested antimicrobial substance against the particular strain of bacteria. This method is crucial in determining the susceptibility of bacteria to specific agents, aiding in the selection of appropriate treatments in clinical and research settings (5, 13, 15, 19).

Results and discussions

The epidemiological examination conducted at the beginning of the flock's placement and throughout the growth of turkey poults on the farm highlighted that the targeted shelter was prepared with some measures necessary for turkey farming technology, such as feeding and watering sources, and lighting. However, technological gaps were identified, notably in terms of temperature fluctuations.

The hybrid flock originates from Hungary, and the transportation of the birds was carried out under appropriate conditions, adhering to protocols and current legislation. Upon arrival, in the first few days of life, the turkey poults were introduced into the designated shelter, equipped with permanent bedding (wood shavings and chopped straw) and provided with lighting and heat sources. The initial purpose of the construction was for feed storage but was later transformed and adapted as a space for raising poultry.

According to the health history provided, the owners reported losses through mortality in previous series of birds maintained in the same shelter, as well as in other farm annexes dedicated to poultry rearing.

Regarding ventilation in the shelter, it was achieved by allowing air to pass through open windows and the entrance door. The consequence of this was the creation of drafts that led to crowding of the turkey poults, especially in the warmer areas of the shelter, representing a stress factor for the birds. This was compounded by interruptions in the lighting regime and, consequently, the heat starting from the first week of the turkeys' lives. In addition to the starter feed administered, the birds were also fed with other types of feed.

All losses through mortality were recorded as an essential parameter of the epidemiological examination. For this purpose, the daily number of dead turkey poults was recorded, and necropsies and bacteriological examinations were conducted. The losses generated by mortality were documented and interpreted (Fig. 1).

A discernible reduction in the daily feed intake per bird has been observed, typically exceeding 1.5 kg during the initial 4-week period of avian development but currently averaging at 0.9 kg. This decrement in nutritional uptake has resulted in a

commensurate deceleration of growth rates, with the avian population attaining an average weight of 900 g at the end of the first month of life.

In the first 2 weeks of life, during the group clinical examination, certain symptoms were observed, such as anorexia, lethargy, ruffled feathers, dyspnea, and diarrhea. In some birds, even nasal discharge was noted. Additionally, clustering of birds, often in shelter corners, was reported, indicating a thermal deficiency in the housing environment.

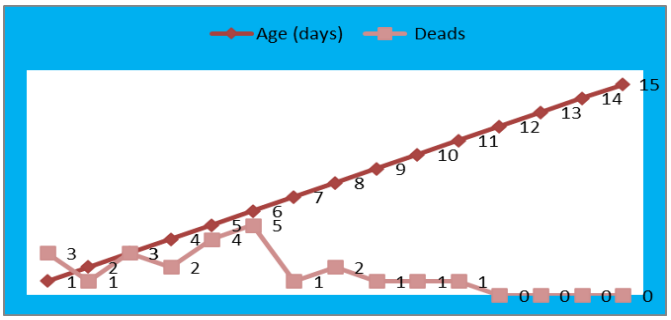

Fig. 1. The dynamics of mortality losses in turkey broilers

Post-mortem examinations revealed characteristic lesions associated with avian colibacillosis, in all 8 bodies examined. Thus, the most frequently encountered were: fibrinous pericarditis (n=7), perihepatitis (n=5), and aerosaculitis (n=4), followed by uric acid deposits on the ureters (n=2), omphalitis (n=1), and pulmonary congestion (n=1), respectively (Fig. 2).

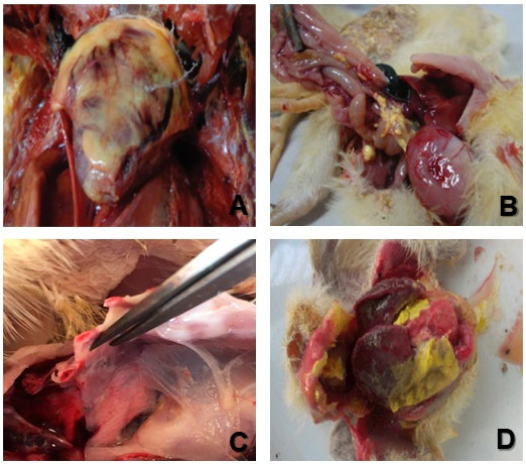

Fig. 2. Fibrinous pericarditis (A), omphalitis (B), aerosaculitis (C), perihepatitis (D)

During the period 2-15 days following bacteriological examinations, multiple strains of *E. coli* were isolated on Columbia agar supplemented with 5% sheep blood and in broth. These isolated strains were subsequently subjected to the multitest system API 20E (Analytical Profile Index), leading to the identification of *E. coli*. This comprehensive analysis enhances our understanding of bacterial isolates, providing valuable information about their characteristics and facilitating more informed decisions in the realm of microbiological diagnostics.

The results obtained from conducting the antibiogram for strains of *E. coli* demonstrate that certain strains exhibit different behavior in response to specific antimicrobials. These strains show resistance to certain substances, leading to therapeutic failure in most cases. This finding underscores the importance of careful consideration in selecting appropriate antimicrobial agents for the effective treatment of *E. coli* infections, as the resistance patterns observed have significant implications for the success of therapeutic interventions.

All strains (n=8) expressed multiple antimicrobial resistance phenotypes (resistance from 3 to 10 antimicrobials). The highest resistance was observed towards tetracycline and doxycycline (87.5%), followed in descending order by erythromycin (75%), spectinomycin (62,5%) amoxiclav (37,5%), and novobiocin (25%). Total susceptibility was noticed against: florfenicol, gentamicin, lincomycin, enrofloxacin, and ciprofloxacin (Table 1). The study demonstrated that avian colibacillosis, produced by *E. coli* with multiple antimicrobial resistance, is an important disease implicated in the mortality of turkey poults.

Colibacillosis caused by APEC strains is recognized as one of the major threats to the poultry industry. The use of antibiotics in poultry for treatment or preventive purposes may result in problems in the treatment of infections because of the selection of resistance among pathogenic bacteria (1, 4, 20). However, the development of resistance especially in zoonotic bacteria can be seen as a public health problem which results in primary treatment failures (5). Antimicrobial resistance may vary according to the antibiotics used. In this study, the highest rate of resistance (87.5%) in APEC isolates was determined against tetracycline and doxycycline, antibiotics of the tetracyclines group. Similarly, high resistance to tetracycline group antibiotics has been reported in other studies around the world. Salehi and Bonab (24) reported 94% tetracycline resistance in APECs in Iran. In another study performed in Iran, 91% tetracycline resistance was determined (11).

Antibiotic resistance of *E. coli* has become an increasing concern in poultry farming worldwide. In the Netherlands, Van den Bogaard et al. (31) obtained the following results in turkey broilers: amoxycillin and oxytetracycline (87%), ciprofloxacin (45%) and neomycin (81%). In Egypt, Abdel-Rahman et al. (2) demonstrated antibiotics resistance profile of *E. coli* isolates from 2014 to 2020: ampicillin (97,8%), tetracycline (94,8%), doxycycline (85,5%) and ciprofloxacin (73,5%).

In Bangladesh was reported 64% resistance to erythromycin and 52% to tetracycline among *E. coli* isolated (6).

Table 1

Antibiogram results

Antibiotics	1	2	3	4	5	6	7	8
Tetracycline	▲	▲	▲	▲	▲	▲	▲	-
Doxycycline	▲	▲	▲	▲	▲	▲	▲	-
Erythromycin	▲	-	-	▲	▲	▲	▲	▲
Spectinomycin	▲	▲	▲	-	-	-	▲	▲
Amoxiclav	-	▲	-	▲	▲	▲	-	▲
Novobiocin	▲	-	▲	-	-	-	▲	-
Florfenicol	-	-	-	-	-	-	-	-
Gentamicin	-	-	-	-	-	-	-	-
Lincomycin	-	-	-	-	-	-	-	-
Enrofloxacin	-	-	-	-	-	-	-	-
Ciprofloxacin	-	-	-	-	-	-	-	-

In Turkiey, a similar result was detected by Egilmez, T. et al. (11), who observed high resistant *E. coli* isolates. The phenotypic antibiotic resistance profile of *E. coli* isolates to ampicillin, tetracycline, and ciprofloxacin were 79%, 77%, and 61% respectively. However, none of the *E. coli* isolates were resistant to novobiocin and spectinomycin, which differs from our findings.

Conclusions

The study provides significant insights into the prevalence and impact of avian colibacillosis, specifically caused by *E. coli* strains exhibiting multiple antimicrobial resistance, on the mortality of turkey poults. The findings underscore the importance of understanding and addressing antibiotic resistance in poultry-associated *E. coli*, as it poses a considerable threat to the health and well-being of turkey populations.

Furthermore, the research highlights the necessity for additional investigations specifically focusing on the zoonotic potential of these *E. coli* strains. Assessing the zoonotic risk is crucial for understanding the potential transmission of antibiotic-resistant strains from poultry to humans, thereby evaluating the public health significance of these isolates. This emphasizes the importance of a comprehensive One Health approach, considering the interconnections between animal health, human health, and the environment, in managing and mitigating the risks associated with antimicrobial resistance in the context of poultry farming.

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SENSITIVITY TO ANTIMYCOTICS OF SOME *MALASSEZIA PACHYDERMATIS* STRAINS ISOLATED FROM DOGS

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Summary

Infection with Malassezia pachydermatis in dogs is treated with systemic or topical antifungal agents along with different antiseptics. Often, the treatment failure and the recurrence of infection occur. The most important fact that determines this is the application of antifungal treatment without carrying out tests to establish the sensitivity to antifungal agents. In addition, this aspect leads to increased resistance to antifungal substances This paper presents results of the sensitivity on different antifungal substances of some strains of Malassezia pachydermatis isolated from dogs, in order to assess the effectiveness of the products that are frequently recommended in the treatment and to monitor the effect of resistance to antifungals of this yeast specie. Of the total strains tested, all showed sensitivity to Clotrimazole. The sensitivity to the other two antimycotic substances, with potential antimycotic efficacy, was different, because there were strains sensitive to Miconazole, but intermediately sensitive to Nystatin. Against Ketoconazole and Amphotericin B, all tested strains were considered resistant, the zone of inhibition being non-existent or very small.

Keywords: *Malassezia*, dogs, antimycotic, sensitivity.

The genus *Malassezia* includes various species of yeasts, some of which are etiological agents of dermatological conditions in humans and animals (3).

Currently, it is known that the genus *Malassezia* includes seven distinct species of yeasts, differentiated on the basis of morphological, ultrastructural, physiological and especially molecular characteristics.

Yeasts of the genus *Malassezia* are characterized by a thick and multilayered cell wall, a unipolar budding process, as well as their ability to use lipids as a carbon source (1, 8).

Among these, the specie considered an opportunistic pathogen of domestic animals, *Malassezia pachydermatis*, is unique in that it does not have an absolute requirement for long-chain fatty acid supplementation in the culture medium. This ability to grow on lipid-restricted media simplifies its recognition and differentiation from other *Malassezia* species, in the microbiology laboratory (2, 10).

M. pachydermatis is a commensal organism that is frequently found on the skin, mucous membranes, especially in the ear of healthy dog (4, 11, 16, 17). Favorable growth conditions in the local environment allow the overgrowth of this organism, which may then function as an opportunistic secondary agent (4, 11, 14).

Based on various *in vitro* tests, some studies have reported high rates of resistance of *M. pachydermatis* strains isolated from dogs to various antifungal

agents (6, 9, 16, 18).

In clinics, prescription of topical or systemic antifungal drugs is done without sensitivity tests, which can lead over time to the development of the resistance phenomenon (19).

Taking into account these aspects, the aim of this study was to test the sensitivity of some strains of *Malassezia pachydermatis* isolated from dogs present in veterinary clinics, in order to assess the effectiveness of the products that are frequently recommended in the treatment and to monitor the effect of resistance to antifungals of this species of yeasts.

Materials and methods

Cultivation, isolation and mycological examination. The sensitivity to certain groups of antifungal substances was performed in the Microbiology laboratory of the Faculty of Veterinary Medicine from Timisoara.

The strains of *Malassezia pachydermatis* isolated and tested for sensitivity to antifungals come from dogs diagnosed with otitis, which were presented in the veterinary clinics in Timișoara.

In veterinary clinics, from dogs with otitis were performed smears from the otic secretions, which were stained by Diff-Quik method and examined using a light microscope and 100× (oil immersion) The highlighting of *Malassezia* cells, with a characteristic peanut appearance, confirmed the presence of yeast at the otic level (Fig. 1). The classic peanut shape is due to the fact that *Malassezia pachydermatis* is characterized by monopolar budding of daughter cells from one site on the cell wall (12).

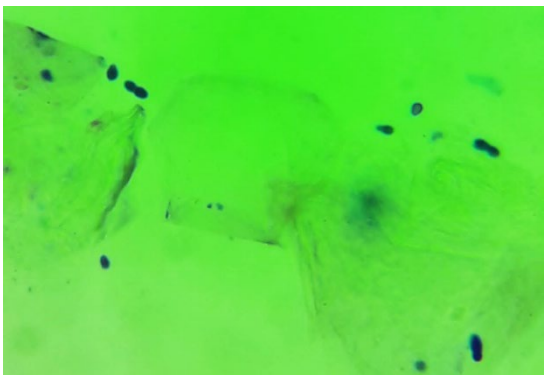

Fig. 1. The classic peanut shaped *Malassezia* yeasts are visible in examination under 100× (oil immersion objective) microscope (original)

From the ears of dogs with otitis, in which the cytological examination showed the presence of characteristic yeast cells of *Malassezia,* samples were

taken with sterile swabs, for cultivation and isolation of *M. pachydermatis* strains in the laboratory. Each sample was identified and brought to the laboratory. Inoculations were carried out by streaking on the surface, on the Sabouraud medium, without added lipids. The plates were incubated at 25 °C for 4 to 5 days. Colonies with a characteristic appearance were then inoculated on Sabouraud medium, without the addition of lipids, to obtain pure cultures. Colonies of *Malassezia pachydermatis*, are convex and smooth initially, but their appearance changes, becoming dry and shriveled, creamy white on the front and the reverse (12).

Each pure culture developed was examined microscopically to confirm the presence of the microorganism (yeast) in a pure state. For the examination, smears were stained by the Giemsa method. These were examined under a cedar oil immersion microscope and 100 objective (Fig. 2).

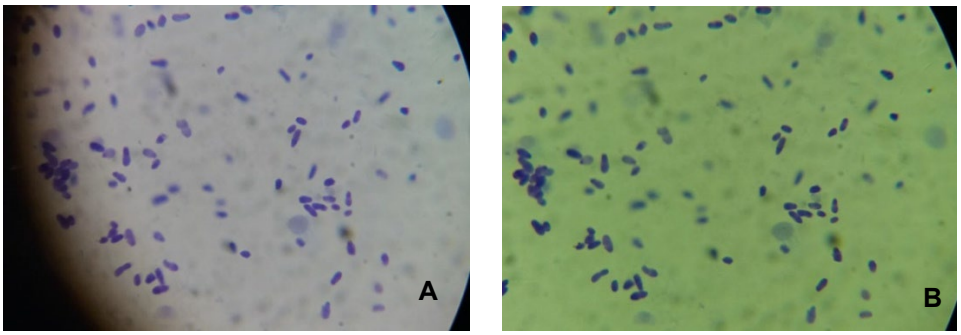

Fig. 2. Yeast cells of *Malassezia pachydermatis*, isolated in the laboratory (x100) (A and B)

Antimycotic susceptibility test

From the total of 50 samples analyzed from dogs with otitis, *Malassezia pachydermatis* species was isolated and identified from only 16 animals.

Each of these isolated strains was tested for antifungal susceptibility.

Five antifungal substances were selected: Ketoconazole, Clotrimazole, Miconazole, Amphotericin B and Nystatin (HI Media Laboratories).

Of these, three are substances included in the *Azole* group (Miconazole, Ketoconazole and Clotrimazole), which act on the ergosterol synthesis pathway, by blocking the activity of the enzyme 14 a dimethylase, changing the structure and permeability of the cytoplasmic membrane, and the other two (Amphotericin B and Nystatin), are part of the *Polyene* group, which act by selectively binding to ergosterol molecules, from the structure of the cell wall, causing in this way both the modification of the structure and the permeability of the fungal membrane.

The tests were carried out by the disk-diffusimetric method. The diffusimetric method is performed on solid media and is based on the ability of the

substances present in the disks to diffuse into the culture medium, from the place where they are deposited, according to the method recommended by the Clinical and Laboratory Standards Institute (20).

In order to carry out the test, fresh cultures were prepared from the 16 strains of *Malassezia pachydermatis* isolated and preserved in the discipline.

For this, each strain was inoculated onto Sabouraud's medium, poured into test tubes, which were incubated for 4 days at 25 °C.

Then 10 ml of sterile nutrient broth was poured over the obtained fresh cultures and the culture present on the surface of the medium was scraped with the help of the microbiological loop. A suspension of yeast cells was thus obtained, in a concentration of 10^6 (at the turbidity of the standard 0.5 Mc Farland), to carry out the inoculation on the solid medium, Sabouraud.

From each test tube, which contained the suspension of yeast cells, 1 ml was taken and was inoculated by flooding on the surface of the solid medium in the plates. The uniform dispersion of the inoculum on the surface of the medium was then ensured by performing rotating movements with the plate slightly tilted. The plates were then left to rest for 15 minutes to ensure good adhesion of the spores to the surface of the medium, and then sterilely, the excess liquid from the surface of the medium was removed with a pipette. At the end, on the surface of the culture medium, from each plate, the antimicotic disks were placed, at the distance of 30 mm between the disks and 15 mm from the edge of the Petri plate. After 10 - 15 minutes (pre-diffusion time) from placing the disks, the Petri dishes went for incubation at a temperature of 25°C. These were read after 4 days of incubation.

For interpretation, the diameter of the inhibition zone was taken into account, measured in mm with a ruler, in two - three directions, including the disk. The results were expressed by transcribing the diameter of the inhibition zone in mm, indicating the corresponding values in qualitative attributes: resistant strains, intermediate resistance or sensitive strains, according to the criteria specified by the manufacturer of the microtablets (HI Media Laboratories) (tab. 1)

Table 1

Criteria for assessing the action of the antifungal substances tested (mm)

Interpretation	Susceptible	Intermediate	Resistant
Diameter of inhibition zone (mm)	≥ 20	10 -19,9	5-9

Results and discussions

The results regarding the sensitivity testing of some strains of *Malassezia pachydermatis* to certain antifungals are shown in Table 2.

Table 2

The effectiveness of some antimycotic substances on some strains of *Malassezia pachydermatis* isolated from dogs

No. of the isolated strain	Diameter of inhibition zone (mm)				
	Ketoconazole	**Miconazole**	**Clotrimazole**	**Nystatin**	**Amphotericin B**
1	5	21	28	10	5
2	5	22	31	14	8
3	5	18	42	12	5
4	5	17	22	15	6
5	5	21	27	14	5
6	8	22	40	12	5
7	7	16	31	20	5
8	11	17	33	19	5
9	8	18	28	20	7
10	6	19	28	17	6
11	7	17	29	20	5
12	5	21	32	11	5
13	10	20	40	14	5
14	6	18	31	14	7
15	5	19	29	18	5
16	5	20	27	13	5
X ± sdx	**6.5±1.88**	**19.12±1.89**	**31.12±5.38**	**15.18±3.37**	**5.56±0.96**

Analyzing the results obtained, it was found that of the five antimycotic substances tested by the diffusimetric method, in terms of effectiveness on the isolated strains of *Malassezia pachydermatis* (n=16), Clotrimazole showed a special activity.

Against all strains tested (16 strains), Clotrimazole showed remarkable efficacy, with the diameter of the induced inhibition zones ranging from 22 mm to 40 mm, with an average of 31.12 ± 5.38 mm. Antimycotic action was also found in two other tested substances, namely: Miconazole and Nystatin.

Miconazole determined zones of inhibition with an average of 19.12 ± 1.92 mm, that is, diameters of inhibition ranged from 16 mm to 22 mm. Out of the total of 16 strains, 7 proved sensitive to this substance, and 9 strains were intermediately sensitive.

Nystatin caused, on average, an inhibition of 15.19 15.18 ± 3.37 mm, but out of a total of 16 strains, only 3 were sensitive to this substance (diameter ≥ 20 mm), the difference of 13 strains of *M. pachydermatis* proving to be intermediately sensitive.

Analyzing in detail, however, the sensitivity of each strain tested, it was found that all 16 showed sensitivity to Clotrimazole, but the sensitivity to the other

two antifungal substances (Miconazole and Nystatin) was different, in the sense that there were strains that were sensitive to Miconazole (7 strains), but intermediately sensitive to Nystatin.

Almost all tested strains can be considered resistant to Ketoconazole and Amphotericin B. The diameter of the zones of inhibition induced by the two antimycotic substances was extremely small or non-existent (microtablet diameter = 5 mm) on average 6.5 ± 1.88 mm for Ketoconazole and 5.56 ± 0.96 mm respectively to Amphotericin B (intermediate sensitive - 10 -19.9 mm and resistant 5-9 mm).

It is known from specialized studies that, in clinical practice, antifungals such as Itraconazole and Ketoconazole are still effective against *Malassezia* infections, which is why they are widely used in current practice. J*esus* et al. (7) using the technique of exposing a microorganism to increasing concentrations of the antifungal substance in the broth, reported a dramatic increase in resistance to Flouconazole, Ketoconazole and Itroconazole in 30 isolates of *M. pachydermatis* (7).

Similar results were obtained in another study, conducted by *Cafarchia* et al. (5) Overall, these findings indicate that *M. pachydermatis* is a microorganism capable of developing resistance mechanisms. It is not yet known, and needs to be investigated, to what extent the in vitro conditions applied to induce antifungal resistance in *M. pachydermatis* mimic what happens to this microorganism when exposed to drugs during the treatment of a dog.

Resistance to treatment with an antifungal substance is considered to be clinical when in a clinical case there is persistent infection or relapses that recur after 4 weeks after the end of treatment, on a correct dosage regimen of the antifungal drug.

To date, no correlation has been reported between the sensitivity of an antifungal substance on the species *Malassezia pachydermatis* and its clinical effectiveness, as well as the occurrence of relapses during a treatment. As a result, investigations must be done in this regard.

Overall, the sensitivity to ketoconazole and itraconazole, shown in specialized studies, was higher than to other types of antifungals, including griseofulvin and terbinafine. However, these results are different from those obtained in our study (13).

Conclusions

The present study revealed that among the five substances tested for antifungal efficacy against some strains of *Malassezia pachydermatis*, Clotrimazole showed a special activity, determining zones of inhibition that were well above the sensitivity limit given by the manufacturer of discs.

Miconazole and Nystatin acted with less effectiveness, causing smaller inhibition zones than Clotrimazole, having from intermediate to susceptible efficiency.

Of the total strains tested, all showed sensitivity to Clotrimazole, but the sensitivity to the other two antimycotic substances, with potential antimycotic efficacy, was different; there were strains sensitive to Miconazole, but intermediately sensitive to Nystatin.

Against Ketoconazole and Amphotericin B, all tested strains were considered resistant, the zone of inhibition being non-existent or very small.

Antifungal susceptibility tests for dermatophytes should become routine in veterinary and human practice both to ensure treatment effectiveness and to prevent the development of resistance.

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CANINE LEISHMANIOSIS: ETIOLOGICAL AND EPIDEMIOLOGICAL REVIEW

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Summary

Leishmaniosis is a parasitic zoonosis, caused by protozoa of the genus Leishmania and transmitted by vectors. The vectors involved in the transmission of the parasitosis are phlebotomines of the genera Phlebotomus and Lutzomya, by means of wich the disease is distributed in the environment. The main natural reservoir of the disease is infested dogs, whose symptoms manifest in three forms: cutaneous, cutaneo-mucosal and visceral. The clinical picture is expressed through skis lesions, weight loss, anorexia, lymphadenopathy, ocular lesions, epistaxis, locomotor dysfunction and muscle atrophy. Canine leishmaniosis has been recorded in about 55 countries of the world, and as far as the European continent is concerned, the area of the interes is the Mediterranean Sea basin. In the last decade there has been an increase in canine leishmasiasis cases in Europe and the possible causes are climate change, increased migration and urbanisation. Studies report that between 2041-2070, based on global warmning predictions, a broad spread of sand fly species is expected in countries as Poland, the Czech Republic, Slovakia and Romania, which will also lead to an increase in the incidence rate of leishmaniosis. With increased animal travel from disease-free areas to endemic areas, the disease may evolve and its incidence may increase significantly.

Keywords: Lesihmaniosis, Phlebotomus, Mediterranean Basin, Zoonosis, Global warming.

Leishmaniosis is a vector-borne parasitic zoonosis caused by protozoa of the genus *Leishmania*. Phlebotomines belonging to the genera *Phlebotomus* and *Lutzomya* are the vectors that contribute to the spread of parasites and the spread of the disease in the environment (34).

The pathogen has a particular importance in veterinary medicine, firstly because of the large number of susceptible animals and secondly, because of the significant number and diversity of vectors. In host organisms, parasites are located intracellularly, in macrophages (8, 35).

Wild animals like rodents, hyracoids, and wild carnivores have been the primary reservoir for leishmaniosis. Subsequently, after the domestication of animals, and until today, dogs have been considered the most important hosts and reservoirs, playing a major role in the epidemiology of the disease (11).

The majority of canine leishmaniosis cases have been identified in China, Brazil, and the Mediterranean basin, with about 50 of the world's 88 countries being affected. The life cycle of vectors is favoured by climate change, leading to an increase in their geographical distribution. Risk factors such as increased migration,

increased deforestation, urbanisation, malnutrition, immune suppression and failed treatments have led to an increase in the global rate of leishmaniosis in humans as well as in animals (2, 15).

Depending on the involved protozoa species, on the host's immune profile and on the clinical signs, leishmaniosis can occur in three forms: cutaneous, muco-cutaneous and visceral, which is both the most common and the most severe form, often even fatal. Clinical signs in dogs are represented by skin lesions, weight loss, anorexia, lymphadenopathy, ocular lesions, epistaxis, locomotor dysfunction, and muscle atrophy (1, 26).

The disease has clinical variations, making it hard to diagnose based on history and symptoms alone. In order to reach a definitive diagnosis veterinarians should turn to serology in order to detect humoral and cellular responses doubled by microscopic examinations used to detect the parasite (8, 24).

Unfortunately, there is currently no active substance available on the market that can completely cure the disease however, most countries have adjuvant treatment protocols which limit its progression. Immunization of animals is a continuous process, and research is being conducted to develop vaccines against Leishmania pathogens. Canine leishmaniosis can be prevented and controlled by using repellents and by detecting infected dogs during early stages (22, 34).

Etiology

The family *Trypanosomatidae* includes the genus *Leishmania* which is renowned for its numerous species. At first, classification was determined according to several factors such as clinical signs observed in affected patients, insect vector species, serological tests, and geographical distribution. Worldwide, cutaneous leishmaniosis was thought to be caused by *L. tropica* and visceral leishmaniosis by *L. donovani*. In 1908, the first change to the original classification was made, and a new species, *L. infantum*, present in the Mediterranean basin and causing visceral leishmaniosis, was included into the nomenclature (19).

A case of disseminated leishmaniosis in Brazil was found to be caused by *L. braziliensis*, which became the following new species identified in 1911. Yakimoff and Schokhor in 1914 believed that *L. tropica* in Asia had two distinct varieties- one was considered the urban form of cutaneous leishmaniosis, known as *L. tropica* var. *minor* and the other was seen as the rural form, *L. tropica* var. *major* (9, 32).

Over the years, several species of parasites from different parts of the world have been described, but the taxonomic position of many, for example *L. colombiensis* and *L. martiniquensis*, isolated from humans, and *L. enriettii, L. equatorensis, L. herrer* and *L. hertigi*, isolated from wild animals, has remained uncertain (12, 33).

The genus *Leishmania* consists of three subgenera: *Leishmania, Viannia* and *Sauroleishmania*, all of which correspond to the section *Euleishmania*, and species that do not fall into any of these subgenera are included in the section *Paraleishmania* (Table 1) (27).

The species identified as part of the *Leishmania* subgenus are: the donovani complex with *L. donovani*, *L. infantum*, the tropica complex with *L. tropica*, *L. killicki*, the major complex with *L. major, L. gerbilli, L. arabica, L. turanica* and the mexicana complex with *L. mexicana* (*L. pifanoi*), L*. amazinesis, L. garnhami, L. aristidesi, L. venezuelensis* and *L. forattinii.*

The subgenus *Viannia* contains species endemic to the New World. The braziliensis complex with *L. braziliensis* and *L. peruviana*, the guyanesis complex with species *L. guyanensis, L. panamensis, L. shawi*, as well as species *L. naiffi, L. lainsoni, L. lindenbergi, L. utingensis.*

The *Paraleishmania* section includes the species *L. colombiensis, L. equatoriensis, L. hertigi, L. herreri* and *L. deanei* (7, 12).

Table 1

Classification of species into the three subgenera of the genus *Leishmania*

Leishmania	***Viannia***	***Sauroleishmania***
L.donovani	*L. braziliensis*	*L. colombiensis*
L. infantum	*L. peruviana*	*L. equatoriensis*
L. tropica	*L. guyanesis*	*L. hertigi*
L. killicki	*L. panamensis*	*L. herreri*
L. major	*L. shawi*	*L. deanei*
L. gerbili	*L. naiffi*	
L. arabica	*L. lainsoni*	
L. turanaica	*L. lindenbergi*	
L. mexicana	*L. utingensis*	
L. amazinesis		
L. garnhami		
L. aristidesi		
L. venezuelensis		
L. forattinii		

To study the phylogeny of the *Leishmania* genus, several species have been studied in the last decade. Fraga et al. have helped to establish a simplified taxonomic framework through DNA sequencing assays and hsp70 analysis, and the classification of species into the genus *Leishmania* in the future should be based on gene sequencing analyses (6, 14).

Leishmania species cannot be distinguished structurally. Non-flagellate (amastigote) forms, which parasitize in the reticuloendothelial system, are ovoid, measuring between 2 and 5 μm in size, with homogeneous cytoplasm, a spherical

nucleus and a rod-shaped or spherical kinetoplast. Infected macrophages are blackberry-shaped and hypertrophied. Amastigotes can be seen both intracellularly and extracellularly when macrophages are lysed or altered by staining techniques (Fig. 1) (5).

Amastigotes are usually located within dermal, splenic, hepatic, bone marrow macrophages and, exceptionally, blood monocytes. Multiplication is achieved by longitudinal binary division (5).

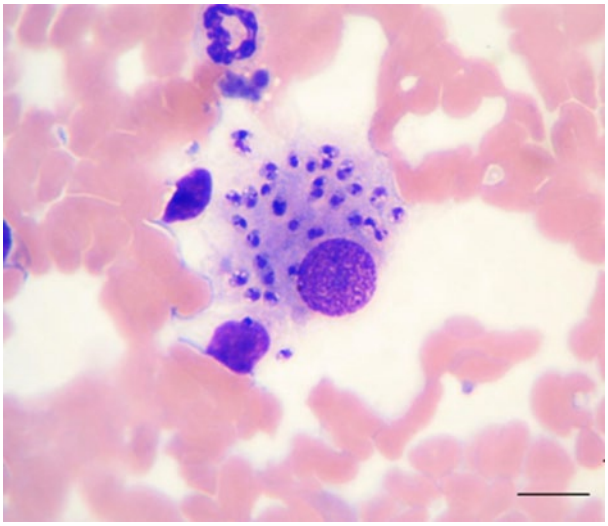

Fig. 1. *Leishmania infantum* in canine macrophage (35)

The promastigote (flagellate) stage develops in the salivary glands and gut of vectors, but also on cell cultures, *in vitro*. The shape is similar to a spindle, with the nucleus positioned centrally and the kinetoplast pointing towards the anterior pole. The size of promastigotes ranges between 2 and 4 μm. In the dermis, promastigotes are phagocytosed by histiocytes, they lose their flagella, divide, and transform into amastigotes (5).

Phlebotomines are the vectors responsible for transmitting leishmaniosis to humans and animals through biting. The feeding of the vectors on the blood of an animal is how they in turn become infested, as these feeding sessions are important for the development of parasites inside the host (3).

The vectors belong to the family *Psychodidae*, subfamily *Phlebotominae*, with almost 800 species identified corresponding to the genera *Phlebotomus, Lutzomya, Sergentomya, Warileya, Hertigia, and Brumptomya*. Species of interest are represented by *Phlebotomum ariasi, P. perniciosus, P. sergenti, P. similis, P. neglectus, P. papatasi, P. perfiliewi, P. alexandri* and *P. tobbi* (8).

Morphologically, phlebotomines are small, brown-yellowish diptera measuring between 1 and 4 mm. The body consists of three segments (head, thorax and abdomen). On the head they have two pairs of antennae, with 16 segments each, and two compound eyes. The female is haematophagous, with mouthparts adapted for stinging and sucking, while the male mouthparts are non-functional. The second segment, the thorax, is composed of three fused segments: pro-, meso- and metathorax. Phlebotomines have a single pair of hairy, lance-shaped wings, dorsally inserted on the metathorax. Ventrally, on each thoracic segment, a pair of pentasegmented legs is inserted. The abdomen consists of ten segments, but only the first eight are visible, the last two fulfilling the role of the hypopygium and oviscape (8).

Distinguishing the sexes is easy because the external genitalia are highly developed in the male (Fig. 2). The digestive tract comprises the following segments: oral cavity, hypopharynx, midgut, goitre, pylorus, hindgut and rectal ampulla. The first portion comprises a blood sucking duct, formed by the labrum approaching the hypopharynx, followed by the oral cavity with dendrites and then the pharynx, dilated in the distal portion. The salivary glands are considered adnexal glands and are identified on the edge of the pharynx. It communicates with the midgut via the cardia, and the pylorus is located between the middle and posterior portions (8).

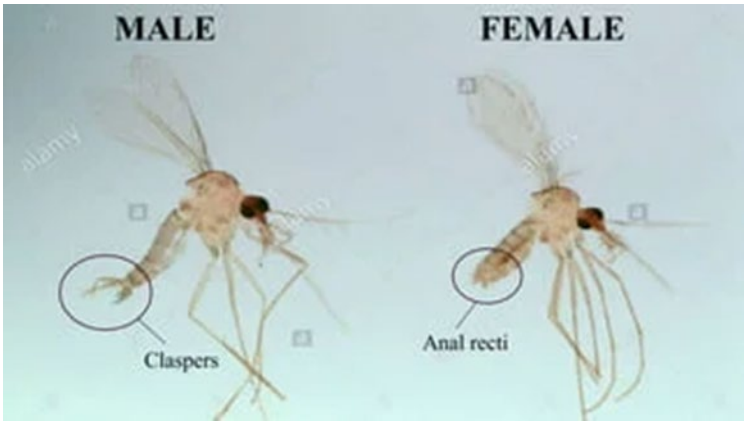

Fig. 2. Differentiation of the sexes in phlebotomines (37)

During feeding, phlebotomins inject chemicals that inhibit blood clotting and stimulate the production of histamine by mast cells, which contributes to the distention of capillaries and promotes blood flow. The multiplication of these diptera is autogenous, they must feed on blood in order to produce eggs. A single meal can support the production of over 100 eggs (37).

The life cycle of these insects is divided into four larval stages that progress over 3-5 weeks depending on environmental abiotic factors. To promote their

development, the ideal temperature is 23°C and relative humidity must be between 80 and 100% (8).

Phlebotomines live in sinanthropic environments and show crepuscular activity. Phlebotomines have two seasons of activity, spring and autumn. In the Mediterranean basin, activity peaks in late summer and early autumn, when the phlebotomine population is mainly composed of adults, which have fed several times and may be infected. In Latin America*, P. longipalpis* is active throughout the year, especially in the periods May-June and October-November. The disease is transmitted through the bite of female phlebotomines. In North America, the disease has arisen through the movement of dogs from one continent to another (5).

The life cycle of the parasite is carried out with the participation of humans, dogs and rodents on the one hand and phlebotomines on the other. The parasites multiply in reticuloendothelial cells, macrophages and polymorphonuclear cells. By feeding on blood from sick individuals, phlebotomins ingest the amastigote forms, which will later multiply in the gut and lead to promastigote development. Depending on environmental conditions, within about 15-20 days the promastigotes, in the digestive tract, become infested. Through the flagella, they move into the vector's body and reach the salivary glands. When feeding, they inoculate the pathogen by biting animals or humans (5, 23).

The feeding mode of phlebotomines is traumatic and creates an area of haemolymph drainage which allows promastigotes to come into contact with macrophages, which will phagocytose the parasites, allowing amastigotes to develop. The multiplication and spread of amastigote macrophages in the host organism are fast.

Direct transmission between two dogs or from dog to human is possible, but only through direct contact between a wound rich in parasite infected exudate and another injured skin area (5, 23).

Epidemiology

Around 800 species of phlebotomines are known worldwide, most of them belonging to the *Phlebotomus* and *Lutzomya* genera. Of these, only 10% can be vectors for various diseases and about 30 species have public health implications. In the Palearctic region, the dominant genus is *Phlebotomus*, including regions such as: Europe, Asia, northern Africa, northern and central parts of the Arabian Peninsula. In North America and Latin America, phlebotomines of the genus *Lutzomya* predominate in number and distribution. Phlebotomines or sand flies (genus *Phlebotomus*) are the main vectors in the emergence and spread of animal and human leishmaniosis and are most often observed in desert and semi-arid ecosystems, while individuals of the genus *Lutzomya* are found in areas near forests and in habitats with abundant vegetation (21).

Sand flies are the only arthropods adapted for the transmission of leishmaniosis, and a relatively low proportion of phlebotomines that are infected with *L. infantum* (0.5-3%) is sufficient to maintain infection in endemic areas. Other

proven routes of transmission have been transfusions from contaminated blood donors, vertical transmission and venereal transmission (29).

The studied species have shown *Phlebotomum ariasi* to occur in Spain, the southern regions of France, England, up to the German-Polish border. The species are expected to spread to countries such as Poland, the Czech Republic, Slovakia, Romania, Moldova and Ukraine between 2041 and 2070. According to studies, *P. perniciosus* is distributed broadly similarly to *P. ariasi*, but will have a major expansion in the future, with presence in countries such as the UK, Germany, Hungary, Romania, Ukraine and Turkey (34).

P. sergenti and *P. similis* are present in the Mediterranean basin (Spain, Italy, Macedonia, Greece and Turkey), the difference being that *P. similis* is specific to the eastern region of the basin. *P. neglectus, P. papatasi, P. perfiliewi* and *P. tobbi* have similar requirements, and as distribution *P. perfiliewi*, unlike *P. papatasi*, is not identified in Spain, while *P. tobbi* has distribution limited to the eastern part of the Mediterranean coast (34).

Mucocutaneous leishmaniosis is caused by *L. panamensis, L. amazonensis, L. major, L. tropica*, *L. infantum* and *L. brasiliensis*. Visceral leishmaniosis in humans is also called Kala-Azar disease and is a severe, fatal disease. This form of the disease is caused by *L. donovani* (East Africa and India) and *L. infantum* (Europe, Latin America and North Africa). In Latin America cutaneous and visceral leishmaniosis are associated with strains of *L. braziliensis* and *L. mexicana* (34).

In European countries, only two species of *Leishmania* are endemic: *L. infantum*, which causes the cutaneous form and has the dog as a reservoir, and *L. tropica*, with sporadic cases in Greece. European countries reporting indigenous cases of leishmaniosis are Portugal, Spain, France, Italy, Greece, Malta, Cyprus, Croatia, Albania, Bulgaria and Turkey. Although most reports include sporadic, endemic forms, recent outbreaks of leishmaniosis have occurred in Spain and Italy, and increased travel has led to the importation of cases of the disease into free countries (23).

The risks of leishmaniosis emerging or re-emerging in Europe are associated with three scenarios:

1. Introduction of exotic species or strains into Europe through increased human and domestic dog travel.
2. Natural spread of visceral and cutaneous leishmaniosis caused by *L. infantum* and *L. tropica* from the Mediterranean region, where the species are endemic, to neighbouring temperate areas.
3. Re-emergence of the disease in the Mediterranean basin due to an increase in the number of immunosuppressed people (25).

Cases of leishmaniosis reported in free areas are usually in animals imported from endemic areas or which have spent time in those areas. Occasionally cases have been reported in phlebotomine free countries in animals that had not left the territory. This could suggest the existence of an alternative vector, a tick or other insect. Only one case of transuterine transmission of infection has been reported

over time, in a free area, in a puppy borned from an infected mother. Transmission is also possible via transfusions, which have been made with blood from infected animals (28).

Most epidemiological studies of canine leishmaniosis have been carried out using serological techniques because of their ease of implementation and efficiency. It is known that these immunological tests are limited because they cannot differentiate between past and present infections and cross-relationships with other pathogens. Direct immunofluorescence assay (IFAT) is one of the most commonly used methods and is the reference test for estimating the prevalence of canine leishmaniosis in the Mediterranean basin. Some researchers prefer the ELISA technique, considering it easier to perform and interpret. Both techniques have been recommended by the World Organisation for Animal Health to study the prevalence of this disease (17).

The Mediterranean basin is an endemic region for canine leishmaniosis, where the disease is a major veterinary issue but also raises human health concerns (Fig. 3). However, the geographical distribution of the disease is heterogeneous and not all countries and regions have been studied (16).

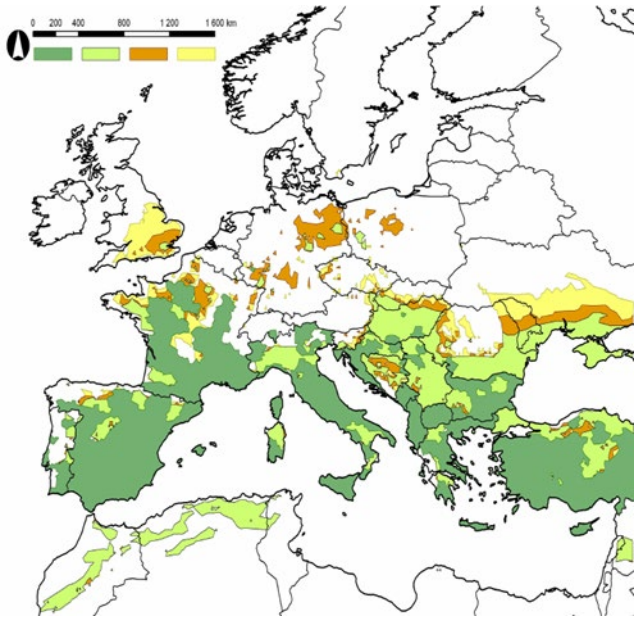

Fig. 3. Current distribution of phlebotomines in Europe (green), projected potential distribution 2011-2040 (orange) and 2041-2070 (yellow) (30)

In southern European countries, the seroprevalence of canine leishmaniosis reaches high levels (Fig. 4). The disease is widespread throughout Spain and considered endemic. Using blood samples from patients in veterinary clinics and from randomly selected dogs, the seroprevalence in the south-eastern region of Spain was found to be 23.7%, with a high incidence in the Axarquia region (Malaga) (18).

The figures dropped considerably when studying the center of the country and the northern area (1-5%), while the north-eastern regions and the Pyrenees mountains showed a prevalence of 19.5%, especially among hunting dogs. The lowest prevalence was recorded in the Canary Islands, 0-2.5% (10).

In other southern European countries, such as Italy, France, Portugal, epidemiological surveys showed prevalence rates of 14% in the Campania region (Italy), an average of 12% in France and an average prevalence of 6.31% in Portugal. Surveys have shown that the area of distribution of *Phlebotominea* is constantly increasing, due to global warming, thus increasing the prevalence of leishmaniosis (16).

Canine leishmaniosis is also reported in Greece, where the pathogens are *L. infantum* and *L. tropica*. In a study carried out between 2005 and 2010, the average seropositivity of dogs was 22.1%, reaching values of over 50% in some places (20).

In the Balkan region, the incidence was found to be lower compared to the above countries, with a prevalence of 3.2% in Albania, 3.1% in Bosnia and Herzegovina, 1.38% in Croatia, 5% in Montenegro, and 1.8-10.6% in Serbia and northern Macedonia (31).

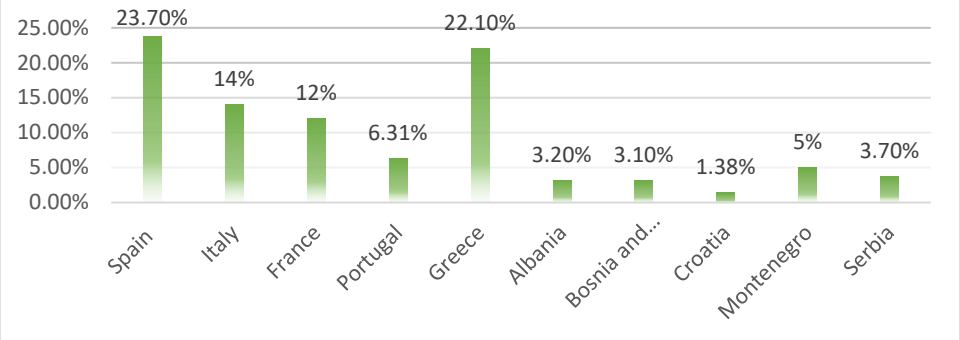

Fig. 4. Prevalence of canine leishmaniosis in some european countries (15, 17, 19, 30)

Although the disease is not endemic in central Europe, vectors such as *P. mascittii* have been identified in Germany and Austria. *P. perniciosus* has been identified in south-west Germany, but with no cases identified so far (16).

Romania is considered a leishmaniosis-free country, but there are historical reports, from 1912-1968, of indigenous cases of *Leishmania infantum* parasitism in dogs and humans in southern Romania. Between 2013 and 2017 there were new reports of *L. infantum* in dogs, a human and a jackal in southern Romania. These reports indicate that there is a risk of canine leishmaniosis becoming endemic in southern Romania. Only one case has been identified in Hungary since 2012 and 10 cases have been reported in Bulgaria in the last decade (4, 13).

Conclusions

Although the disease is not endemic in central Europe, vectors such as *P. mascittii* have been identified in Germany and Austria. *P. perniciosus* has been identified in south-west Germany, but with no cases identified so far.

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THE EPIGENETICS PROCESSES AND THEIR IMPORTANCE IN VETERINARY MEDICINE

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Summary

Epigenetics studies the possible mechanisms by which gene expression can be altered without affecting the nucleotide sequence of the gene. These changes can be transferred by mitosis, but also by meiosis and they are dynamic throughout the life. The main epigenetic changes can take place at DNA level, at histones level, through chromatin remodelling, or posttranslational through non-coding RNA. All these changes are important because they can influence cell fate and also plasticity of the phenotype, by mediating the influence of environmental factors (exposome) on the genotype. The purpose of this article is to present some aspects regarding the history evolution of the epigenetics concept, the mechanisms of epigenetics, the importance of epigenetic changes in gametogenesis, embryonic development, in animal production (such as the quantity and quality of milk, the quality of meat, the production of eggs, or the quality of wool fibre), in the appearance of pathologies, such as cancerous changes, in heritable disorders, but also in livestock immunity. In this article, we also discused epigenetic biomarkers and their possible applicability in diagnosis, prognosis, and even in epigenetic therapy of animal pathologies in order to improve animal health.

Keywords: epigenetics, DNA methilation, gene impriting, biomarkers, livestock.

History of Epigenetics

Epigenetics is a word that comes from the ancient Greek term “epigenesis”, where “epi” means *on* or *after* and “genesis” is translated as *origin, to be produced*. The epigenesis is a theory according to which the embryo is formed gradually in the egg by the successive formation of new parts (37). In the seventeenth century, this theory competed with the theory that "the living organism is completely formed in the germ" (38). It is the physician and physiologist William Harvey that, in approximately 1650 described the concept of epigenesist by that of development as a gradual and complex process starting from initially uniform material within the egg. This notion can be traced back to Aristotle's original proposal (7).

Today, the term epigenesis no longer has the same meaning as it did at the time, and in fact refers to: "the action of external, non-genetic factors that influence the course of embryogenesis and of various biological, psychological and other processes" (according to the Larousse). So, the term is old and has drastically changed its meaning over time.

There have been many debates over the centuries about the veracity of epigenetics. Many famous researchers, such as Maupertuis and Descartes, have examined the question. Descartes, for example, proposed an epigenetic theory

based on his own mechanism, imagining, during fertilization, particle movements and shocks leading to a kind of self-organization, which he believed could be explained in a purely material way. However, this theory was soon put aside and it was Maupertuis who succeeded in bringing epigenetics to the fore, epigenetics itself being rarely attested before the 1740s. "the history of embryology, from the 1740s onwards, was to be that of a constant progression of epigenesis, until its triumph (after much resistance) in the first decades of the 19th century" (24).

As for epigenetics proper, Conrad Hal Waddington was a leading embryologist and geneticist from the 1930s to the 1950s and plays an important role in the epigenetics domain. He is remembered mainly for his concepts of the 'epigenetic landscape' and 'genetic assimilation'. The first time he talked about the epigenetic landscape was in his book named “Organizers and Genes”, which was published in 1940. But the main book which describes more in detail the subject is named “The strategy of the Genes”, and was published 17 years later in 1957. According to Arizona state university’s article about Embryo Project Encyclopedia: “The epigenetic landscape is a visual metaphor of a ball signifying a cell, traveling down a landscape of ridges and valleys where multiple factors influence the cell to take a certain course toward a final tissue type” (33).

Waddington's investigations in genetics led him to develop the concept of genetic assimilation, which he proposed as a mechanism aligned with Darwinian principles. This idea posits that certain acquired characteristics could become inheritable. Waddington specifically directed his attention to the crossveinless trait in *Drosophila* as a focal point of his genetic assimilation studies. *Drosophila* are flies which are widely used in genetics researches, because of their characteristics: have a short life-cycle, a small size, simple genetics and a well-characterized genome (∼180 Mb genome size and encodes ∼13,600 genes) (33).

Conrad Waddington was aware, based on his developmental research, that altering the environmental temperature or introducing a chemical stimulus could induce Drosophila embryos to exhibit varying thorax and wing structures. The diagram below (Fig. 1, 2) may help us to better understand his intentions. We can see that, originally, there were several possible routes for the marbles to take. However, one route will be chosen more than another, showing that development can be canalized to follow different route, which has become more favorable than another depending of the environmental conditions. As a result, adults with the same genotype could express different physical traits or phenotypes (17).

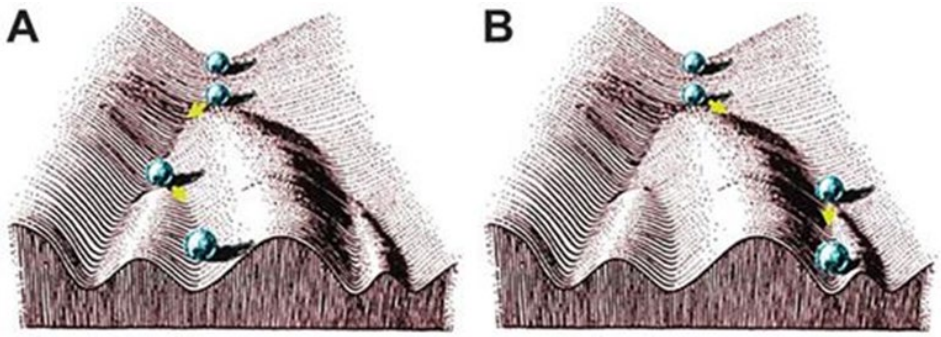

Fig. 1. Waddington's developmental landscape diagram, the development can follow different routes A or B (17)

The crossveinless trait in Drosophila, typically manifesting at a higher frequency in heat-treated flies, eventually established itself within the population over several generations without the need for continued heat exposure. This transition was attributed to the utilization of latent genetic variations that had undergone a process of assimilation (33).

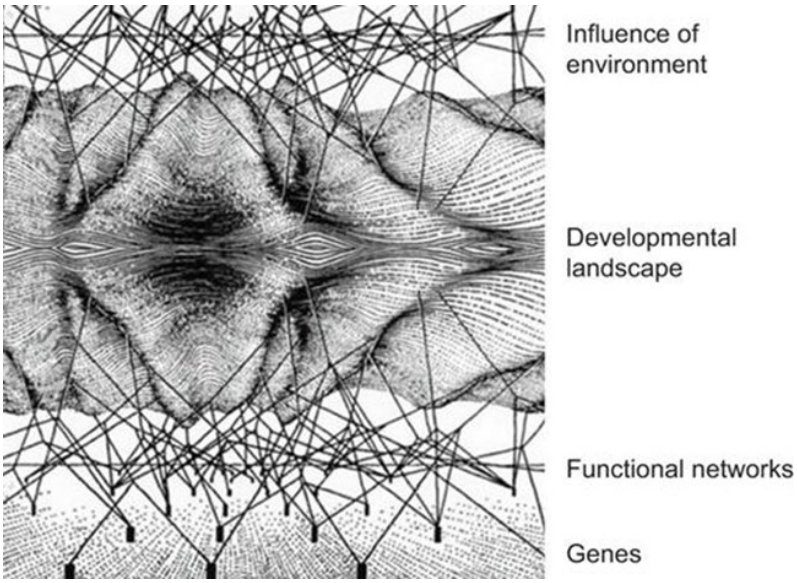

Fig. 2. Waddington's diagram to show how the developmental landscape relates to individual genes through networks of interactions in the organism (17)

Epigenetic mechanisms

Epigenetics refers to the study of heritable changes in gene expression or cell phenotype that do not involve changes in the underlying DNA sequence. These changes can be influenced by environmental factors and have a significant impact on various biological processes. Epigenetic mechanisms include several key processes that alter gene expression and play a fundamental role in development, cell differentiation, and disease (1).

These mechanisms function as cellular memory, being the source of diversity, the interaction between genes and extrinsic factors leading to the emergence of phenotypes. The first epigenetic reprogramming occurs with fertilization, when the inherited genetic material undergoes changes to reveal the activity of genes that are essential for embryonic development. Due to the significant loss of epigenetic signs with fertilization, this reprogramming, necessary for the harmonious development of the embryo, takes place.

The mechanisms of epigenetics refer to certain signs placed at the genome level, which lead to the organization of inherited genetic material into active, respectively inactive, domains that order the genomic reading. Epigenetic marks imprint lasting changes in gene expression, but without altering DNA. They are fixed in response to an external stimulus, persist despite its disappearance and are hereditary and stable. Epigenetics is a response given to the cell to ensure the selection of the genetic information (12).

The main epigenetic mechanisms include:

DNA methylation: DNA methylation is one of the most well-studied epigenetic mechanisms. It involves adding a methyl group (-CH_3) to a cytosine base in the DNA molecule. DNA methylation typically results in gene silencing, as it can block the binding of transcription factors and other regulatory proteins to the promoter region of the gene. This methylation is controlled by a family of enzymes called DNA methyltransferases (DNMTs). DNA methylation is a reversible process, achieved by passive/active demethylation, but with each mitosis there is a progressive loss of epigenetic information.

Histone changes: Histones are proteins that help package DNA into a compact structure called chromatin. Post-translational histone changes, such as acetylation, methylation, phosphorylation, and ubiquitination, can influence RNA polymerase and DNA accessibility to transcription factors. Histone-specific changes can activate or suppress gene expression.

Noncoding RNAs: Noncoding RNAs, including microRNAs (miRNAs) and long noncoding RNAs (lncRNAs), are sequences consisting of less or more than 200 nucleotides that do not encode genetic information but can regulate gene expression at the post-transcriptional level. For example, miRNAs can bind to messenger RNAs (mRNAs) and inhibit their translation or promote mRNA degradation.

Chromatin remodeling: The chromatin structure can be modified by chromatin remodeling complexes. These complexes use energy to eject or

restructure nucleosomes, making regions of DNA more or less accessible for transcription.

Histone code: The combination of different histone changes on a given piece of DNA is often referred to as the "histone code". Different combinations of histone changes can convey specific regulatory information about gene expression.

Epigenetic inheritance: Some epigenetic changes can be passed from one generation of cells to another, which can lead to hereditary changes in gene expression. This is especially important in processes such as differentiation and cell development.

Imprinting: Imprinting involves epigenetically marking certain genes in a manner specific to the parent of origin. For example, genes inherited from the mother can be differentially marked compared to those inherited from the father.

X chromosome inactivation: In females, one of the X chromosomes is epigenetically inactivated in each cell to balance gene expression between the sexes.

Environmental influences: Environmental factors, including diet, exposure to toxins, and stress can influence epigenetic changes. These changes can have both short- and long-term effects on gene expression and health.

Understanding these epigenetic mechanisms is important for strengthening our knowledge about the development, the disease and the potential therapeutic interventions. Epigenetic research continues to uncover the complex regulatory processes that govern gene expression and cell function (34).

Epigenetics in germ cells

A germ cell represents the sexual reproductive cell at any stage from the primordial cell to the mature gamete. Germ cells, unlike somatic cells, possess the unique capability to give rise to an entirely new organism upon fertilization. To enable this remarkable process, germ cells undergo a highly specialized cellular differentiation process called gametogenesis. This intricate process leads to the formation of distinct oocytes and sperm, both in terms of their structure and function, which are essential for successful fertilization and subsequent development (9).

Germ cells are both genetically and epigenetically important. Genetically speaking, they will be used to transmit the genetic information, carrying the genetic material from one generation to the next; they contribute to the genetic diversity, which is essential to the adaptation and evolution of species; and helps the preservation of the genome, by minimizing mutations and DNA damages. From an epigenetic point of view, germ cells also carry epigenetic information and so plays a role in epigenetic inheritance, epigenetic reprogramming (crucial to ensure that the epigenetic information is appropriately reestablished during early embryonic development, allowing for proper differentiation and development of the organism), and also has a disease and an environmental impact (in the sense that these changes may have long-lasting effects on the health and development of future generations) (23).

The central concept in this field is that an organism's exposures, like for example diet, stress, chemicals, and so on, affect the composition of germline non-DNA molecules. All of these exposures could then affect descendants' phenotypes. Epigenetic molecules present in sperm and oocytes will be passed on during fertilization. These epigenetic factors within germ cells are subject to potential changes based on an individual's life experiences and exposures. Consequently, abnormalities in the epigenetic profiles of sperm and oocytes can have a substantial impact on the risk of disease in future generations. So, epigenetics can be a good thing for future generations, but it can also be a bad thing depending on what is passed on (23).

Some environmental exposures, resulting in epigenetic changes in gametes include: cigarette smoke, caloric restriction in utero which induce histone retention, glyphosate (a broad-spectrum system herbicide and crop desiccant) can induce DNA methylation in sperm cells, early life trauma can induce miRNA and lncRNA modifications in sperm cells, obesity can induce miRNA modification in sperm cells or restraint stress with histone acetylation, methylation, phosphorylation on oocytes (5).

Cigarette smoke has an effect on histones in germ cells, by inducing histones retention, which normally it should be around 8% from the sperm epigenome. During spermiogenesis, that is a distinct process that take place after spermatogenesis, morphological changes and chromatin compaction take place in order to prevent paternal genome from damage. So, the majority of histones are replaced first by testis-specific histone and transition proteins (TPs) and in the end, they are replaced by protamins. Epigenetic modifications, such as acetylation, ubiquitination, methylation or phosphorylation were identified in order to facilitate the histone-protamine transition (28).

The sperm epigenome through histones have a possible role in transmitting epigenetic memory from the sperm to the embryo. Histone retention appears in genome region that contains 3K4me3, H3.1K27me3 and H3.2K27 and have a reduced intrinsic affinity for protamins (27). Then in the following factor, caloric restriction in utero, so the fact that hey restrain food during the pregnancy, will implies, in addition to histone retention, DNA methylation. According to the National Library of Medicine: "DNA methylation is a heritable epigenetic mark involving the covalent transfer of a methyl group to the C-5 position of the cytosine ring of DNA by DNA methyltransferases (DNMTs)".

The early life trauma factor triggers an epigenetic change at the level of the long non-coding RNAs (lncRNAs). It is a subtype of non-coding RNAs (ncRNAs). They are defined by their length and consist of more than 200 nucleotides. Typically situated in intergenic regions without protein-coding capabilities, lncRNAs may occasionally overlap with coding genes. LncRNAs might play a role in both silencing and activating protein-coding genes, thereby influencing their translation (15). As for microRNAs (miRNAs), which we can see that they suffer epigenetic changes in case of both obesity and early life trauma situations; they constitute a

category of small, noncoding RNA molecules ranging from 18 to 28 nucleotides in length. They primarily serve in posttranscriptional regulation, influencing protein expression. Their participation has been evidenced in both normal and pathological cellular processes. In the context of tumors, certain miRNAs act as oncogenes, while others function as tumor suppressors. The elevation of oncogenic miRNAs, termed oncomiRs, has been observed in cancer cells (19).

In the situation of restraint stress, which represent a method used to induce physiological responses in an animal by restricting its free movement, the consequences are histone acetylation, methylation and phosphorylation. Unlike the other cases, this factor affects oocytes rather than sperm cells. Histone acetylation is the process consisting of the neutralization of the charge on histones, which will therefore influence transcriptional regulation. Methylation is the introduction of a methyl radical into a substance. Unlike acetylation, it does not alter histone charge or directly impact histone-DNA interactions. Histone phosphorylation plays a crucial role as an intermediate step in various cellular processes such as chromosome condensation during cell division, transcriptional regulation, and DNA damage repair. In contrast to acetylation and methylation, histone phosphorylation initiates interactions with other histone modifications, acting as a platform for effector proteins. This interaction, in turn, triggers a downstream cascade of events.

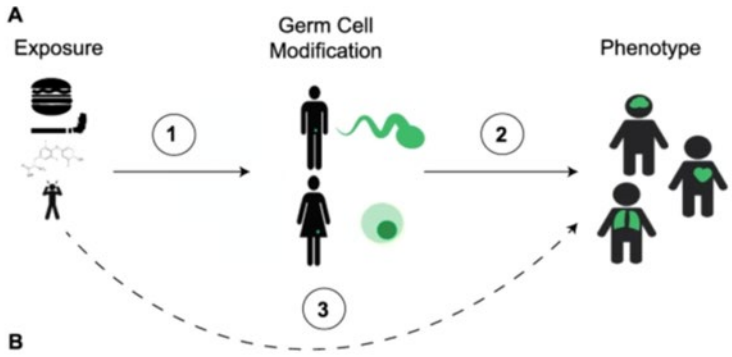

Fig. 3. Sheme expressing the germline-mediated non-genetic inheritance in humans (23)

To conclude about epigenetics in germ cells, comprehending this field is essential for elucidating how gene expression is managed and how environmental factors influence the developmental process (Fig. 3). Epigenetic mechanisms, including DNA methylation, histone modifications, non-coding RNA, and chromatin structure, play a crucial role in regulating gene expression. Therefore, a comprehensive understanding of development and biological processes necessitates the integration of both epigenetics and genetics. Unfortunately, the

process of transgenerational epigenetic inheritance, which involves manipulating epigenetics during gametogenesis to obtain epigenetically modified sperm and eggs, is not well understood yet. Futures researches have still to figure out whether and how this information in germline from parents could pass onto their progenies; and if so, whether somatic cells and/or germ cells of the progenies inherit this information. So, since the understanding of the epigenetic regulation of gametogenesis is a relatively recent area of investigation, there's still a lot more to discover on this subject in the future (5).

Epigenetic in the embryo

In the context of embryonic development, epigenetic mechanisms play a crucial role in regulating gene expression and determining cell fate. Understanding the role of epigenetics in embryonic development is key to unraveling the complexities of cell differentiation and tissue formation. Epigenetic changes not only shape the developing embryo, but also help program patterns of gene expression that persist into adulthood.

Influence of mitochondria on fertility

The major implications of mitochondria for the viability of oocytes and sperm are known. Due to their ability to generate the energy needed by a cell, any mutation in mitochondrial DNA, which is a small circular genome, can be the cause of various pathologies. The dependence of oocytes and sperm on mitochondria is related to the high energy requirement for chromosome segregation and fertilization. An inadequate number of copies of mitochondrial DNA has been shown to have major implications for fertility rates. Mitochondrial DNA mutations and epigenetic changes in mitochondria play important roles in various biological processes and can impact health and disease.

Vertebrates inherit mitochondria through the maternal line. In animals, the subjection of females to oxidative stress, but also the old age are correlated with decreased viability of oocytes and fertility. In this regard, the technique of mitochondrial supplementation has been used, based on partial or even total cytoplasm transfer from oocytes collected from young females.

Epigenetic changes in mitochondria

The number of mtDNA copies is high in mature oocytes, on average between 200.000-400.000, which indicates their importance for the beginning of embryogenesis. DNA methylation: While nuclear DNA is highly methylated, the role of DNA methylation in mitochondria is still an area of active research. Recent studies have suggested the presence of low levels of DNA methylation in mitochondrial DNA, and changes in mitochondrial DNA methylation have been associated with certain diseases and aging.

MtDNA methylation is reduced in oocytes, blastocysts, granulosa cells and is found in the CG regions, but also in the CHG, CHH regions. This mtDNA methylation seems to have a role in gametogenesis and can be influenced by environmental factors, but also by the age of the female (25). This reduction of methylation during oogenesis is carried out by the TET (Ten-eleven translocation

methylcytosine dioxygenase) family of enzymes, so that during the pre-implantation period of the embryo, mtRNA methylation is carried out by DNMT3A and DNMT3B enzymes and is then maintained by DNMT1. Methylation of mtDNA seems to be consistent with methylation of nuclear DNA involved in factors that ensure mtDNA replication, but also of genes involved in development (26).

Changes in histone-like proteins: Mitochondria contain proteins with histone-like functions that can regulate gene expression in the mitochondrial genome. These proteins help package and organize mitochondrial DNA, influencing its accessibility for transcription.

Mitochondrial ribonucleic acid (mtRNA) changes: Epigenetic changes, such as RNA methylation and changes in its bases, play a role in regulating the stability and function of mitochondrial RNA. These changes can affect the expression and function of mitochondrial genes.

The assisted reproduction technique is widely used today, but exposure to this method results in diminished competence in oocyte development. Studies in fertilized mouse oocytes have shown this as a consequence of inducing new epigenetic changes.

Another factor that influences epigenetic changes at the embryo level is controlled ovarian stimulation, which aims to increase the likelihood of obtaining a gestation. There has been loss of genomic fingerprint, associated with Beckwith-Wiedemann syndrome, which correlates with excessive growth in bovine fetal tissues. Demethylation of imprinted genes in the bovine genome has also been reported (8).

The studies carried out on models of female mice in which mutations were induced in mtDNA ($Polg^m$ mice) highlighted a reduced fetal growth and placental dysfunctions. The cause being a global loss of oocyte DNA methylation, which determined after fertilization an insufficient demethylation of the zygotic genome and thus dysfunctions in embryonic gene expression (11).

In vitro embryo cultivation – epigenetic changes

Regarding the development of early embryos, there are known mechanisms that have the role of regulating factors in the oviducts of the female reproductive system, where local hormones, nutrients or cytokines act (21). The vulnerability of *in vitro* cultured mammalian embryos is considerably increased, influenced by culture conditions. Aberrant genetic material demethylation kinetics have been detected in embryos cultured *in vitro* compared to those of *in vivo* origin. In addition, oviductal extracellular vesicles have shown to host miRNAs that regulate specific mRNA, which can also alter gene expression. This exchange of information from the oviduct to the embryo is missing *in vitro*, having important roles in regulating the embryonic epigenome.

Cryopreservation

In terms of germ cell preservation, two main methods are used, namely slow freezing and vitrification, which aim to prevent ice crystals from forming inside the cells. Vitrification is a method used especially in the case of assisted

reproduction in cattle, combining rapid increase in medium viscosity and concentration to be dissolved, followed by rapid freezing in liquid nitrogen. As a method, vitrification significantly reduces the ATP content in the oocytes of many mammalian species (29).

In cattle, vitrification of oocytes is connected with a significant reduction in the expression profile of three epigenetic genes, namely DNMT1, DNMT3B and HDAC1. In pigs, in vitrified embryos, a much reduced expression of key epigenetically associated genes was observed, leading to altered epigenetic reprogramming, respectively decreased rates of obtaining the blastocyst stage. (16). Moreover, the presence of cryoprotective agents in the vitrification media may negatively affect the epigenetic profile of the embryos, as dimethylsulfoxide (DMSO) is responsible for the massive DNA methylation and decrease in ATP content observed in vitrified human heart tissue (8).

Nutritional epigenetics

The major implications of nutrition for the epigenome during embryonic development are not new. Prolonged dietary selenium supplementation in rats was observed to affect both global and specific DNA methylation in liver and colon tissue. Malnutrition can also epigenetically induce obesity in offspring, usually manifested in adulthood. Studies have been conducted in mice and cattle that have confirmed the implications of maternal diet on oocyte DNA methylation. Postpartum, in cows exposed to metabolic stress, maternal imprinting of several genes was observed in their hypomenthylated oocytes.

Female obesity and overnutrition affect mitochondrial function, inducing epigenetic changes in mitochondrial DNA. An excessively high-fat or high-carbohydrate maternal diet has been associated with increased mitochondrial DNA, ultimately leading to depletion of the ovarian follicular reserve resulting in young and mature females (8).

Epigenetic and animal production (milk and meat)

From a genetic point of view, the phenotypic variability of the traits of interest in order to obtain productions can be partially explained, because they are influenced by epigenetic changes. Although genomic selection and animal breeding aim to improve traits and productions, in general, performance is particularly influenced by environmental factors, thus limiting the effectiveness of genomic selection. Therefore, it is considered that these epigenetic mechanisms translate the effects of environmental factors on the genome. At present, unfortunately, when considering a genomic selection, this phenotypic variation is neglected (4).

An increase in the productivity of animals is aimed for economic reasons, so, in many cases, the overpopulation of the breeding spaces is reached, implicitly limiting the maintenance conditions. In this regard, the harmful effect of heat stress on lactating dairy cattle and beyond has been proven. Exposure of cattle in the last period of gestation to thermal stress modifies development programming with

negative effects prolonged over 4 years from the actual exposure of the animal to the stressful thermal factor.

In order to obtain beef cattle, it must be emphasized that, in the development of skeletal muscles, a crucial role is played by the intrauterine environment, which is also dependent on maternal resources. In the case of overfeeding or feed restriction, the changes will influence the final meat quality of the draft product. In particular, the genetic effect of SNPs within epigenetic genes, related to beef quality and carcass traits, is currently being studied, starting from epigenetic changes that can be transmitted from one generation to another (10).

Interventions of epigenetics are also known, especially in animal reproduction, therefore different epigenetic markers are pursued in the selection of breeding animals, such as DNA methylation, histone modifications and non-coding RNAs. Epigenetic changes that influence meat production and quality can be related to muscle growth and development, fat deposition, tenderness, flavor or meat marbling (31).

Researchers are exploring ways to use genetic interventions to improve animal production. For example, epigenetic editing techniques such as CRISPR-Cas9 can be used to modify specific epigenetic marks in animals, potentially improving desired traits (30).

Meat quality is influenced by a combination of genetic and environmental factors, but epigenetics plays a role in regulating the expression of these genes that influence the quality. Although a complex and multifactorial process, specific genes known to be epigenetically regulated and affect meat quality in cattle, pigs and chickens include:

Myostatin (MSTN): Myostatin is a gene that inhibits muscle growth. Epigenetic changes can affect its expression. A reduced expression of myostatin leads to increased muscle development and improved meat quality in terms of tenderness.

Fatty acid metabolism genes: Epigenetic changes can influence the expression of genes involved in fatty acid metabolism, affecting meat marbling and fat content. Genes related to lipogenesis and lipolysis, such as FABP4 (fatty acid binding protein 4) and FASN (fatty acid synthase), are among those epigenetically regulated.

Desaturase genes: Genes encoding desaturases, such as FADS1 (fatty acid desaturase 1), are involved in the production of unsaturated fatty acids, which contribute to meat flavor and tenderness. Epigenetic regulation of these genes can influence the fatty acid composition of meat.

Meat sensory perception genes: Genes associated with taste and sensory perception, such as TAS1R and TAS2R (taste receptor genes), can be epigenetically regulated and can influence the overall taste and flavor of meat.

HSP (Heat Shock Protein) genes: Epigenetic changes can affect the expression of heat shock protein genes. These proteins play a role in meat quality, influencing the texture, color and tenderness of the meat.

Collagen genes: Collagen is a protein that mainly affects the texture of meat. Epigenetic regulation of collagen genes can influence collagen content in muscle tissues and, consequently, meat quality.

Heme oxygenase (HO-1): Heme oxygenase is an enzyme involved in the degradation of heme. Epigenetic regulation of HO-1 can affect oxidative stability and meat color (24).

The epigenetic regulation of these genes is very complex and can be influenced by various factors, including nutrition, stress or management practices. In addition, the specific genes and the involved epigenetic changes may vary between different animal species and breeds.

Researchers continue to study the epigenetic regulation of genes related to meat quality to improve breeding programs and production practices in the meat industry. Epigenetics can have a significant impact on milk production in animals.

Milk production is influenced by various genetic and environmental factors, and these epigenetic changes may play a crucial role in regulating lactation-related gene expression and milk quality. The following implications are:

Lactation gene expression: Epigenetic changes, such as DNA methylation or histone modifications, can influence the expression of genes involved in lactation. For example, genes responsible for milk protein synthesis, such as the casein and lactoglobulin genes, are epigenetically regulated. Appropriate epigenetic control of these genes is essential for high-quality milk production.

Mammary gland development: Epigenetic regulation of genes involved in mammary gland development and differentiation is essential for milk production. Epigenetic changes can affect the growth and differentiation of mammary epithelial cells, which are responsible for milk production.

Milk fat and protein composition: Epigenetic factors can influence milk composition, including milk fat and protein content. Genes related to milk fat synthesis, such as fatty acid desaturase genes, can be epigenetically regulated.

Epigenetic inheritance: Epigenetic changes can be passed from one generation to another, affecting the lactation and milk production characteristics of the offspring. This is particularly relevant in breeding programs where the aim is to produce animals with specific milk production traits.

Dairy researchers and practitioners continue to explore the potential of epigenetics to improve milk production practices, given that milk quality is influenced by a combination of genetic, nutritional and environmental factors. Although a complex and multifactorial process, some specific genes known to be epigenetically regulated and affect milk quality in dairy animals such as cows and goats include:

Casein genes (CSN1S1, CSN2, CSN1S2, CSN3): Caseins are the major proteins in milk, and their expression is vital for milk quality. Epigenetic changes can influence the expression of casein genes, affecting the protein composition and nutritional value of milk.

Lactoglobulin gene (LGB): Lactoglobulin is another important milk protein that plays a role in milk quality. Epigenetic regulation of the LGB gene can affect its expression and consequently the protein content and characteristics of milk.

Lipogenic genes: Genes involved in milk fat synthesis and fatty acid metabolism can be epigenetically regulated. These genes include FASN (fatty acid synthase), DGAT1 (diacylglycerol O-acyltransferase 1), and SCD (stearoyl-CoA desaturase). Epigenetic changes can affect milk fat composition and its nutritional properties.

Mammary gland developmental genes: Epigenetic regulation of genes related to mammary gland development and function may influence milk quality. Genes involved in mammary epithelial cell differentiation and milk synthesis, such as GATA3, ESR1 (estrogen receptor alpha), and PRLR (prolactin receptor), are among those that can be epigenetically regulated.

Inflammatory response genes: Epigenetic changes can affect genes related to the inflammatory response in the mammary gland. This can influence the quality of the milk, controlling the number of somatic cells and the presence of inflammatory compounds in the milk.

Immune genes: Epigenetic regulation of genes involved in the immune response can influence milk quality by affecting mammary gland health and milk production.

Stress-related genes: Stressors such as environmental conditions, management practices and diseases can induce epigenetic changes that affect the expression of stress-related genes. These genes can affect milk quality by influencing dairy animal welfare and milk production.

It is important to note that the epigenetic regulation of these genes is complex and can be influenced by various factors, including nutrition or stress. Furthermore, the specific genes and the involved epigenetic changes may vary between different breeds and individual animals. Dairy researchers continue to study the epigenetic regulation of genes related to milk quality to improve breeding programs and production practices, ultimately providing consumers with high-quality dairy products (20).

Epigenetics and cancer

“Cancer is a disease caused when cells divide uncontrollably and spread into surrounding tissues” by the National Cancer Institute definition. From a genetic and epigenetic point of view, cancer is the result of mechanisms in which, in addition to genetic mutations that affect the gene sequence or genome, there are other modifications known today as epimutations that alter the genes but do not affect the sequence, making up the epigenome (32). Cancer can develop when epigenetic changes go wrong. They can affect the two main groups of genes with cancer connections -- oncogenes and tumor suppressor genes. Oncogenes can become too active, and tumor suppressor genes may change or go away (36).

Throughout life, our cells accumulate epigenetic alterations; it is not yet established to what extent they play a passive role in senescence or whether they

are a causative agent. By altering the expression of genes involved in cell regulation, epigenetic modifications play a fundamental role in the initiation and progression of tumours. But, unlike genetic mutations, they are potentially reversible (14). Cancer cells often divide anarchically and uncontrollably: they proliferate to the detriment of the organism. Some cancerous tumours do not stop their progression once they have invaded the tissue of origin. They may lose their ability to adhere to neighbouring cells, escape the original tissue and progressively invade other tissues or even spread throughout the body. At each stage in this progression towards metastasis, the cell undergoes changes (32).

The cancer epigenome exhibits widespread changes in DNA methylation, histone modifications, and non-coding RNA (ncRNA) mediated regulation of gene expression, providing a growth advantage to tumor cells and fostering cancer development. DNA methylation acts as a regulator of gene expression, by switching genes between active and inactive states. Notably, hypermethylation of promoters in CpG islands stands out as a well-recognized mechanism of epigenetic alterations in cancer cells, playing a crucial role across various cancer types. Both genome-wide and gene-specific DNA methylation alterations are critical events in the initiation and progression of tumors (Fig. 4) (18). Cancer cells can be distinguished from their healthy counterparts by this epigenetic modifications of opposite tendencies: a global methylation deficit on the one hand, and foci of aberrant hypermethylation located in the promoters of tumour suppressor genes on the other. These two phenomena coexist in all the types of cancer analysed. Cancer cells are usually characterised by an overall methylation deficit of around 25% (14).

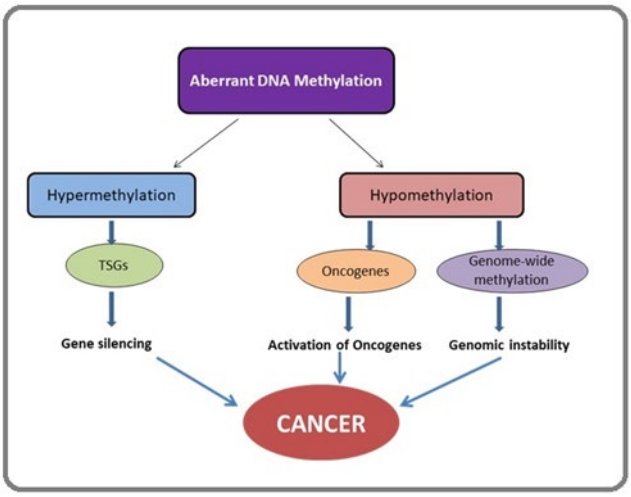

Fig. 4. Sheme of the cascade of events leading to cancer (18)

Epigenetic changes are a ubiquitous presence in all human cancers and collaborate with genetic alterations to drive the cancer phenotype (18).

The consequence of hypomethylation is the increased expression of oncogenes, which are genes promoting cancer. Conversely, hypermethylation works to suppress the expression of tumor suppressor genes, contributing to the overall initiation and progression of cancer. These processes collectively play an essential role in the complex landscape of cancer development (35).

The clinical uses of epigenetics include defining the epigenomic profile of tumours (for example, their methylation profile), which opens the way to the development of so-called epigenetic diagnostic tests. These tests can be based on a PCR (polymerase chain reaction) test that specifically detects the DNA methylation profile in the cell samples studied.

Drawing a link with previous research into the influence of environmental factors on the body, cigarette smoke is by far the most studied external factor when it comes to cancer: several epigenetic marks have in fact been associated with the development of breast and lung cancers, and with lung cancer survival prognoses (2).

Moreover, epigenetic changes are not only implicated in the onset of cancer but also hold significance in predicting cancer prognosis. Reduced levels of histone post-translational modifications, whether through methylation or acetylation, are associated with unfavorable outcomes in prostate, lung, and kidney cancers. On the contrary, heightened levels of a specific histone modification, H3K9ac, are linked to lower survival rates in lung cancer. This underscores the intricate relationship between epigenetic alterations and the clinical prognosis of cancer patients (35).

Concerning human cancers, abnormal epigenomic processes are recognized as contributors to diverse stages of neoplastic development, including initiation, promotion, invasion, metastasis, and resistance to chemotherapy. Recent propositions suggest that over 300 genes and their products undergo epigenetic alterations in different human cancers. These modifications are implicated in proliferative changes, cellular atypia (a cell presenting abnormalities), dysplasia (the abnormal growth or development of a tissue or organ), carcinoma in situ (group of abnormal cells), invasive malignancy, metastatic malignancy, and resistance to therapy. Carcinogenesis, which is the initiation of cancer formation, involves the activation of genes that enhance cell growth (oncogenes) or inhibit cell death, alongside the inactivation of tumor-suppressor genes. Tumor suppressor genes can be rendered inactive through mutations that disable their functions, loss of the gene itself, or epigenetic changes that switch off the gene in a heritable somatic manner, distinct from DNA sequence mutations (18). The silencing of genes through epigenetic mechanisms can impact cancer progression at various stages. The epigenetic changes in gene expression and their pathologic correlation is a result of overlapping changes in genes expression. However, some of these

changes may be specifically associated with particular stages of cancer development (13).

Major epigenetic changes during cancer development (reprogramming of the methylome) are carried out by hypermethylation and hypomethylation of DNA. DNA hypermethylation occurs especially in CpG regions, which are normally not methylated and have a role in controlling anticancer mechanisms, but which become inactive if they are hypermethylated. Unlike these CpG islands where cytosine is not methylated, in other non-CpG regions cytosine is heavily methylated normally, but shows hypomethylation during neoplastic transformation (6).

Epigenetic events leading to gene silencing play a crucial role in tumorigenesis (Fig. 5). The initial phases of tumorigenesis are characterized by abnormal clonal expansion, which arises during the stresses of cell renewal. It is often induced by factors like aging and chronic inflammation. These cell clones are particularly susceptible to subsequent genetic and epigenetic alterations that may drive the progression of tumors (3).

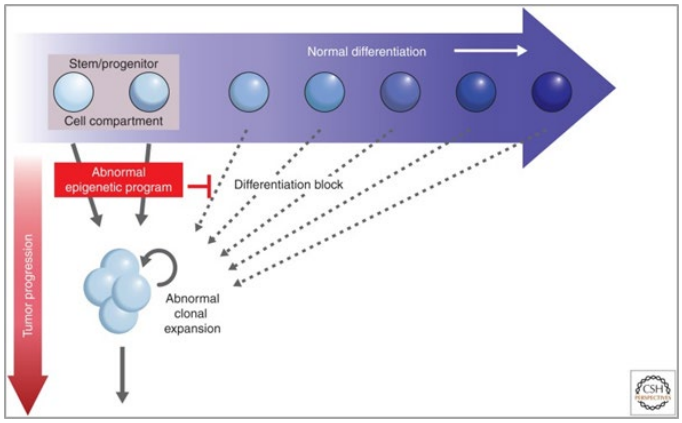

Fig. 5. How an abnormal epigenetic program could affect a cell (3)

The treatment of cancer with chemotherapy is based on the destruction of tumour cells, generally by apoptosis. Epigenetic therapy works by modifying the gene expression profile within tumour (14). The market for anti-cancer epimedicines is presented as very promising. According to a recent prospective study, the global market for this type of drug and for diagnostics should increase fourfold by 2027. Epigenetics therefore appears to be an opportunity on which both pharmaceutical companies and biotech start-ups are positioning themselves (2). There are already drugs or epidrogens that target these modifications. Two of them, for example, azacitidine and entinostat, are mainly used to treat patients with certain blood cancers (32). The convergence of epigenetic therapies with conventional cancer treatments, encompassing chemotherapy, immunotherapy,

and targeted therapy, has emerged as an appealing approach in cancer therapy. This integration supports the notion that epigenetic drugs (epi-drugs) can synergistically interact with other anticancer agents, potentially reversing resistance to cancer therapies in preclinical models (Fig. 6) (18).

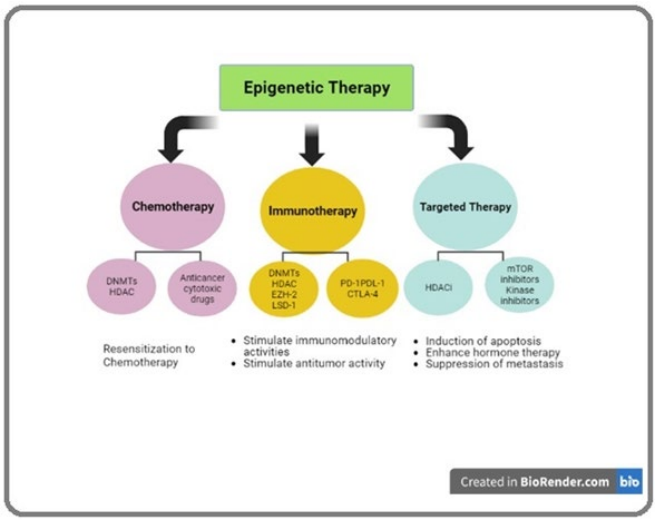

Fig. 6. Sheme of the functionement of the epigenetic therapy (18)

The cancerous cell contains gene mutations, as well as epigenetic changes, compared to a healthy cell. The role of medical research is to better understand how epigenetic changes can influence cancer development and tumor growth (Edith Heard, Professor at the Collège de France and Director of the Genetics and Developmental Biology Unit) (32).

In cancer therapy, epigenetic biomarkers have a promising role, for example test for multi-cancer early detection (stMCED). These tests have the role of creating a panel of biomarkers from circulating tumor DNA (ctDNA), which are found in biological fluids, are stable and early detectable (6).

Conclusions

This retrospective study presented some implications of epigenetics in different important fields in veterinary medicine such as reproduction, animal production or cancer pathology. The role of epigenetics as a new discipline in medicine is to improve diagnostic accuracy, to provide new epigenetic biomarkers, as well as the investigation of new therapeutic targets.

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CORRELATIVE STUDY REGARDING THE ANEMIC STATUS IN THE HEMATURIC PATIENT

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Summary

In the pathology of companion animals, hematuric diseases of renal and non-renal origin, due to their complexity, diversity and incidence, constitute a dominant one whose research of clinical-evolutionary aspects and paraclinical changes require a focused multidisciplinary and correlative-analytically evaluated approach. Hematuria by severity and usually by serious physiopathological insults at the systemic level or at the level of the affected urinary tract components is mainly a clinical expression that impresses the owner by the presence and sometimes by the drama of the clinical expression, calling for a thorough evaluation by the specialist veterinarian. The clinical investigations aimed at assessing the systemic, functional and physical changes in the urinary system aimed at achieving a characteristic dominant clinical framework for the presumptive clinical diagnosis of hematuria in pets. The hematological, biochemical blood, urinary and ultrasonographic evaluations were carried out in order to confirm the presumed clinical diagnosis and to assess the systemic or functional co-affection at the level of the urinary system. In some cases, due to chronic blood loss through the lower and upper urinary tract or the genital organs (vagina, uterus, prostate), hematuric patients may present varying degrees of anemia, but especially in the case of chronic kidney disease coexists with the decrease of erythropoietin synthesis.

Keywords: anemia, hematuria, dogs, cats.

The diseases of the urinary system through diversity, the polymorphism of clinical expressions and the numerous systemic and organ physiopathological interferences, together with the extremely diverse and intricate etiopathogenetic context involved in their induction - represent one of the dominant pathology of companion animals (2, 4).

On these coordinates, hematuria of different degrees (macrohematuria and microhematuria), with distinct origins and mode of induction, offers an extremely vast investigative field, often representing a challenge for the specialist veterinarian (6, 7, 8).

It is appreciated that the current specialized literature in human medicine as well as in animal medicine, underlines the overwhelming importance of the clinical examination in establishing the diagnosis, but, more than that, of the modern paraclinical investigations, which with maximum relevance and accuracy allow obtaining a scientific model of interpretation of symptoms and diagnosis (6, 17).

The extremely diverse ensemble of lower (especially) but also upper urinary tract conditions, with inflammatory, lithiasis and neoplastic etiology, require a distinct clinical and paraclinical approach, with imaging investigations, blood and urine

biochemistry, hematological examinations and special urine examinations , whose relevance and clinical-diagnostic and prognostic significance, assessed in an integrated way, can allow the creation of the premise of a therapeutic orientation with maximum accuracy.

The idea is unanimously accepted that the presence of hematuria and its early identification (in the stage of microhematuria) offer, in most situations, real chances of limiting the local/systemic hematuric condition (6, 7, 8).

In general, systemic and organ diseases with hematuric potential are part of groups of urological and nephrological diseases with at least reserved evolution and prognosis.

Minimal periodic screening of adult patients and especially geriatric patients allows very easily the identification of hematuria and its possible causes. General and specific evaluations of potentially hematuric conditions, in the form of well-defined and judiciously recommended protocols, allow improving the medium and long-term prognosis of patients thus affected (9, 12).

Materials and methods

The present study was carried out on a number of 50 companion animals (Table 1) belonging to several breeds, of different ages and sexes, including conditions that assume in their pathogenesis congestive or inflammatory phenomena that were clinically or subclinically expressed with macroscopic or microscopic hematuria.

The study was conducted over a period of 1 year (from 2022 to 2023), analyzing a total of 552 samples (125 samples of lime honey, 114 samples of acacia honey, 96 samples of sunflower honey, 89 samples of rapeseed honey and 128 samples of polyfloral honey).

Table 1

The structure group of hematuric patients included in our study

TOTAL SUBJECTS TAKEN INTO STUDY (n=50)						
DIAGNOSIS	**SPECIES**	**AGE**				**SEX**
		1-5	**6-10**	**11-15**	**16-20**	
PRIMARY ACUTE CYSTITES *(n=9)*	Canine ***(n=5)***	1	1	2	1	M=2 F=3
	Feline ***(n=4)***	1	-	2	1	M=2 F=2
	Canine ***(n=4)***	1	2	1	-	M=2 F=2

SECONDARY CHRONIC CYSTITES ***(n=5)***	Feline ***(n=1)***	-	-	1	-	M=1
ACUTE RENAL FAILURE ***(n=3)***	Feline ***(n=2)***	1	1	-	-	M=2
	Canine ***(n=1)***	1	-	-	-	F=1
CHRONIC RENAL FAILURE ***(n=11)***	Canine ***(n=5)***	1	1	3		M=3 F=2
	Feline ***(n=6)***	-	4	1	1	M=4 F=2
BENING HYPERPLASIA OF THE PROSTATE ***(n=5)***	Canine ***(n=5)***	1	2	1	1	M=5
PROSTATITIS ***(n=9)***	Canine ***(n=9)***	1	3	3	2	M=9
PARAPROSTATIC CYSTS ***(n=2)***	Canine ***(n=2)***	1	-	1	-	M=2
INTRAPROSTATIC CYSTS ***(n=3)***	Canine ***(n=3)***	2	1	-	-	M=3
ANTICOAGULANT POISONING ***(n=2)***	Canine ***(n=2)***	-	1	1	-	M=1 F=1
PYELONEPHRITIS ***(n=1)***	Canine ***(n=1)***	-	1	-	-	F=1

The research activity was carried out in the period october 2022 - october 2023, within the Clinic of the Faculty of Veterinary Medicine Bucharest. The studies and investigations initiated and carried out started from the suspicion and confirmation of the presence of hematuria, which presupposed the performance of some preliminary stages of the clinical examination - the anamnesis and the history of the condition in order to highlight some pre-existing pathologies and concurrent comorbidities, data on the nature of urination, the frequency of urination and the amount of urine eliminated during a micturition in 24 hours, evaluation of the clinical status and subsequently paraclinical methods translated by performing the urine summary, confirmation of hematuria through the microscopic examination of the urinary sediment, biochemical and hematological profile and imaging methods.

The urine sample collection technique consisted of collection by classical methods according to standard protocols - urine stream (obtained following spontaneous urination or manual abdominal compression), urethral catheterization or cystocentesis in a volume of 5-10 mL, with macroscopic evaluation and recording data and unitary processing of the results obtained and subject to the preliminary investigation protocol (summary examination of urine and urinary sediment).

The preliminary urine biochemical examination was carried out with the help

of quantitative and qualitative tests such as urine strips or Urispec Plus type diagnostic strips (Fig. 1) or interpreted with the help of an automatic analyzer respectively, IDEXX VetLab UA Analyzer (Fig. 2.)

In the classic method, the examination involves evaluating the reaction of the reagents with changes in color following the concentration of the analyte present. The colors generated by each pad are visually compared to a range of colors on the strip tube (9, 15).

Urine density is determined by means of the REC-300ATC refractometer, which involves the transfer of a few drops of urine, closing the lid of the prism and appreciating the resulting value through the eyepiece in the direction of the light source (Fig. 3).

The evaluation of the urinary sediment was carried out microscopically with Eurostar III Plus microscope (Fig. 4) having previously been centrifuged for 5 minutes at 5000x in a combined centrifuge. After centrifugation, the supernatant is decanted and the sediment is aspirated with a pipette, placed on the slide and covered with a slide. It is examined microscopically at 400x in order to confirm the presence of red blood cells in the preparation. Hematuria is confirmed at five or more red cells per microscopic field (6, 17).

Fig. 1. Urine reagent strips Urispec Plus (original photo

Fig. 2. Urine reagent strips – IDEXX UA (original photo)

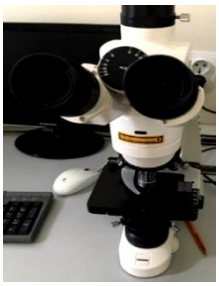

Fig. 3. Refractometer model REC-300ATC (original photo)

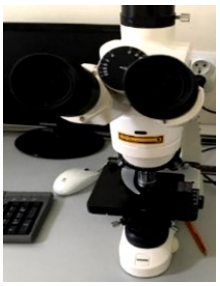

Fig. 4. Light microscope Eurostar III Plus (original photo)

The hematological parameters were determined with IDEXX VetAutoread Hematology Analyzer (Fig. 5), using samples collected in special tubes with anticoagulant. The blood count makes a quantitative and qualitative assessment of the cellular elements in the blood - the number of red blood cells - erythrocytes (RBC), the number of white blood cells or leukocytes (WBC), the total amount of hemoglobin in the blood (ie HGB), the percentage of red blood cells (hematocrit, HCT), mean blood cell volume (MCV), red blood cell size, mean blood hemoglobin (MCH), mean hemoglobin concentration (MCHC), platelet count (PLT) (5).

Fig. 5. IDEXX VetAutoread Hematology Analyzer (original photo)

The imaging evaluation in the case of the present study was carried out with the help of ESAOTE Veterinary MyLab 30, the animal being prepared in advance by trimming the area of choice and positioning it in right or left lateral recumbency, dorsal recumbency and quadrupedal decubitus (depending on the organ addressed).

Results and discussions

In the study and investigations regarding the presence and level of hematuria in renal and non-renal pathologies, this objective was achieved on 50 patients, belonging to different races.

Following the centralization, hematuria was identified in 13 felines and 37 canines, predominantly in males at a percentage of 72% (n=36), and in females 28% (n = 14).

The individuals included in our investigations recognized a classification according to age, being in percentage 22% between 1-5 years (n=11), 34% between 5-10 years in 17 patients, 32 % between 11-15 years in 16 individuals and in

percentage of 12% (n=6) in the age group between 16-20 years.

All patients were divided according to diagnosis, and we will present the hematological values of all patients included in the study (Table 2, 3, 4, 5, 6, 7, 8, 9, 10, 11).

Table 2

Hematological parameters in the patients with primary acute cystities

Parameters	Dog, 7 years, F	Dog, 12 years, M	Cat, 17 years, F	Cat, 3 years, M	Dog, 4 years, F
HGB (g/dL)	**11.7**	**12.8**	**19.8**	**19.3**	**20.1**
HCT (%)	**35.8**	**41.3**	**45.7**	**37.9**	**36.2**
Grans (K/µL)	**14**	**8.9**	**16.9**	**11.4**	**19.9**
WBC(mii/mm^3)	**16.5**	**21.9**	**28.1**	**10.6**	**24.6**
PLT (K/µL)	**518**	**463**	**621**	**501**	**489**

Table 3

Hematological parameters in the patients with secondary chronic cystitis

Parameters	Dog, 13 years, M	Dog, 5 years, M	Dog, 9 years, F	Dog, 5 years, M	Cat, 14 years, M
HGB (g/dL)	**13.7**	**15.7**	**13.0**	**17.8**	**11.3**
HCT (%)	**37.8**	**33.3**	**30.9**	**41.9**	**25.6**
Grans (K/µL)	**15.1**	**18.9**	**20.2**	**9.7**	**17.5**
WBC(mii/mm^3)	**11.2**	**19.6**	**13.8**	**10.4**	**14.7**
PLT (K/µL)	**389**	**250**	**360**	**299**	**73**

Table 4

Hematological parameters in the patients with acute renal failure

Parameters	Dog, female, 5 years	Dog, female, 5 years	Dog, female, 5 years
HGB (g/dL)	**13.7**	**14.8**	**16.8**
HCT (%)	**39.2**	**42.4**	**21.2**
Grans (%)	**13.8**	**10.9**	**18.3**
Grans (K/µL)	**26.5**	**17.8**	**18.2**
PLT (K/µL)	**432**	**526**	**136**

Table 5

Hematological parameters in the patients with chronic renal failure

Parameters	Dog, female, 5 years	Dog, female, 5 years	Dog, female, 5 years		
HGB (g/dL)	**12.1**	**10.8**	**13.0**	**17.8**	**10.9**
HCT (%)	**38.2**	**29.4**	**25.8**	**40.1**	**20.8**
Grans (%)	**14.3**	**19.1**	**22.3**	**10.5**	**19.2**
Grans (K/µL)	**10.66**	**18.7**	**12.9**	**9.9**	**13.8**
PLT (K/µL)	**323**	**401**	**29**	**154**	**168**

Table 6

Hematological parameters in the patients with benign hyperplasia of the prostate

Parameters	Dog, female, 6 years	Dog, male, 9 years	Dog, male, 17 years	Dog, male, 13 years
HGB (g/dL)	**14.5**	**15.7**	**16.8**	**12.9**
HCT (%)	**37.6**	**49.2**	**43.2**	**36.8**
Grans (%)	**12.9**	**15.3**	**19.1**	**20.3**
Grans (K/µL)	**14.8**	**10.1**	**19.3**	**22.7**
PLT (K/µL)	**480**	**378**	**592**	**122**

Table 7

Hematological parameters in the patients with prostatitis

Parameters	Dog, male, 3 years	Dog, male, 6 years	Dog, male, 6 years	Dog, male, 9 years	Dog, male, 13 years	Dog, male,15 years
HGB (g/dL)	**14.2**	**15.8**	**16.2**	**20.1**	**18.6**	**10.1**
HCT (%)	**38.2**	**29.4**	**25.8**	**40.1**	**20.8**	**21.5**
Grans (%)	**14.3**	**19.1**	**22.3**	**10.5**	**19.2**	**25.6**
Grans (K/µL)	**10.66**	**18.7**	**12.9**	**9.9**	**13.8**	**15.8**
PLT (K/µL)	**323**	**401**	**29**	**154**	**168**	**101**

Table 8

Hematological parameters in the patients with paraprostatic cysts

Parameters	Dog, male, 4 years	Dog, male, 12 years
HGB (g/dL)	**16.8**	**17.3**
HCT (%)	**40.5**	**43.8**
Grans (%)	**10.8**	**14.7**
Grans (K/µL)	**8.6**	**12.8**
PLT (K/µL)	**368**	**411**

Table 9

Hematological parameters in the patients with intraprostatic cysts

Parameters	Dog, male, 3 years	Dog, male, 6 years	Dog, male, 6 years
HGB (g/dL)	**18.1**	**20.3**	**19.2**
HCT (%)	**44.8**	**46.8**	**39.5**
Grans (%)	**12.2**	**14.8**	**16.9**
Grans (K/µL)	**11.2**	**9.9**	**14.2**
PLT (K/µL)	**352**	**416**	**169**

Table 10

Hematological parameters in the patients with anticoagulant poisoning

Parameters	Dog, male, 4 years	Dog, male, 12 years
HGB (g/dL)	**9.2**	**8.3**
HCT (%)	**21.5**	**20.7**
Grans (%)	**10.8**	**8.44**
Grans (K/µL)	**17.2**	**3.7**
PLT (K/µL)	**362**	**402**

Table 11

Hematological parameters in the patients with pyelonephritis

Parameters	Dog, female, 7 years
HGB (g/dL)	**17.3**
HCT (%)	**40.8**
Grans (%)	**15.8**
Grans (K/µL)	**8.6**
PLT (K/µL)	**83**

By analyzing the values of the hematological examination, it is found in a proportion of 26% the absence of a paraclinically identified systemic echo at the level of the internal organs, especially on the renal functionality. Through the paraclinical picture performed, 24% of conditions with an insidious evolution from a clinical point of view that can create serious homeostasis disturbances, represented exclusively by anemia, and 30% by granulocytosis and/or neutrophilia, which highlights the presence of an inflammatory/infectious systemic process.

Conclusions

The synthetic and comparative analysis of the results obtained in our investigations allowed the outline based on the correlated association of clinical and paraclinical elements constituting genuine diagnostic protocols/algorithm of upper and lower urinary tract conditions in disorders dominated and/or accompanied by hematuric syndromes.

The set of researches included in the study groups carried out, we appreciate that it has considerable importance in the fundamental research on the incidence, the primary induced complex or responsible for the pathogenic vulnerabilities useful in clinical activity in the structured and correlated screening of hematuric syndromes in companion animals.

The purpose of our work was to present multifactorial etiology in the induction of hematuric diseases with various degrees of damage, varied morphology and functional modifications among the most severe, which through a multidisciplinary approach, can be prevented, limited and even treated successfully.

This study forms the premise of exhaustive multidisciplinary collaborations in the field of research in some hematuric diseases in dogs and cats.

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STUDY REGARDING THE ULTRASONOGRAPHIC FINDINGS OF EXOCRINE PANCREATIC INSUFFICIENCY (EPI) IN DOGS

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Summary

Exocrine pancreatic insufficiency is a syndrome characterized by insufficient synthesis of pancreatic enzymes leading to pancreatic dysfunction, and finally, to maldigestion and malabsorption. Ultrasound examination is a non-invasive technique, that is often chosen as the first diagnostic method in this type of diseases. The purpose of this study was to describe ultrasound features of the pancreas during EPI in dogs. This study was performed in 12 dogs (8 males and 4 females of various breeds, ranging from 4 to 7 years of age), with clinical signs of maldigestion. The most common clinical signs of EPI, present in more than 90% (n=11) of affected dogs, have been reported to be weight loss, poor digestion, flatulence, polyphagia, increased fecal volume and defecation frequency. The conclusive diagnosis for EPI is performed by measurement of serum canine trypsin-like immunoreactivity (cTLI). In all our patients from this study (n=12), cTLI values were lower than 5 µg/L (the reference range in healthy dogs is between 5.0 and 35 µg/L). During ultrasound examination, in all pacients were valued the size, shape, echostructure and echogenicity of the pancreas. The mean pancreatic thickness in all our patients was significantly lower than the mean reference values of healthy dogs. In 66% of dogs (n=8), the pancreas had a normal ultrasound appearance. Additional sonographic intestinal findings were recorded and so, ultrasonographic intestinal abnormal findings were identified in 83% of dogs (n=8) and were suggestive of inflammatory bowel disease.

Keywords: exocrine pancreatic insufficiency, abdominal ultrasound, dog.

The pancreatic dysfunction due to the insufficient synthesis of pancreatic enzymes, is characterized by series of clinical signs among which we mention, weight loss, flatulence, poor digestion, polyphagia, increased fecal volume and increased defecation frequency (11, 16).

To establish a definite diagnosis for EPI, the measurement of serum canine trypsin-like immunoreactivity is performed, because very low serum cTLI concentrations (<2.5 µg/L) associated to typical pancreatic dysfunction clinical signs, are considered highly diagnostic for EPI (10, 13, 16).

Is important to mention that a normal cTLI value (>5.0 lg/L) does not rule out the presence of mild pancreatic dysfunction and because of this, an ultrasonographic evaluation of the pancreas/abdomen could be useful to establish a conclusive diagnosis for exocrine pancreatic insufficiency (1, 3, 14). The ultrasonographic findings in EPI were decreased pancreatic size, irregular widening of the pancreatic ducts and variable echogenicity of pancreatic parenchyma (4, 6, 8). The literature

mentions that in many cases of EPI ultrasound signs of inflammatory bowel disease are present (9, 17, 19).

The aim of this study is to establish the diagnosis of EPI in cases where ultrasound examination detects the thinned pancreas associated with signs of intestinal inflammation, due to the pancreatic dysfunction.

Materials and methods

The present study was carried out in the University Emergency Hospital Prof. Univ. Dr. Alin Bîrțoiu, Bucharest. The study was performed over a period of two years (April 2020 – May 2022), in 12 dogs (8 males and 4 females of various breeds, ranging from 4 to 7 years of age), with clinical signs of maldigestion.

The diagnosis of EPI had been establish based on the clinical signs of the selected animals, and by measuring the decreased pancreatic secretion capacity, using pancreatic function tests (serum cTLI concentration). Values of cTLI<5 are considered cases of EPI (10, 12, 19). In the same time, the pancreas ultrasound examination had to confirm the diagnosis of EPI with a complete report of the size, shape, echogenicity and echostructure (2, 5, 7)

In this study the ultrasonographic exams were performed using a My Lab 30 machine, with 7.5 -10 MHz microconvex probe (Fig. 1).

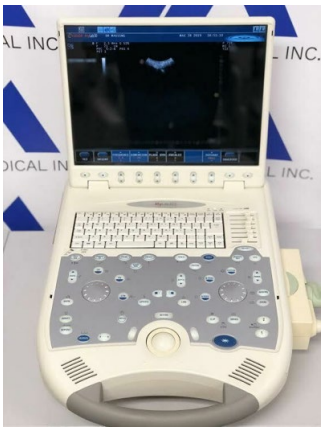

Fig. 1. My Lab 30 machine

Results and discussions

The canine breeds included in this study were, mix breed dogs (*n* = 4), German Shepherd (*n* = 3), Collie (*n* = 2), Boxer (*n* = 1), Jack Russel Terrier (*n* = 1), Labrador Retriever (*n* = 1). The clinical signs (type and frequency) reported in our study are comparable to those described in the specialty literature (11, 13, 20).

In our study we observed, that pacients with cTLI concentrations in range of 2.5 to 5.0 µg/L, may not show symptoms over a long period of time or have only rare or chronic gastrointestinal signs. The main clinical signs observed in the studied pacient (belonging to various breeds, ranging from 4 to 7 years of age) are present in the (Table 1 and Fig. 2).

Table 1

Clinical signs in dogs with exocrine pancreatic insufficiency

No.	Clinical signs of the pacients	Number of pacients	% of pacients
1	**Weight loss**	8	66.6%
2	**Polyphagia**	4	33.3%
3	**Diarrhea**	10	83.3%
4	**Vomiting**	7	58.3%
5	**Flatulence**	6	50%
6	**Steatorrhea**	7	58.3%
7	**Other associated disorders**	5	41.6%

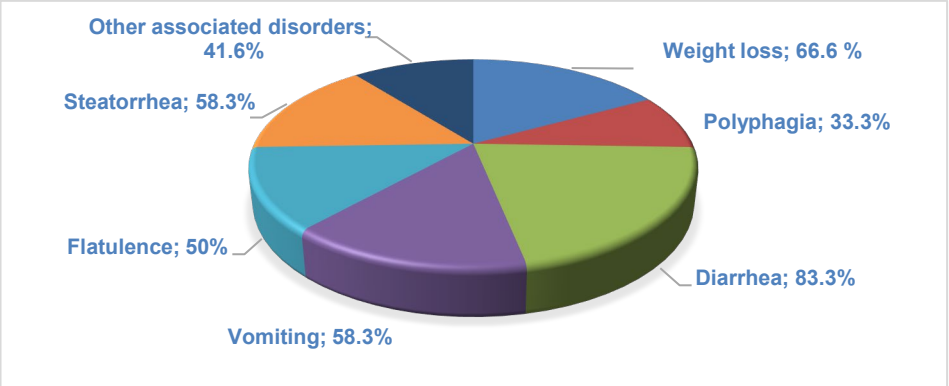

Fig. 2. Graphic representation of the main clinical signs in 12 dogs with exocrine pancreatic insufficiency

The pancreas and abdominal ultrasound examination in cases with EPI is very important because this thechnique could show a diminution of the pancreatic parenchyma, partial pancreatic atrophy, or chronic pancreatitis (Fig. 3-5).

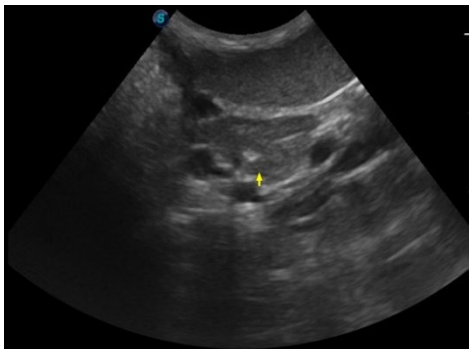

Fig. 3. Pancreatic sclerosis. Sclero-atrophic aspect of the pancreatic lobe – hyperechoic pattern (arrow)

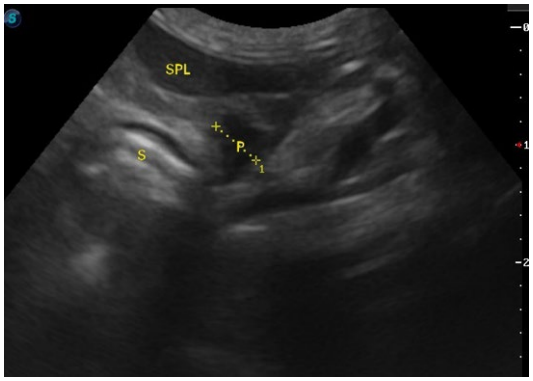

Fig. 4. Pancreatic atrophy. Reduced volume of the pancreas (right lobe). Pancreatic duct (arrow) and sclero-infiltrative aspect of the pancreatic lobe – hyperechoic and hyperechoic micronodules

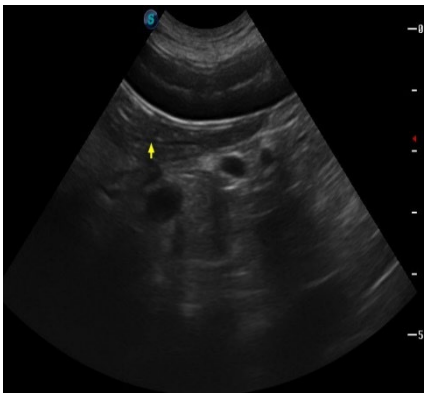

Fig. 5. Pancreatic sclerosis and peri-pancreatic oedema. Obvious reduction of pancreas (*P*) volume with sclerotic aspect and peripheral hipoechoic aspect (oedema)

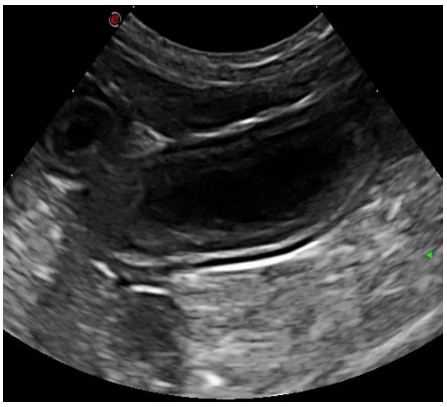

Fig. 6. Chronic enteritis. Important reaction of the intestinal mucosa with hypotonic aspect

In our study we observed that the pancreatic parenchyma atrophy begins in the early stages of the disease, although the onset of clinical manifestations started late, the explanation may be due to the large functional reserve capacity of the pancreas.

The ultrasonographic exam shows also gastrointestinal problems (in a large percentage of pacients – 83%) and the most common gastrointestinal findings were enlargement of the intestinal loops, hyperechogenicity of the intestinal mucosa (Figure 6) and reduced peristalsis, which determines maldigestion due to a reduced pancreatic enzyme production, and failure of intraluminal digestion.

Conclusions

In most of the patients with exocrine pancreatic insufficiency (EPI), the pancreas was thinner than normal, with sclero-atrophic or with sclero-infiltrative aspect of the pancreatic lobe, and in many cases with peripheral hipoechoic aspect (oedema).

In 83% of cases, the ultrasonographic exam shows gastrointestinal abnormalities like important reaction of the intestinal mucosa with hypotonic aspect, and reduced peristalsis, enlargement of the intestinal loops, hyperechogenicity of the intestinal mucosa.

This study, demonstrates the fact that the ultrasonographic exam should be considered among the tests for the diagnosis of exocrine pancreatic insufficiency, but is important to mention that it cannot replace functional evaluation of the pancreas, in order to establish a definite diagnosis.

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COR TRIATRIATUM DEXTER IN DOG: CLINICAL CASE

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Summary

Cor triatriatum dexter in dogs represents a congenital condition with a low frequency. It is characterized by the persistence of the right embryonic valve in the form of a membrane, leading to the division of the right atrium into two chambers. This condition progresses with cardiac symptoms, affecting blood hemodynamics and the overall health of the organism. The presentation of a clinical case in this context represents a novelty in the field of veterinary medicine due to the complex diagnostic process and its low prevalence within the canine population. Clinically, the dog in the present study, suffering from Cor triatriatum dexter, exhibited clinicalsymptoms consisting of a holosystolic cardiac murmur of V/VI intensity, with maximum intensity at the right tricuspid focus, moderate exertional cough, and brief post-exertional syncopal episodes. To establish a correct diagnosis, paraclinical investigations were employed, including lateral and dorsoventral thoracic radiography, which revealed the alteration of the right atrium. Furthermore, electrocardiography and echocardiography in various modes such as B-mode, B+M-mode, color Doppler, spectral Doppler, confirmed the presence of a membrane within the right atrial cavity resembling a valve. Additionally, tricuspid regurgitation was identified with certainty.

Keywords: cor triatriatum dexter, echocardiography, canine, congenital cardiac diseases, tricuspid valve dysplasia

Cor triatriatum dexter (CTD) is a rare congenital cardiac defect of dogs accounting for 0.3% of all congenital heart diseases in this species (1). It is characterized by the presence of an abnormal membrane that divides the right atrium into two chambers: a high-pressure caudal chamber (CaRA) and a low-pressure cranial chamber (CrRA) (2). Cor triatriatum dexter derives from the persistence of the embryological Eustachio's valve or embryologic right sinus venosus. This is a structure that has a physiological presence in embryonic life inside the right atrium, in order to direct oxygenated blood from the cranial vena cava, through the oval foramen, and into the left atrium. In some cases, this structure fails to regress and causes abnormal right atrial chamber bipartition, impeding normal venous return from the caudal vena cava and coronary venous sinus (3). This membrane can be imperforated or perforated. The size and number of the perforations underlie the clinical expression of this pathology. The imperforated forms or those characterized by small perforations will result, for patients at a very young age, in a typical pathological picture marked by signs of right-sided congestive heart failure (4, 5).

In veterinary medicine, the currently described anatomical variants depend on the location of the coronary venous sinus outlet, which can take place at the level of the CaRA or the CrRA (6). Echocardiography is the gold standard for CTD diagnosis since it can be used to visualize the pathological membrane, measure the orifice membrane, record the turbulent flow between the CaRA and CrRA, and evaluate concomitant congenital heart disease (7).

Tricuspid valve dysplasia represents approximatively 2-7% off all congenital cardiac malformations in dogs (15).

Tricuspid valve dysplasia represents approximatively 2-7% off all congenital cardiac malformations in dogs (15). The most common effects of tricuspid valve insufficiency is the increased systolic inflow that generates right atrial volume overload (2, 16). The increased right ventricular end diastolic volume causes eccentric hypertrophy of the right ventricle and allows the right ventricle to eject a larger volume to compensate for the decreased stroke volume caused by the regurgitation over the tricuspid valve. When the diseases progress, the right heart keeps on increasing, determining the widening of tricuspid valve annulus as well (9). It has be proven in other animal species that reactivation of the embryonic pathways for leaflet growth may result in adaptation of the leaflets, but, obviously, this increase in size does not compensate adequately to prevent further worsening of the regurgitation (4). This vicious circle ultimately results in a massive regurgitation with loss of compensatory mechanisms, an increased right atrial pressure and right-sided congestive heart failure, in combination with systemic congestion and a decreased forward flow into pulmonary vasculature.

Materials and methods

Clinical examination was focused on specific cardiac examination.

Auscultation was done with a Littman Eko Core digital stethoscope on both sides of the thorax, and at the same time was recorded the phonocardiogram by connecting the stetoscope to a computer.

An ECG was recorded (PolySpectrum) with the animal restrained in right lateral recumbency using 6 standard leads. There were done several recordings over a 5 minutes periods.

Thoracic radiography was performed in right lateral, left lateral and dorsoventral recumbency.

Echocardiographic examination was performed with an Mindray Vetus E7 machine equipped with a phased array, multifrequency transducer (5-7,5 MHz). The echocardiac examination was done after clipping hair from both sides of the thorax with the animal gently restrained on a cut-out table. For the echocardiographic examination was used the two-dimensional imagines, M mode and Doppler evaluation.

Results and discussions

A 2 years old, crossbreed male has referred for to our clinic for a complete cardiac examination. The owner report that the dog present coughing at effort with syncopal episodes. The dog had a good body condition score (4/10), no jugular vein distention or pulse, and no ascites or peripheral edema. Heart auscultation revealed a holosystolic murmur grade V/VI on tricuspid area on the right side of the thorax, also seen on a phonocardiogram as a crescendo-descrescendo murmur between S1 and S2 (Fig. 1).

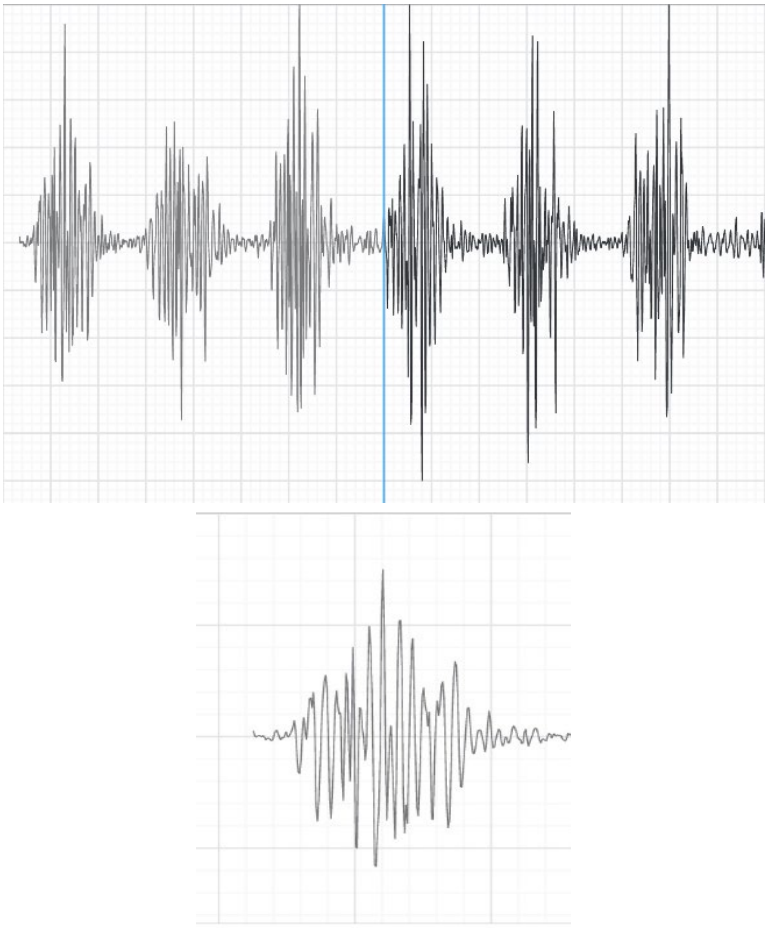

Fig. 1. Phonocardiogram recorded at auscultation

Thoracic radiography was performed in three orthogonal views (right lateral, left lateral and dorso-ventral). Thoracic radiographs showed mild right heart cardiomegaly, and especially an atrial enlargement (Fig. 2). Because of the enlargement of the right atrium the apex of the heart is pushed to the left side of the thorax.

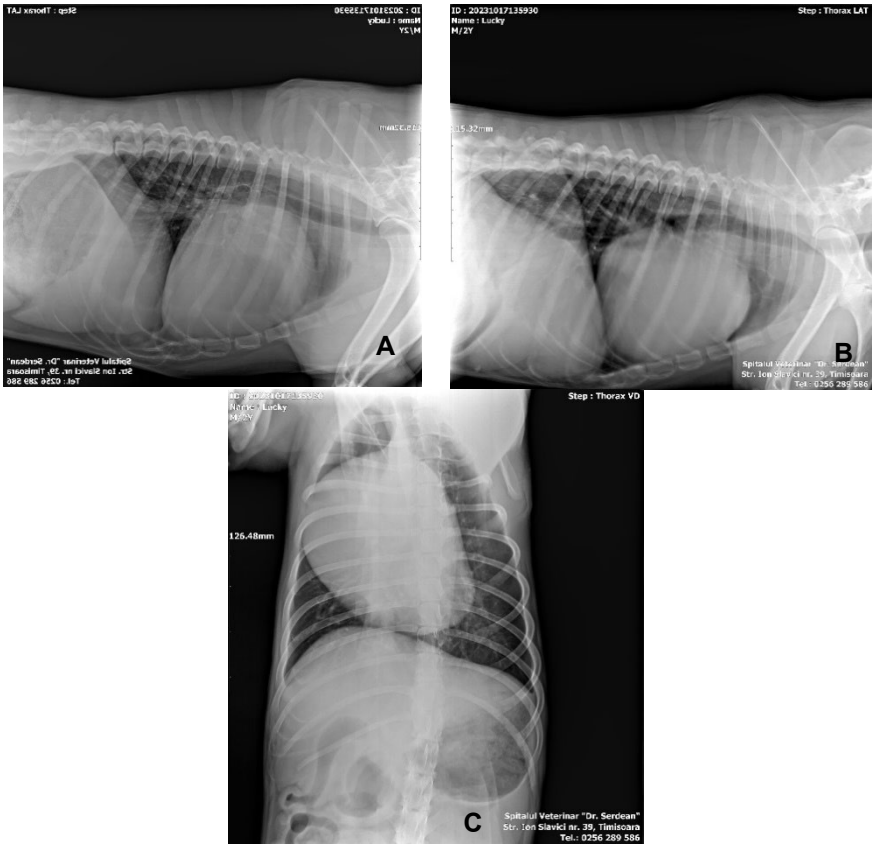

Fig. 2. (A) Right lateral thorax radiograph. The cardiac silhouette is moderately widened, measuring four intercostal spaces in with. There is an increased sternal contact and the apex of the heart is retracted dorsally. The right side of the cardiac silhouette has a rounded appearance. (B) Left lateral thorax radiograph. The same appearance as on the right side. (C) Dorsoventral thorax radiograph. The cardiac silhouette has a reverse D shape and an enlargement right atrium. The pulmonary parenchyma shows a moderate diffuse broncho-interstitial pattern

On electrocardiogram there was a sinus rhythm with signs of right atrial enlargement represented by tall P waves in DII (>0,4 mV)(50 mm/sec) and a normal mean electrical axis of the QRS complex (Fig. 3).

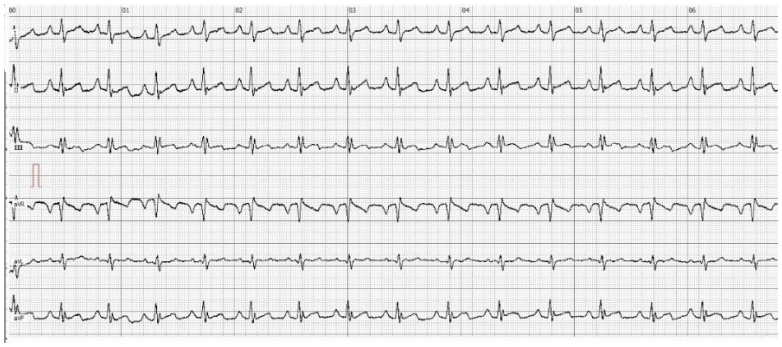

Fig. 3. ECG trace (50 mm/sec) (P – 74 msec/0,6 mV, PQ – 112 msec, QRS – 78 msec, QT – 234 msec, QTc – 356 msec, MEA - 76°, HR 139 beats/min)

A complete echocardiographic examination was performed including two-dimensional, and Doppler evaluations. On the long axis four chamber view was observed an eccentric hypertrophy of the right ventricle and an enlarged right atrium. The right volume overload was more obvious than the right ventricular volume overload. Also was observed a slight right ventricular pressure overload characterized by flattening of the interventricular septum (Fig. 4). The dimensions and function of the left heart were within normal limits.

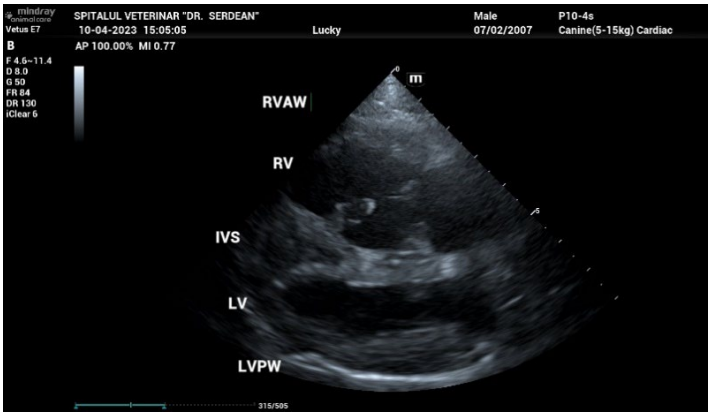

Fig. 4. Right long axis 4 chamber view – right ventricular volume and pressure overload

By angling the probe more caudally toward right atrium was observed a pathological thick hyperechoic membrane dividing the right atrium into two chambers, one larger cranial (CrRA) - true right atrium and the other one shorter caudally (CaRA) - accessory right atrium (Fig. 5).

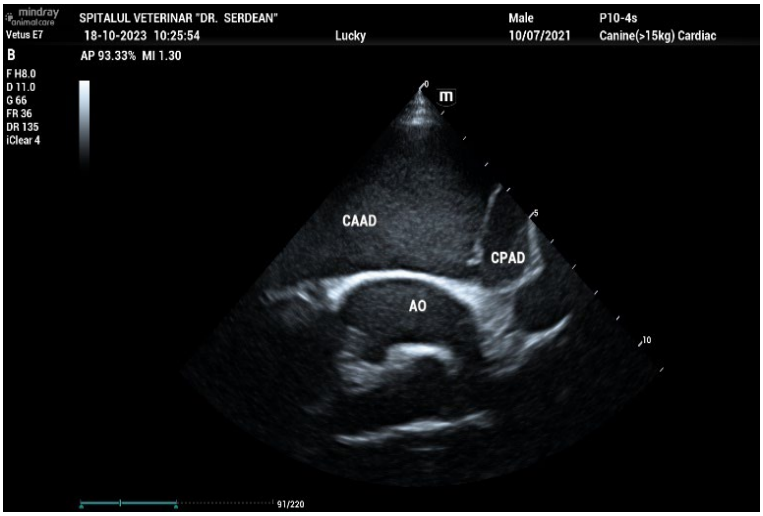

Fig. 5. Two-dimensional echocardiographic - right parasternal oblique view focused on the right atrium and caudal vena cava showing the right atrium divided by a membrane in two chambers: the caudal chamber (CaRA) and cranial chamber (CrRA)

This condition is compatible with Cor Triatriatum Dexter. The membrane from the right atrium was perforated, so the flow could pass from right atrium to right ventricle. The communication between the atrial chambers was demonstrated by a contrast bubble study using agitated saline solution injected in the left saphenous vein. The passing of the bubbles from CaRA to CrRa demonstrated a perforated Cor Triatriatum Dexter. Considered the age of the patient, lack of ascites and no evidence of right-sided heart failure did not meet the criteria for a typical Cor Triatriatum Dexter.

The tricuspid valve leaflets were thickened, with an incomplete coaptation. Color flow Doppler evaluation of the tricuspid valve identified a turbulent flow across this valve (Fig. 6) and also a continuous turbulent flow through the membrane perforation (Fig. 7).

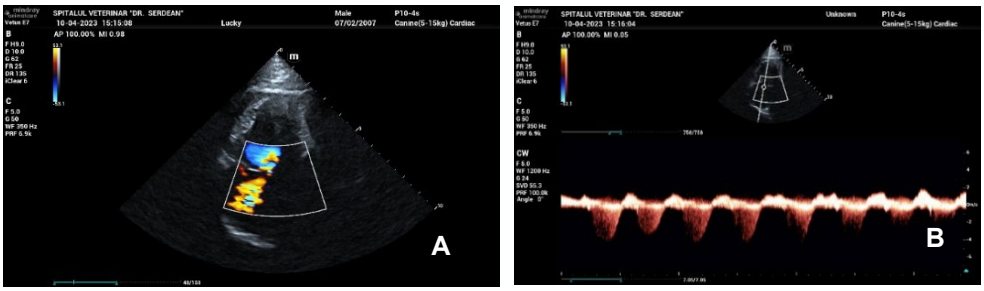

Fig. 6. Tricuspid regurgitation – Left apical 4 chamber view; A. Color Doppler mode B – Continous wave Doppler

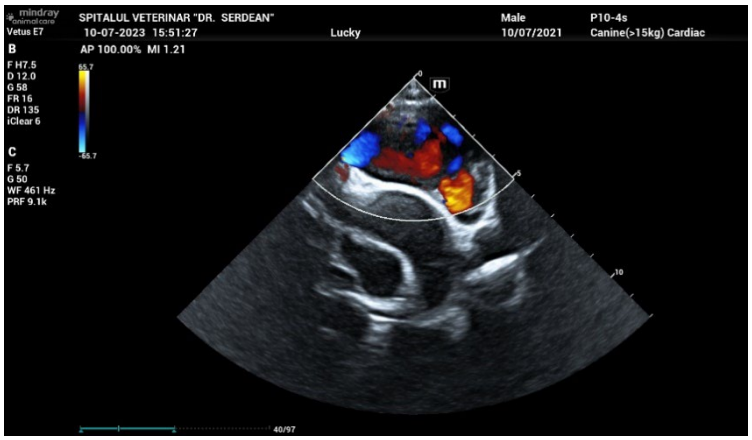

Fig. 7. Color Doppler on modified right parasternal long axis, four chamber view shows the flow through the perforation of the membrane with a mild regurgitation at this level

The embryologic abnormality that causes Cor Triatriatum Dexter is also thought to be involved in the development of tricuspid valve dysplasia. In fact, failure of apoptosis of embryological myocardial cells could induce both incomplete separation of the tricuspid valve cusps from the right ventricular wall and persistence of the embryological sinus venosus valve, conditions that caused tricuspid valve dysplasia and CTD, respectively (12). This mechanism could be a plausible explanation for the frequent correlation between the two congenital diseases (7). However, Cor Triatriatum Dexter in dogs has been described together with other congenital diseases such as pulmonary valve stenosis, mitral valve dysplasia, two-chambered right ventricle, patent ductus arteriosus, perimembranous interventricular septal defect, and persistent left cranial vena cava (11, 12).

It has been reported that around 40% of dogs with Cor Triatriatum Dexter do not need any treatment because the ostium is of a sufficient size to determine a low or no hemodynamic impact. On the other hand, with one small membrane perforation, resistance to blood flow increases the pressure in the proximal chamber with direct consequences for venous return to the right heart. The resulting clinical syndrome is referred to by some authors as the “Budd–Chiari-like syndrome”. It is characterized by the development of ascites in the absence of pleural effusion and jugular distension and pulse.

Conclusions

Cor Traitriatum Dexter is a congenital condition with low prevalence in canine population (less than 0.3%). In dogs with Cor Traitriatum Dexter, the presence of symptoms depends mainly on the presence or absence of communication between the two chambers (caudal and cranial), as well as the size of the perforation of the membrane itself. In sufficiently large perforations, as in the present case, a diagnosis of Cor Triatriatum Dexter can be made only incidentally and no therapeutic or surgical intervention is required.

The ultrasound examination is the gold standard diagnostic of Cor Traitriatum Dexter.

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